Encapsulation-Free Local Delivery of BDNF Enhances Recovery in Stroke-Injured Rats

by

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Institute of Biomaterials and Biomedical Engineering
University of Toronto

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Abstract

Until the early 1990s it was common belief that the adult brain did not possess the capacity to change or regenerate. We now know that the adult brain is not static and is able to repair itself to a small degree. Through examination of endogenous processes, brain-derived neurotrophic factor (BDNF) has emerged as a key modulator of plasticity in both the healthy and stroke-injured brains, and benefits to recovery have been demonstrated upon its administration; yet, current delivery strategies are highly invasive or inefficient. The work presented herein describes an effective, biomaterial-based strategy for minimally invasive local delivery of BDNF using a revolutionized approach to control protein release.

BDNF incorporation into a composite consisting of poly(lactic-co-glycolic acid) (PLGA) nanoparticles and a hyaluronan and methyl cellulose (HAMC) hydrogel provided sustained release of the protein, using electrostatics to control release without encapsulation. When delivered epi-cortically to a rat endothelin-1 model of stroke injury, BDNF diffused from the HAMC/PLGA composite into the lesioned tissue and resulted in improved early forelimb recovery and delayed hindlimb performance enhancement. Tissue analysis revealed synaptic
plasticity in the contralesional hemisphere at the time of sacrifice, suggesting that these behavioural effects were due to increased plasticity in the uninjured tissue. These studies validate the use of BDNF for plasticity-mediated stroke treatment and provide a minimally invasive strategy for effective, local delivery to the brain.
Acknowledgments

None of this work would have been possible without the amazing support I’ve had throughout. I would first like to thank my supervisor, Dr. Molly Shoichet for allowing me the honour and privilege of working with her and learning from her throughout this degree. I could not have chosen a better supervisor - she knew exactly when to push and when to support. She has taught me to question everything and to break through all of the glass ceilings I encounter. I would also like to thank my committee members, Dr. Michael Sefton and Dr. Cindi Morshead for their time, expertise, guidance and thoughtful discussion throughout the course of this degree.

My parents, Susan and Mark Obermeyer, prepared me for this challenge before any of us knew it would come to pass. As a little girl, bedtime discussions about astrophysics and the nature of the universe with my Dad instilled in me a sense of wonder and curiosity while showing me how fun science can be. Countless hours spent practicing violin with my Mom taught me dedication, confidence and perseverance while developing a much-needed outlet for creative expression. I could never thank them enough for these gifts or for the support they have provided throughout my academic journey. My sister and other half, Robyn, has been my sounding board throughout and has brought me back to earth when I’ve been in danger of floating off into the ether. My Aunt, CJ Berg, has kept me believing that I am capable of defending this degree and that I am worthy of the title. My narwhal, Daniela Mejia, picked me up off the ground, re-charged me and taught me to watch out for the footstools.

Anup and Sam are the other two legs of the tripod - I would not still be standing without them. From back-to-back 16-hour surgery days to MacGyver-ing rehab cages, they made being in the trenches not only bearable, but something to look forward to. They have been there for me in ways I can’t even put into words during the fender benders of the past 6 years and I have learned so much about science, life and myself from both of them. They continue to inspire me to think critically, and love unconditionally. Stroke team forever.

It was really the people of the Shoichet lab that made this one of the most powerful experiences of my life. Thank you to all of the remaining Gurls that I have had the honour of working with for making this treacherous journey into the adventure of a lifetime – together.
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<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>aCSF</td>
<td>artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>AIS</td>
<td>axon initial segment</td>
</tr>
<tr>
<td>AKT</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>AMPA</td>
<td>$\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variables</td>
</tr>
<tr>
<td>AP</td>
<td>anteroposterior</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>BDA</td>
<td>biotinylated dextran amine</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>chABC</td>
<td>chondroitinase ABC</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CREB</td>
<td>cyclic AMP-responsive element-binding protein</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>CSPG</td>
<td>chondroitin sulfate proteoglycan</td>
</tr>
<tr>
<td>DAG</td>
<td>diacyl glycerol</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DDS</td>
<td>drug delivery system</td>
</tr>
<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglion</td>
</tr>
<tr>
<td>DV</td>
<td>dorsoventral</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbant assay</td>
</tr>
<tr>
<td>EPO</td>
<td>erythropoietin</td>
</tr>
<tr>
<td>ET</td>
<td>endovascular thrombectomy</td>
</tr>
<tr>
<td>Et-1</td>
<td>endothelin-1</td>
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<td>fibroblast growth factor-2</td>
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<td>FUS</td>
<td>focused ultrasound</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
</tr>
<tr>
<td>HA</td>
<td>hyaluronan</td>
</tr>
<tr>
<td>HAMC</td>
<td>hyaluronan and methyl cellulose hydrogel</td>
</tr>
<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin-like growth factor 1</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol triphosphate</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MC</td>
<td>methyl cellulose</td>
</tr>
<tr>
<td>MCAO</td>
<td>middle cerebral artery occlusion</td>
</tr>
<tr>
<td>MEK</td>
<td>mitogen-activated protein kinases</td>
</tr>
<tr>
<td>ML</td>
<td>mediolateral</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<tr>
<td>np</td>
<td>nanoparticle</td>
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<tr>
<td>NPC</td>
<td>neural precursor cell</td>
</tr>
<tr>
<td>NSC</td>
<td>neural stem cell</td>
</tr>
<tr>
<td>NT-3</td>
<td>neurotrophin-3</td>
</tr>
<tr>
<td>NT-4/5</td>
<td>neurotrophin-4/5</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>OB</td>
<td>olfactory bulb</td>
</tr>
<tr>
<td>PACAP</td>
<td>pituitary adenylate cyclase-activating polypeptide</td>
</tr>
<tr>
<td>PAP</td>
<td>perisynaptic astrocytic process</td>
</tr>
<tr>
<td>PBCA</td>
<td>poly(butyl cyanoacrylate)</td>
</tr>
<tr>
<td>pBDNF</td>
<td>pro-BDNF</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PDMS</td>
<td>polydimethylsiloxane</td>
</tr>
<tr>
<td>PEI</td>
<td>polyethyleneimine</td>
</tr>
<tr>
<td>PGA</td>
<td>poly(glycolic acid)</td>
</tr>
<tr>
<td>PLA</td>
<td>poly(lactic acid)</td>
</tr>
<tr>
<td>PLCγ1</td>
<td>phospholipase Cγ1</td>
</tr>
<tr>
<td>PLGA</td>
<td>poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PVA</td>
<td>polyvinyl amine</td>
</tr>
<tr>
<td>RMS</td>
<td>rostral migratory stream</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>SGZ</td>
<td>subgranular zone</td>
</tr>
<tr>
<td>SH3</td>
<td>SRC homology 3</td>
</tr>
<tr>
<td>SSRI</td>
<td>selective serotonin reuptake inhibitor</td>
</tr>
<tr>
<td>SVZ</td>
<td>subventricular zone</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>tPA</td>
<td>tissue plasminogen activator</td>
</tr>
<tr>
<td>TrkB</td>
<td>tyrosine kinase B</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>XMC</td>
<td>crosslinked methyl cellulose</td>
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Figures and Tables

Figure 1. Cascades initiated by BDNF that influence plasticity. Phosphorylation of tyrosine residue 785 on TrkB recruits PLCγ to the receptor, and activates the enzyme with tyrosine phosphorylation. PLCγ hydrolyses phosphatidylinositol 4,5-biphosphate to give a diacylglycerol (DAG), which activates protein kinase C (PKC). This also activates inositol triphosphate (IP3) that releases Ca^{2+} from intracellular stores and acts with DAG to activate TRC channels, causing an influx of Ca^{2+}. Phosphorylation of tyrosine residue 515 on TrkB mediates the interaction with Src homology 2-containing protein (Shc). Shc recruitment to the Trk receptor is followed by activation of the phosphatidylinositol 3-kinase (PI3K) pathway, which shuttles Akt (protein kinase B) to the plasma membrane. Akt then downregulates tuberous sclerosis complex (TSC1) that complexes with TSC2, inactivating Rheb and allowing activation of mTOR, permitting mRNA translation. The Shc/Trk receptor interaction also causes phosphorylation of an adaptor protein, leading to activation of Ras/Erk signaling pathway through recruitment of Grb2 and SOS. A phosphorylation cascade with kinases Raf, Mek, Erk, and Rsk ultimately phosphorylates cAMP response element binding protein, a transcription factor that regulates the expression of BDNF [66], [67].

Table 1. Summary of additional endogenous plasticity modulators. .................................................................8

Table 2. Summary of exogenous plasticity-modulating biomolecules.................................................................13

Figure 2: Sustained local delivery to the brain can be achieved using drug-loaded polymeric particles suspended in hyaluronan/methylcellulose (HAMC) hydrogel. (A) Coronal view of stroke injured brain with drug delivery system shows that HAMC is injected directly onto the cortex. (B) Drug delivery system in expanded view shows that HAMC is held in place by both gelation and a casing comprised of polycarbonate discs. Adapted from work by Wang et al. and Tuladhar et al. [143], [144]...........................................................................................................................................19

Figure 3. Two different PLGA np systems are compared for controlled protein release. (A) Protein encapsulated in PLGA np dispersed in a hydrogel. (B) Protein and blank PLGA np dispersed in a hydrogel. For the latter, protein adsorbs to the PLGA np but is not encapsulated with- in them........................................................................................................................................25
Figure 4. Controlled sustained release of positively charged proteins from PLGA np does not require encapsulation. (A to C) Three different proteins show nearly identical release profiles whether encapsulated within PLGA np or simply mixed with blank PLGA np in a hydrogel: (A) SDF (pI 10.9, molecular mass of 8 kD, 200 ng/release), (B) NT-3 (pI 9.5, molecular mass of 27 kD, 1000 ng/release), and (C) BDNF (pI 10.9, molecular mass of 27 kD, 300 ng/release). The cumulative percentage released is significantly greater for soluble proteins with no PLGA np compared to soluble proteins with blank PLGA np or proteins encapsulated in PLGA np at all time points (P <0.05, n = 3 for all releases, mean ± SD plotted). Asterisks indicate significant differences between release of soluble protein with PLGA np and release of protein encapsulated in PLGA np (*P <0.05, **P < 0.01, ***P < 0.001). Curves for soluble SDF with no PLGA np and SDF encapsulated in PLGA np were taken with permission (15). (D) EPO (pI ~4, molecular mass of 30 kD, 84 ng/release) shows no attenuated release when mixed into HAMC with PLGA np versus just HAMC alone (without PLGA np), indicating that the phenomena observed with SDF, NT-3, and BDNF are based on short-range electrostatic interactions between positively charged proteins and negatively charged PLGA (n=3, mean ± SD plotted).

Figure 5. Sustained release of BDNF from agarose containing PLGA np is disrupted by increased salt. (A) BDNF shows the same delayed- and sustained-release profile from agarose containing PLGA np as from HAMC with PLGA np while diffusional release from agarose alone is still fast. (B) Almost all soluble BDNF is adsorbed to PLGA np after incubation, even at short times. (C) NaCl (0.5M) completely disrupts the interaction of BDNF with PLGA np, resulting in purely diffusional release (P >0.05 for all timepoints, n = 3 for all releases, mean ± SD plotted).

Figure 6. Release of positively charged proteins from composite HAMC can be tuned by changing the available nanoparticle surface area. (A) Cumulative percentage release of BDNF from composite HAMC is higher with 1000-nm-diameter PLGA np than with 300-nm-diameter PLGA np while keeping PLGA np mass constant. (B) (i) Release of NT-3 from composite HAMC with 0, 0.1, 0.5, 1, and 10 wt % PLGA np. Cumulative percentage of NT-3 released is significantly lower for 10 wt % PLGA np than for all other curves at t >1day (P < 0.05). Cumulative percentage of NT-3 released is significantly higher with 0 wt % PLGA np than with 0.1 wt % (t = 1 day, P < 0.05), 0.5 wt % (1 day ≤ t ≤ 3days, P <0.05),and 1wt%(t >3hours, P < 0.05). (ii) Release of 0.5, 1, or 10 mg of NT-3 from composite HAMC with 10 wt %PLGA np.
The concentration of NT-3 incorporated can be increased up to 20 times with virtually no change in release profile. Cumulative percentage of NT-3 released is significantly higher for 10 mg than for 0.5 mg at 10 and 14 days (P < 0.05) and is significantly lower for 0.5 mg than for 1 and 10 mg at 21 and 28 days (P < 0.05) (n = 3 for all releases, mean ± SD plotted).

Figure 7. Release of positively charged proteins can be tuned by changing the environmental pH. (A) Release of SDF from composite XMC into media at pH 3, pH 5, or pH 7. Cumulative percentage release of SDF is significantly greater at pH 3 than at pH 5 (t < 10 days, P < 0.01) and pH 7 (t > 0 days, P < 0.0001), likely because PLGA carboxylate anions are protonated to carboxylic acids, thereby reducing electrostatic interactions with positively charged proteins. (B) Release of BDNF from HAMC with PLGA np with or without encapsulated magnesium carbonate (MgCO₃). Release is delayed with encapsulated MgCO₃ (a basic salt), which neutralizes acidic degradation products and thereby maintains a higher/neutral local pH (n = 3 for all releases, mean ± SD plotted).

Figure 8. Adsorption may be rate-limiting for the release of positively charged proteins from PLGA np. (i) Initially, the protein is fully adsorbed to the negatively charged nanoparticle surface. (ii) As the nanoparticle begins to degrade, acidic components build up and decrease the local pH. (iii) At a certain threshold, the nanoparticle surface becomes neutral, weakening the electrostatic interactions with the positively charged proteins and initiating release.

Figure 9. Local delivery of BDNF enhances early forepaw recovery following stroke injury. Data plotted as individual animal staircase task scores with mean ± SD shown, *p ≤ 0.05. (A) Paradigm for staircase task training and testing relative to stroke injury and composite delivery. (B) BDNF-treated rats demonstrated significantly improved forelimb performance at week 3 relative to the week 1 injured timepoint (n = 10). (C) Vehicle treated rats (n = 11) and (D) injury only rats (n = 9) did not exhibit significant recovery at any time examined. (E) Sham group staircase performance remained consistent throughout (n = 8).

Figure 10. Local delivery of BDNF and vehicle enhances hindlimb recovery following stroke injury. Data plotted as individual animal beam task scores with mean ± SD shown, #p < 0.1, *p < 0.05, **p < 0.01. (A) Behavioural training and testing paradigm for tapered beam task. (B) BDNF-treated rats show some hindlimb recovery at week 3 with a #p < 0.1 (p-value = 0.0879) relative to week 1 and significantly (*p < 0.05) improved hindlimb performance at week 7 relative
to the week 1 injured timepoint (n = 9). (C) Vehicle-treated rats exhibited a deficit at week 1 and a significant improvement in hindlimb function at week 3 compared to week 1 that was not maintained to week 7 (n = 10). (D) Injury-only rats did not exhibit any significant recovery relative to the week 1 injured timepoint (n = 4). (E) Sham group staircase performance remained unchanged throughout the testing period (n = 11).

Figure 11. Lesion volume, a proxy for tissue degeneration, is decreased in animals that received the vehicle with or without BDNF. Data plotted as individual mean ± SD, *p≤0.05, **p≤0.01. (A) Lesioned tissue was defined as NeuN− and traced to quantify the area it occupied and regions of interest, R1 and R2, were defined on either side of the lesion for additional immunohistochemical quantification. (B) Cortical lesion (CL) volume was significantly decreased in BDNF- and vehicle-treated animals relative to injury only animals. (C) Striatal lesion (SL) volume was unaffected by treatment with vehicle or BDNF. (D) Cortical and striatal lesion volumes were combined to give a total lesion volume, where a significant decrease was observed in BDNF- and vehicle-treated animals relative to injury animals.

Figure 12. Injured animals experience loss of mature neurons in peri-lesional ROI. Data plotted as individual mean + SD, *p<0.05, **p<0.01. (A, B) No differences in the number of neurons were observed between groups in the homotopic regions of interest in the contralesional hemisphere. (C) In the ipsilesional regions of interest, injured animals experienced a significant loss of neurons in the peri-infarct area while animals that received the vehicle either alone or with BDNF did not exhibit this loss. (D) No differences were observed between the groups in ipsilesional region of interest R2.

Figure 13. Local BDNF delivery results in significantly increased synaptophysin expression in the homotopic contralesional hemisphere. Data plotted as individual mean + SD, *p<0.05, **p<0.01. (A) Contralesional homotopic region of interest R1 in BDNF-treated animals exhibited significantly increased synaptophysin expression compared to vehicle and injury groups. (B) Contralesional homotopic region of interest R2 in BDNF-treated animals exhibited significantly increased synaptophysin expression compared to vehicle, injury and sham groups. (C,D) No significant differences between groups were observed in ipsilesional regions of interest.
Figure 14. BDNF diffuses into stroke-injured rat brain tissue from the PLGA-HAMC composite deposited on the surface of the cortex, superficial to the lesion. (A) Total mass of BDNF detected in stroke-injured rat tissue at 1, 4, 7 and 21 days post-delivery, as measured by BDNF tissue ELISA. (B) Depth diffusion profiles demonstrate that BDNF accumulated in the stroke-injured rat brain between 4 and 21 days. (C) No BDNF was detected above endogenous levels at 1 day post-delivery. Spatial distribution analyses at 4, 7 and 21 d timepoints show that BDNF diffuses to a depth of at least 3 mm from the brain surface. (Mean + SD reported, n = 4).

Figure 15. Potential pathways of BDNF influence. Contralesional effects could be governed by interhemispheric axonal sprouting initiated by BDNF treatment in the ipsilesional hemisphere, anisotropic diffusion guidance of delivered BDNF along the corpus callosum, and/or re-distribution of the delivered BDNF via CSF flow in the subarachnoid space.
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Figure A3. (A) Activity of released NT-3 was assessed using a DRG neurite outgrowth assay. Released NT-3 elicits significantly increased neurite outgrowth for up to 10 days compared to a 0 ng/mL control, with a trend towards increased outgrowth for 21 days (n = 3, mean ± standard deviation plotted). (B) SDF activity was assessed using a neurosphere migration assay. Released SDF caused significantly higher neural stem/progenitor cell migration for up to 7 days compared to a 0 ng/mL control, with a trend towards increased migration for 28 days (n = 5 independent releases, mean ± standard deviation plotted). (C) Activity of released BDNF was assessed using a DRG neurite outgrowth assay. Released BDNF elicits significantly increased neurite outgrowth for 42 days compared to a 0 ng/mL control (n = 3, mean ± standard deviation plotted). (* p < 0.05, ** p < 0.01, *** p < 0.001). ........................................................................................................ 108

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Figure A5: HAMC with 10 wt% PLGA np has a significantly higher swelling ratio than HAMC alone between 3 and 28 days (p < 0.05, n = 3, mean ± standard deviation plotted) ................. 110

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Figure A10. BDNF diffuses into stroke-injured rat brain tissue from PLGA-HAMC composite deposited epi-cortically, 4 days post-stroke (n = 4). Data presented as mean ± standard deviation.

Figure A11: Failure to demonstrate benefit of rehabilitation therapy in study performed at the University of Toronto under the guidance of the Corbett lab. (A) Study paradigm, indicating when training, testing and rehabilitation was performed. (B) Standard care animals were housed in standard rat cages and did not receive any rehabilitation sessions, n = 7. (C) Enriched rehabilitation animals were housed in larger cages with extra environmental stimulation, n = 11. Data presented as mean ± standard deviation.
1 Background and Rationale

Parts of this chapter have been submitted as an invited review:


J.M.O., E.H, A.G. and M.S.S. wrote and edited the manuscript. J.M.O. wrote the introduction and sections on defining neuroplasticity, clinical strategies for stroke treatment, and endogenous response to stroke injury. J.M.O. and A.G. wrote sections on exogenous plasticity modulators. J.M.O. and E.H. wrote sections on biomaterials for plasticity modulation.

1.1 Introduction

Neuroplastic processes underlie every aspect of our health and wellbeing. Though we see evidence of this plastic nature in our daily lives, we have only recently acknowledged the potential regenerative capacity it offers. This shift in understanding is timely; increases in metabolic risk factors, such as obesity and diabetes mellitus in conjunction with the ageing population, have made stroke the second most common cause of death and the third leading cause of adult disability worldwide [1]. To add insult to injury, advancements in stroke treatment are severely lacking. Tissue plasminogen activator (tPA) remains the sole clinically-used therapeutic for patients presenting with ischemic stroke. Since the first use of tPA for stroke treatment in 1996 [2], the only significant standard of care improvement has been to widen the timeframe for administration of this drug via endovascular thrombectomy (ET) in large-vessel occlusions [3]. While ET has dramatically increased the population eligible to receive tPA, this intervention still only limits the extent of the damage and does not address the chronic aspects of the illness. Rehabilitation therapy remains the gold standard for management of the memory loss and functional deficits that affect stroke patients, yet it only restores partial function in most cases [4].

The inherent plasticity of the brain may be the key to pushing past this stagnation in stroke therapy innovation. By developing strategies that support and augment natural reparative mechanisms, we can take advantage of the existing architecture of the brain, which may result in
more robust long-term changes. Biomaterials have been fundamental to these strategies, owing to their tremendous versatility and tunability. These biocompatible, and often biodegradable, materials typically serve as the vehicles for sustained therapeutic factor release and cell delivery [5], [6].

1.2 Defining Neuroplasticity

While the term neuroplasticity can be used to describe any changes in nervous system structure or function [7], plasticity at the synaptic level governs the majority of these transformations. Synaptic plasticity refers to the strengthening or weakening of synapses through the expression of neurotransmitters, as well as the creation and destruction of synapses, and is activity-dependent. With the recurring use of certain connections, long-term potentiation (LTP) is initiated, strengthening these synapses to make information transfer more efficient for processes that are frequently used. This concept, originally introduced in 1973 by Bliss and Lomo [8], has helped to explain why repetitive use and coordination of neural pathways is crucial to memory formation and skill development. Early studies by Levy and Steward furthered this understanding, showing that LTP has cooperative and associative properties. A strong input will induce cooperative LTP by recruiting many excitatory synapses to fire together. Associative LTP occurs when simultaneous activation of two separate inputs, one strong and one weak, induces LTP in the weak input that would not experience LTP on its own [9], as is the case in Pavlovian fear conditioning [10]. Though N-methyl-D-aspartate (NMDA) receptor-dependent LTP in hippocampal CA1 neurons has been the most heavily investigated form, LTP takes on different characteristics depending on the area of the brain in which it is observed [11]. Advances in optogenetics are shedding light on the mechanisms that contribute to long-term memory formation and have enabled the deactivation and reactivation of associative memories in a mouse model [12].

To allow synaptic plasticity to occur, neurons must undergo morphological modifications that can include the number and shape of dendritic spines, dendrite and axon arborization, axon initial segment (AIS) length and position, and axon bouton number [13], [14]. Recent studies have also identified changes in other cell types that support synaptic plasticity. Astroglia, for example, possess perisynaptic astrocytic processes (PAPs) that envelop excitatory synapses to an extent that is dependent on a variety of different factors, one of which being the frequency of
Increased synaptic activity increases Ca$^{2+}$ transients in the PAPs and results in greater synapse coverage and longevity [16]. Neurogenesis, angiogenesis, and changes in the extracellular matrix (ECM) occur in conjunction with alterations to existing neural and glial cells. Adult neural stem cells, located in the subventricular zone of the lateral ventricles and the subgranular zone of the dentate gyrus, can be stimulated to rejuvenate neural circuitry or mitigate the effects of injury [17],[18]. With increased or decreased usage of particular regions of the brain, demands for oxygen and nutrients fluctuate, dictating the required degree of vascularization and initiating angiogenesis [19],[14]. The ECM must accommodate and support all of these architectural changes, and so will be catabolized and restructured accordingly [20].

In the event of a major traumatic injury, such as stroke, large regions of neural tissue are compromised and compensatory mechanisms must recruit the remaining tissue to take on functions that would otherwise be lost. This results in a rewiring of entire neural systems that involves extensive synaptic plasticity and has been observed both in animal models [21] and in humans [22], [23]. There is evidence of both ipsilesional [24] and contralesional [25] tissues contributing to this compensation, though it remains unclear precisely what determines the redistribution to one hemisphere over the other [26]. There is also an ongoing debate surrounding which of these two scenarios is preferred when developing intervention strategies, or whether the goal should be to restore more bilateral balance between the hemispheres [27]–[29]. A multifaceted and complex phenomenon, many aspects of neuroplasticity are yet to be fully understood. Still, in recognizing synaptic plasticity as a main driver of structural and functional changes to brain tissue, we can inform our therapeutic strategies such that they influence plasticity at the most basic level and encourage the supportive processes on which these changes depend.

### 1.3 Clinical Strategies for Stroke Treatment

Stroke is generally broken down into two main types: hemorrhagic and ischemic. Hemorrhagic stroke is caused by blood vessels rupturing and is often fatal. The loss of blood supply combined with increased intracranial pressure and infiltration of inflammatory cytokines and matrix metalloproteinases typically results in severe and rapid tissue damage [30]. In ischemic stroke, a blockage such as a blood clot obstructs blood flow to one or more areas, interrupting the supply of oxygen and nutrients. The ischemic injury forms over a longer period
of time than the hemorrhagic, and can be diminished if the blockage is transient. Due to the severity and usual fatal outcome of hemorrhagic stroke, most regenerative strategies are aimed at treating ischemic stroke [31].

With the exception of tissue plasminogen activator administration or endovascular thrombectomy (ET) [32] in the acute phase of injury, no other therapeutic strategies have come close to augmenting stroke recovery in the same way as rehabilitation therapy. While ET or tPA have helped many stroke patients, both significantly increase risk of hemorrhage by disrupting the cerebrovascular physiology [33], [34]. tPA transforms plasminogen into the proteolytic enzyme plasmin when both are bound to fibrin, a major component of blood clots. This complex acts to dissolve the clot, thereby restoring blood flow to affected areas of the brain tissue [2]. ET, a mechanical strategy for removing the thromboembolus from the blood vessel, can be used alone or in conjunction with fibrinolytic treatment. A number of ET devices have been developed and tested in clinical trials in recent years, and while they increase the effective window of tPA, they also greatly increase the rates of asymptomatic hemorrhagic transformation [34].

At present, the gold standard of ischemic stroke treatment is rehabilitation therapy, which draws on the capacity of the brain for endogenous neuroplasticity. Extensive research in rodent models has demonstrated that major cortical remapping can be achieved through rehabilitation training following injury to the motor cortex. Nishibe et al. explored the temporal aspect of this reorganization in a rat endothelin-1 (ET-1) model using intracortical microstimulation in perilesional regions. Several kinematic measures were assessed, revealing a rapid improvement in rehabilitation-trained (rehab) animals that was sustained past the training period. While the animals’ performance in behavioural tasks plateaued [35], reorganization of the forelimb representation area of the brain tissue continued and was more substantial in rehab animals than controls at 38 days post-injury. This has spurred additional studies into the underlying mechanism of rehab therapy and the consequent neuroplasticity.

1.4 Endogenous Response to Stroke Injury

We highlight key events that are relevant to plasticity-based recovery and direct readers to excellent reviews of the processes that are initiated by stroke injury [36], [37]. By examining
the endogenous factors that the brain naturally releases at different stages of injury, we can inform our design of plasticity-enhancing strategies to support healing processes.

1.4.1 Injury Progression

Following ischemic stroke injury, oxygen and nutrient supply to the brain tissue is interrupted, causing neurons and glial cells in the affected regions to undergo apoptosis within minutes. The damage increases as immune cells infiltrate, and waves of neuronal excitotoxicity propagate. The brain responds rapidly to this sudden assault by containing the damage, protecting surviving cells, and re-routing the circuitry to compensate for necrotic tissue. The lesion typically reaches its maximum size by 6 hours post-injury [38], but in the following hours to days, cell death and necrosis continue to spread into the region surrounding the lesion core, termed the penumbra. This penumbra is one of the main targets of neuroprotective strategies, as the tissue here is damaged, but potentially salvageable.

Once the injury has stabilized, the brain shifts to repairing and reorganizing the penumbral tissue. The loss of neurons in the ischemic core triggers an increase in neural progenitors in the subventricular zone (SVZ) that gives rise to neuroblasts. In rodents, the neuroblasts normally migrate from the SVZ, along the rostral migratory stream (RMS) to the olfactory bulb. After injury, their path is altered as they are recruited to the area surrounding the infarct [39]. Angiogenesis and neurogenesis are initiated and act synergistically, with blood vessels providing directional cues to neural progenitor cells and acting as a scaffold for these cells as they migrate to the region of injury [36]. In addition, microvascular endothelial cells secrete growth factors and cytokines that support neurogenesis [40], [41]. While these factors help to create a more favourable environment for post-stroke plasticity, the regenerative capacity of the neural progenitors is minimal due to the limited number of progenitors produced after the injury, and the low survival of new neurons. For example, Arvidsson et al. demonstrated that in adult rats, less than 1% of the lost neurons were replaced by progenitors from the SVZ [42]. Weeks to months after the initial injury, the chronic phase is characterized by decreased plasticity at every level [43], [44]. Recognizing how the ischemic environment fluctuates over time is crucial to developing appropriate interventions.
1.4.2 Reactive Astrocytes and the Glial Scar

Glutamate, the main excitatory neurotransmitter, enables the rapid transmission of signals between neurons in the healthy brain [45]. It exerts its function primarily through the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and NMDA ionotropic receptors that operate in synchrony; AMPA receptors are specialized for fast excitation, acting as gates for sodium and potassium cations, and NMDA receptors are calcium-permeable when activated by glutamate. Binding of glutamate to AMPA receptors causes depolarization of the membrane, enabling calcium influx through NMDA receptors. Subsequently, AMPA receptor expression and trafficking to the cell membrane is increased. This feed-forward mechanism is crucial for LTP to occur [46], but becomes a problem in the acute stroke microenvironment that is flooded with excess glutamate, which is released when neurons undergo apoptosis. In this scenario, the positive feedback loop perpetuates the hyperexcitability of the peri-infarct tissue as neurons fire rapidly, and without order, in response to this excitatory molecule [47],[48]. To break the cycle, reactive astrocytes are recruited to the injury site, where they release gamma-aminobutyric acid (GABA), an inhibitory neurotransmitter that acts against glutamate to quench the excitotoxicity in the lesioned tissue. In an uninjured brain, GABA has two main functions: (1) phasic (synaptic) signaling, where GABA is released by presynaptic boutons in response to interneuron action potentials, which then inhibit depolarization of the postsynaptic neuron transiently; and (2) tonic (extrasynaptic) signaling, where GABA maintains the overall neuron membrane potential and capacity to fire. Clarkson et al. reported that increased tonic GABA currents persisted for more than one month following stroke in mice, with the peri-infarct tissue exhibiting hypoexcitability. While beneficial in the acute phase, the peri-infarct tissue will have a diminished capacity for plasticity (and therefore recovery) if hypoexcitability persists chronically [49].

Reactive astrocytes and pericytes deposit chondroitin sulfate proteoglycans (CSPGs), which contribute to the formation of the glial scar [50]. The glial scar has been shown to inhibit axonal growth after injury in the brain, spinal cord and retina [51], [52]. By degrading the CSPGs, one of the main chemical barriers to regeneration is diminished, enhancing recovery in central nervous system (CNS) injury [53]. However, not all CSPGs are inhibitory and may support some aspects of regeneration. The glial scar is a crucial part of CNS injury response, and manipulating the relative levels of different CSPGs may be more effective for regeneration than eliminating them completely [54], [55]. CSPGs are an integral part of the normal CNS
extracellular matrix [53], forming perineuronal nets with other ECM proteins during brain and spinal cord development to support and stabilize circuits [56]. Thus, the role of CSPGs in injury is complex and one that is being actively investigated.

1.4.3 Brain-Derived Neurotrophic Factor

Gene expression in the peri-infarct tissue following stroke resembles that of early brain development in terms of neuronal growth, axonal sprouting, synaptogenesis and dendritic spine proliferation [57], [58]. A brain in either the developing or acutely injured state lacks perineuronal nets and thus has the ability to create new connections. A homodimer with a molecular weight of 27 kDa, brain-derived neurotrophic factor (BDNF) is an endogenously produced protein that has been identified as a principal agent of plasticity in both the developing and adult brain [59][60]. In normal circumstances, BDNF is widely expressed throughout the parenchyma by neurons and ependymal cells and its expression is immediately upregulated following stroke (4 h) [61]. BDNF is first expressed in a pro-form (pBDNF) that binds the p75 receptor and triggers neuronal apoptosis [62]. It is only with enzymatic cleavage of pBDNF to the mature form (BDNF) that it is able to bind to tyrosine receptor kinase B (TrkB) and initiate intracellular signaling cascades that support survival, neuronal differentiation and plasticity. The largely opposing roles of these two forms underline the importance of the relative amounts of each in the brain, and warrant the investigation of the factors that affect this ratio. Age may be one of the most significant mediators, with multiple groups demonstrating that with age, the brain naturally shifts this ratio to favour the pro-form of the protein [63],[64]. Particularly interesting in the context of stroke is that the cleavage of pBDNF to BDNF is dependent on the presence of extracellular tPA, as shown by Farrell et al. [65]. In their mechanistic study, tPA homozygous null (tPA−/−) mutant mice exhibited severely restricted late-phase LTP in hippocampal slices that could be rescued with application of mature BDNF.

The modulation of plasticity by mature BDNF occurs through three main intracellular signaling cascades that operate in tandem (Figure 1). Binding of BDNF to TrkB causes dimerization and autophosphorylation of tyrosine residues on the receptor and initiates the mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and phospholipase Cγ (PLCγ) pathways. The MAPK pathway regulates protein synthesis-dependent plasticity through activation of cAMP response element binding (CREB) transcription factor and
eukaryotic initiation factor 4E (eIF4E). PI3K influences cell survival and protein translation via activation of Akt and the mammalian target of rapamycin (mTOR), while also regulating the transport of synaptic proteins to maintain synaptic plasticity. With the activation of PLCγ, calcium is both released from intracellular stores as well as pumped from the extracellular space into the cell via plasma membrane channels [66]. This increase in intracellular Ca^{2+} lowers the threshold for neuronal depolarization and allows increased amplitude and frequency of excitatory post-synaptic currents [67]. The PLCγ pathway in particular demonstrates why BDNF is key to LTP, and explains its involvement in rehabilitation therapy. Recent studies linking exercise with the upregulation of BDNF [68], [69], combined with evidence that blocking BDNF expression negates the beneficial effects of rehabilitation [70], demonstrate the synergism of these physical and chemical elements of recovery.

![Figure 1. Cascades initiated by BDNF that influence plasticity. Phosphorylation of tyrosine residue 785 on TrkB recruits PLCγ to the receptor, and activates the enzyme with tyrosine phosphorylation. PLCγ hydrolyses phosphatidylinositol 4,5-biphosphate to give a diacylglycerol (DAG), which activates protein kinase C (PKC). This also activates inositol triphosphate (IP3) that releases Ca^{2+} from intracellular stores and acts with DAG to activate TRC channels, causing](image-url)
an influx of Ca\textsuperscript{2+}. Phosphorylation of tyrosine residue 515 on TrkB mediates the interaction with Src homology 2-containing protein (Shc). Shc recruitment to the Trk receptor is followed by activation of the phosphatidylinositol 3-kinase (PI3K) pathway, which shuttles Akt (protein kinase B) to the plasma membrane. Akt then downregulates tuberous sclerosis complex (TSC1) that complexes with TSC2, inactivating Rheb and allowing activation of mTOR, permitting mRNA translation. The Shc/Trk receptor interaction also causes phosphorylation of an adaptor protein, leading to activation of Ras/Erk signaling pathway through recruitment of Grb2 and SOS. A phosphorylation cascade with kinases Raf, Mek, Erk, and Rsk ultimately phosphorylates cAMP response element binding protein, a transcription factor that regulates the expression of BDNF [66], [67].

1.4.4 Additional Endogenous Plasticity Modulators

In addition to BDNF, a host of other endogenous plasticity modulators are generated following stroke. Among these are erythropoietin (EPO), vascular endothelial growth factor A (VEGF-A), and insulin-like growth factor (IGF-1) [71]. EPO is predominantly responsible for erythropoiesis, but also plays an important role in protecting and regenerating the brain following ischemic injury. Subsequently, neurons and astrocytes increase their production of EPO from basal levels, and other cell types also contribute to EPO production at various stages of injury progression [72]. This factor stabilizes mitochondrial membranes in neurons, limits the formation of reactive oxygen species and reduces inflammatory cytokine production and neutrophil infiltration [73]. Moreover, EPO encourages angiogenesis, neurogenesis, and white matter protection/regeneration. Involvement in both protective and regenerative pathways makes EPO a powerful contributor to endogenous plasticity processes. Plastic processes require oxygen and nutrients to be delivered by the vasculature, thus the brain increases expression of VEGF-A in an attempt to circumvent the blockage [74]. With ischemic injury, reperfusion of the peri-infarct tissue is critical and faster reperfusion results in greater recovery [75]. With an immediate increase in VEGF expression following injury, reperfusion is enhanced and lesion volume is diminished [76]. IGF-1 is another key contributor to neuroprotection following injury. Similar to BDNF, IGF-1 activates the PI3K-Akt and MEK-ERK pathways and inhibits apoptosis, thereby aiding neuronal survival in the excitotoxic environment [77]. Even with the brain’s endogenous production of these plasticity modulators, spontaneous recovery is rarely seen in stroke patients, emphasizing the need for therapeutic intervention. Understanding the different niche functions of these molecules and how they work in synergy endogenously can help identify combinations of exogenous therapeutic factors to support and strengthen naturally occurring plasticity. Table 1 provides a summary of these additional exogenous plasticity modulators.
**Table 1.** Summary of additional endogenous plasticity modulators.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Stroke-associated mechanism of action</th>
<th>Target cell type</th>
<th>References</th>
</tr>
</thead>
</table>
| Erythropoeitin (EPO)          | • Stabilizes mitochondrial membrane  
• Limits formation of reactive oxygen species  
• Has an anti-inflammatory effect  
• Promotes angiogenesis, neurogenesis, white matter protection/regeneration | Neurons, astrocytes, endothelial cells | [73]       |
| Vascular Endothelial Growth Factor (VEGF) | • Induces angiogenesis  
• Enhances reperfusion  
• Decreases lesion volume | Neurons, astrocytes                  | [74], [76] |
| Insulin-like growth factor (IGF-1) | • Inhibits apoptosis and supports neuronal survival  
• Stimulates neurogenesis, neuronal myelination and angiogenesis | Neurons, astrocytes                  | [77]       |

### 1.5 Exogenous Plasticity Modulators

Exogenous factors, including both biomolecules and cells, are delivered to the brain to supplement the endogenous factors already present and promote tissue repair and recovery. In this section, we review these exogenous factors with a focus on plasticity and summarize their effects and intended targets in Table 2.

#### 1.5.1 Exogenous Biomolecules

Of the endogenously produced plasticity modulators we have discussed, BDNF exhibits the greatest potential as an exogenously delivered factor. Owing to its size and short half-life in vivo, the largest obstacles to BDNF delivery are overcoming the BBB and maintaining effective concentrations in the parenchyma. Most investigators have shown that BDNF does not cross the BBB [78],[79], making systemic delivery largely ineffective and potentially causing undesirable
side effects [80]–[83]. Intraventricular infusion enables local BDNF delivery to the brain with reported benefits to tissue [84],[85], but the invasive nature of these procedures combined with the need for multiple injections or implants pose a risk of infection and are not ideal for clinical translation. Some recent, less invasive biomaterial-based strategies for delivering BDNF will be discussed in greater detail in the following sections.

Alternatively, instead of delivering the protein itself, BDNF-mediated pathways can be triggered. For example, Clarkson et al. systemically delivered the BDNF-inducing ampakine, CX1837, resulting in BDNF expression in the peri-infarct cortex and behavioural recovery [46]. Interestingly AMPA receptor activation was beneficial when achieved one week after injury, turning its delivery from harmful to beneficial by delaying administration by 5 days from the time of stroke injury. The temporal aspect of the effect is noteworthy, and underlines the importance of determining the most appropriate timing for exogenous factor delivery. Niacin (nicotinic acid, vitamin B3) has also been shown to modulate neuroplasticity by upregulating BDNF expression. Cui et al showed that when Niaspan, a prolonged release formulation of niacin, was orally administered 24 hours after injury and daily for two weeks in a rodent model, there was improved synaptic plasticity, axon growth and greater angiogenesis [86]. These exogenous BDNF strategies are promising in pre-clinical stroke models, but have yet to be transferred to the clinic.

Another endogenously produced factor, EPO has shown some promise with exogenous delivery. Chau et al treated rodent models with the trophic factor EPO with observed neuroprotective effects [87]; however, Ehrenreich et al demonstrated unfavourable effects, including patient death, with intravenous delivery of EPO [88]. The poor outcome of this clinical study was associated with the high dose of EPO administered over a relatively short time frame of 6 to 48 h. As with BDNF, biomaterials can be used to address these delivery issues and tailor the administration of EPO appropriately.

To date, serotonergic and dopaminergic drugs have demonstrated the most potential clinically. Serotonin, a neurotransmitter that controls cognitive processes such as memory consolidation, is closely tied to plasticity. In animal studies, selective serotonin reuptake inhibitor (SSRI) drugs have been shown to increase neurogenesis and expression of neurotrophic factors, particularly BDNF [89], [90]. Subsequent human studies have highlighted the role of these SSRI drugs in improving motor and non-motor functions after stroke via plastic
mechanisms [91], [92]. Dopamine, another neurotransmitter, is well known for governing reward-based behaviour and has also been implicated in the modulation of LTP [93]. The latter function is still not entirely understood, though we are learning that dopamine may elicit heterogeneous LTP effects that are dependent on the target of action [94]. This location dependency may explain the contradictory results obtained in studies that delivered L-Dopa to stroke patients. Scheidtmann et al. showed that in a randomized study in patients within six months of stroke, a combination of L-Dopa and physical therapy resulted in improved motor recovery as compared to the placebo when L-Dopa was delivered daily for 3 weeks [95]. Other studies that used a single dose of L-Dopa combined with physical therapy in stroke patients showed inconsistent results in motor function recovery [7], [96]. The inconsistent results of these two studies suggest that repeated delivery of L-Dopa might be required to achieve functional recovery. Amphetamine, a psychomotor stimulant that modulates levels of neurotransmitters such as dopamine and serotonin, has also been extensively studied in the context of stroke with mixed results. Several clinical studies have used different treatments of amphetamine combined with physiotherapy to evaluate recovery though, according to a meta-analysis by Martinsson et al., there is no definitive indication for the routine use of amphetamine. Further studies with larger study groups may be needed to investigate this effect [97].

The excitotoxicity generated by an ischemic event presents another opportunity for exogenous drug intervention. Increased extracellular glutamate from apoptotic neurons leads to overstimulation of the glutamate receptor, NMDA, which in turn causes the release of additional glutamate and creates an environment that inhibits neuronal growth and plasticity [45]. NMDA agonists, such as Memantine and Flupirtine, prevent the activation of NMDA receptors, thus breaking the excitotoxicity cycle. Memantine was shown by Wang et al. to enhance neurological recovery and plasticity after stroke by improving motor coordination and spatial memory in a mouse model [98] and Flupirtine demonstrated a similar effect in mice, as reported by Jaeger et al. [99]. The sigma-1 receptor has also been targeted for excitotoxicity because it regulates calcium signalling and is involved in membrane trafficking, neurotransmission and cell survival [100]. A study in rodents by Rushcher et al. demonstrated that the sigma-1 receptor was upregulated after stroke in astrocytes in peri-infarct tissue. Two days after injury, rats were subcutaneously injected with a sigma-1 receptor agonist and this treatment was continued for several weeks, which resulted in enhanced functional recovery, even after the treatment was discontinued [101].
Inhibitory cues are abundant in the lesion environment, especially in chronic stroke. The glial scar, composed mostly of CSPGs, inhibits axonal migration and neurorepair [102], [103], and can be overcome by digestion of the glycosaminoglycan (GAG) side chains by the exogenous factor chondroitinase ABC (chABC). Hill et al, who administered chABC a week after stroke injury, demonstrated this in a rodent model [104]. With this intervention, they reported reduction in the thickness of the glial scar and improved motor recovery at 10 and 14 days post-injury [104]. As with CSPGs, the Nogo-A protein restricts the outgrowth of neurites, thereby stabilizing neural networks that are required for memory formation and skill learning [105]. Following injury, Nogo-A is seen as a hindrance to recovery as it blocks plastic processes that could allow rewiring of circuits and compensation for lost tissue. Delivery of anti-Nogo A blocks the function of Nogo-A and allows axonal and dendritic remodelling, while also increasing dendritic spine density [106]. Interestingly, Lindau et al. demonstrated that this effect was more prominent in the contralesional sensorimotor cortex following stroke when they delivered the antibody locally for two weeks following injury. The observed tissue effects were accompanied by enhanced behavioural recovery [107].

**Table 2. Summary of exogenous plasticity-modulating biomolecules.**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Stroke-associated mechanism of action</th>
<th>Target cell type</th>
<th>References</th>
</tr>
</thead>
</table>
| Serotonergic drugs                 | • Reduce neural inflammation  
• Increase neurogenesis  
• Enhance neurotrophin activity                                                                 | Neurons, astrocytes      | [89]–[92]  |
| Dopaminergic drugs                 | • Regulate expression of growth factors such as FGF-2, BDNF, GDNF                                      | Neurons, astrocytes      | [93]–[95]  |
| NMDA receptor antagonists          | • Reduce astrogliosis  
• Promote capillary formation  
• Increase growth factors such as BDNF, GDNF, VEGF                                                   | Neurons, astrocytes      | [45]       |
| Sigma-1 receptor agonist           | • Stimulates neurite outgrowth  
• Modulates membrane raft dynamics                                                                       | Neurons, astrocytes      | [100], [101]|
| Niacin- associated compounds       | • Improve synaptic plasticity, axon growth                                                              | Neurons                   | [86]       |
### 1.6 Biomaterials for Plasticity Modulation

The plasticity-modulating potential that exogenous drugs and cells possess can only be fully realized if they are effectively delivered. Recent advances in biomaterials technology have generated novel tools that have heightened the efficacy of these exogenous agents by providing a greater degree of control over delivery parameters. For many of the drug-based therapeutic strategies, sustained concentrations are desired without high doses associated with bolus delivery, multiple injections, or invasive surgeries associated with implants. Moreover, crossing the BBB requires innovative strategies beyond traditional intravenous, intramuscular or intraperitoneal injections. For cell delivery, survival is the first and foremost design consideration, dictating foundational biomaterial characteristics. Although not as common, biomaterials themselves have also been used in the absence of drugs and cells, to direct and enhance plastic processes.

#### 1.6.1 Biomaterials for Exogenous Factor Delivery

The BBB is the largest obstacle to exogenous factor delivery to the CNS. Systemic delivery strategies typically use intravenous, oral, or olfactory administration, and thus are well suited to clinical translation, but generally do not achieve therapeutic concentrations of exogenous agents in the brain [124–126]. Local delivery targets the injured tissue, reducing the delivered dose and minimizing unwanted side effects commonly seen with systemic delivery. This efficiency comes at a cost though, as local delivery strategies tend to be significantly more

<table>
<thead>
<tr>
<th>Biomaterial</th>
<th>Functions</th>
<th>Target Cells</th>
<th>References</th>
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<tbody>
<tr>
<td>Chondroitinase ABC (ChABC)</td>
<td>• Promotes angiogenesis</td>
<td>Neurons, astrocytes and oligodendrocytes</td>
<td>[104]</td>
</tr>
<tr>
<td></td>
<td>• Improves neuronal differentiation and plasticity</td>
<td>Neurons, astrocytes and oligodendrocytes</td>
<td>[104]</td>
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<tr>
<td>Anti-Nogo A</td>
<td>• Enables axonal and dendritic remodeling</td>
<td>Neurons, oligodendrocytes</td>
<td>[105]–[107]</td>
</tr>
<tr>
<td></td>
<td>• Increases dendritic spine density</td>
<td>Neurons, oligodendrocytes</td>
<td>[105]–[107]</td>
</tr>
<tr>
<td>Brain derived neurotrophic factor (BDNF)</td>
<td>• Promotes synaptic plasticity</td>
<td>Neurons, ependymal cells, astrocytes, microglia</td>
<td>[84], [85]</td>
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<tr>
<td></td>
<td>• Stimulates neurogenesis</td>
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<td></td>
<td>• Supports survival and neuronal differentiation</td>
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invasive, requiring catheters, minipumps, or syringes to inject therapeutics directly into the brain parenchyma or ventricles [108]. Damage to healthy tissue is unavoidable in this scenario, and implants required for multiple doses pose greater risk of infection. Injectable biomaterials reconcile the advantages of the two routes of administration by making minimally invasive, local delivery strategies possible.

1.6.1.1 Systemic Delivery Using Biomaterial Nanoparticles

The use of polymeric nanoparticles has been somewhat successful for systemic delivery to the CNS as they have increased the longevity of the therapeutics in the blood. The wide array of fabrication techniques and materials that can be used to create nanoparticles has made this possible, as the design parameters have been manipulated to achieve a specific size, morphology, surface charge, chemical composition, hydrophobicity, and topographical features, thereby tailoring the particles to their application. Though many types of nanoparticles exist, we will focus our discussion on liposomal and polymeric configurations, as these are the most widely used in CNS delivery.

Conventional liposomes, phospholipid bilayers enclosing an aqueous core, are efficient at encapsulating both hydrophobic and hydrophilic compounds in their lipid bilayers and aqueous core, respectively [111], yet liposomes are quickly cleared by the reticuloendothelial system (RES), and thus have much shorter half-lives compared to other nanoparticles. Still, nanoliposomes have been effective carriers for drug delivery to the CNS in some cases. For example, citicoline, which has been shown to improve functional recovery and plasticity after experimental stroke [112], [113], was able to reach much higher levels in the brain when delivered intravenously in nanoliposomes compared to free drug [114], [115]. Similar to nanoliposomes, nanoassemblies that are formed by conjugation of adenosine to the lipid squalene, allow prolonged circulation of the nucleoside, providing neuroprotection in mouse model of stroke and improved neurological severity scores [116]. Functionalization of nanoliposomes, with compounds like PEG and glutathione, have reduced their high clearance rate and allowed better longevity in the blood [117]. Site-specific ligands also offer a means of improving BBB crossing through receptor-mediated transport. Zhao et al. showed that PEGylated liposomes conjugated with transferrin effectively target the BBB and deliver a VEGF plasmid, which increases vascular density and improves neurological recovery in a model of
stroke injury [118]. Additionally, manipulating the charge of the liposome can affect BBB targeting. Campos-Martorell et al delivered Simvastatin, known to upregulate BDNF and VEGF expression, intravenously in charged and neutral liposomes. They observed that positively charged liposomes did not significantly accumulate in the infarct while neutral and negatively charged particles increased the transportation of Simvastatin to the brain [119]. Though these studies demonstrate our ability to modify liposomal formulations to address certain challenges, they are not the ideal candidate for CNS delivery due to their instability and limited tunability.

Polymeric nanoparticles have also been used for systemic delivery to the CNS. These formulations can be easily functionalized and offer the advantage of being more stable than nanoliposomes while still being biocompatible/degradable. During the fabrication process, compounds of interest can be adsorbed to, encapsulated by, or chemically bonded to the particles, giving this biomaterial strategy another degree of tunability. Of the natural polymers used to create nanoparticles (chitosan, alginate, gelatin, and collagen), chitosan has shown great potential. A recent investigation by Yemisci et al. loaded chitosan nanoparticles with basic fibroblast growth factor (bFGF) and a small peptide inhibitor of caspase-3 for systemic delivery to the stroke-injured mouse brain [120]. By conjugating antibodies to the transferrin-1 receptor to the surface of these particles, they achieved BBB permeability and elicited a neuroprotective effect with their therapeutics. Though some additional caution is required when delivering synthetic materials to avoid over-accumulation in the kidneys, liver, and spleen [121], they can also be effective carriers when used appropriately. Synthetic polymers, such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(lactic co-glycolic acid) (PLGA), poly(ethylene glycol) (PEG), and poly(n-butyl cyanoacrylate) (PBCA), can be easily fabricated as nanoparticles with specific release profiles that can also protect molecules from degradation or elimination and improve their solubility. For example, Saucier-Sawyer et al. formed nanoparticles using a block copolymer of PLA and hyperbranched poly(glycerol), and modified the surface of the particles with adenosine (PLA-HPG-Ad). These particles, administered intravenously, were modestly effective at penetrating the BBB to deliver the chemotherapeutic agent, camptothecin. In addition to overcoming the BBB, the nanoparticles released camptothecin in a sustained manner, dictated by the degradation rate of the particles, that lasted several days [122]. Harnessing electrostatics between protein and polymers, Harris et al. formulated BDNF-loaded PEG-PGA block-copolymer nanoparticles that spontaneously formed upon mixing the components in water [78].
They delivered these particles intravenously and achieved enhanced bioavailability of BDNF in the brain, and improved neuroprotection and memory in stroke-injured mice. Previous studies have shown that these polyion complexes accumulate in brain vasculature surrounding the lesion [123].

### 1.6.1.2 Local Delivery of Biomaterial Particles

Even with additional targeting ligands and functionalization, one of the main limitation of nanoparticles for systemic delivery is the off-target effects that can result from the high drug loadings required to reach a therapeutic dose in the parenchyma [124]. Local delivery of nano- and microparticles, injected into the lesion site, peri-lesional tissue or intraventricularly, side-step this issue, placing the therapeutic directly at or near the site of interest. Osteopontin, a key mediator in bone formation, has also been shown to be upregulated after stroke, acting as an anti-inflammatory and chemoattractant compound [125]. It was delivered locally to the ischemic rat brain encapsulated in gelatin microspheres, where the release profile was significantly extended compared to free drug, resulting in lower neurological deficits and long-term neuroprotection [126]. Nakaguchi et al. also utilized local delivery of gelatin microspheres, delivering either insulin-like growth factor 1 (IGF-1) or hepatocyte growth factor (HGF), to the stroke injured mouse brain, improving neurogenesis and regeneration after stroke compared to free drug alone [127].

### 1.6.2 Scaffolds

Implantable scaffolds, like hydrogels or sponges, have also been studied in stroke treatment. Scaffolds are ideally biodegradable or bioresorbable, obviating the need for removal and limiting chronic inflammation [128]. Due to the intimate contact with brain tissue in local delivery, the host response must be tightly regulated. In addition to the immunogenicity of the biomaterial, other considerations are stiffness and swelling, as the brain is one of the softest organs in the body [129], and increases in intracranial pressure are a major cause of secondary damage after the initial stroke injury [130]. Implantable scaffolds can be synthesized with a multitude of biocompatible materials, like hyaluronan, methylcellulose, collagen, and agarose. The choice of material for these scaffolds can impact stroke repair. For example, high molecular weight hyaluronan is anti-inflammatory in the CNS [131], [132], and collagen enables cell attachment and migration [133], [134]. Their physical properties can be easily tuned to mimic the
brain’s physical properties, such as stiffness and morphology, while their chemical properties can be modified with binding peptides and trophic factors. Specifically, ECM mimetic hydrogels with specific microstructures, functional proteins, and topographical cues can be designed to drive proliferation, differentiation, and maturation of transplanted neural cells to promote tissue repair after cerebral ischemia. Nih et al. showed that microporous HA gels, functionalized with the RGD peptide and two factor XIIIa substrates, improved migration of NSCs in the infarct and also reduced inflammatory response and glial scar formation [135]. This cellular infiltration was also seen with electrospun poly(e-caprolactone) implanted in the adult rat brain, which enabled neurite infiltration and extension [136]. The stiffness and rheological properties of the material are important parameters to optimize in 3D culture systems, especially if the system will eventually be translated to in vivo use. Stiffness influences cell phenotype in vitro: moderately stiff (100-1000 Pa) gels are associated with neuronal cells, while stiffer (1000-10,000 Pa) gels are associated with astrocytes. Additionally, if the gel is too stiff (>100,000 Pa) or too soft (<10 Pa), neural stem cell survival may be limited [137]. Biomaterial scaffolds can be used to deliver drugs to enhance endogenous plasticity-induced remodelling, serve as the vehicle for cell transplantation to improve their survival, and sometimes enhance recovery with the physical properties of the material itself, as elucidated below.

### 1.6.2.1 Scaffolds as Delivery Vehicles

In addition to their mechanical properties, scaffolds can be combined with drugs or cells to further enhance their therapeutic effect. For drug delivery applications, scaffolds can be designed to achieve sustained release of encapsulated therapeutics to affect the plastic processes of endogenous repair like axonal sprouting, synaptogenesis, and angiogenesis. For example, a hydrogel composed of hyaluronan was used by Cook et al. to deliver BDNF locally over three weeks by injection directly into the infarct. This treatment improved motor recovery of stroke-injured mice at nine weeks post-injury through increased neuronal survival and axonal sprouting [138]. Similarly, Wang et al. was able to deliver EPO in a hydrogel composed of hyaluronan and methylcellulose (HAMC) in order to increase neurogenesis and brain remodelling (Figure 2) [6], [139]. Release rate can be controlled by encapsulating proteins in nanoparticles or microparticles prior to their dispersion within the gel to extend release and improve the longevity of the treatment [6], [156–158].
Figure 2: Sustained local delivery to the brain can be achieved using drug-loaded polymeric particles suspended in hyaluronan/methylcellulose (HAMC) hydrogel. (A) Coronal view of stroke injured brain with drug delivery system shows that HAMC is injected directly onto the cortex. (B) Drug delivery system in expanded view shows that HAMC is held in place by both gelation and a casing comprised of polycarbonate discs. Adapted from work by Wang et al. and Tuladhar et al. [143], [144].

Another method to control release is through affinity binding, such as with heparin and heparin-binding proteins or through specific design, such as that of fusion proteins with Src homology 3 (SH3) and SH3 binding peptides [145], [146]. A recent study by Clarkson et al. demonstrated that these local delivery systems can also be used in conjunction with systemic delivery to significantly improve treatment. Previous work showed that the ampakine CX1837 promoted functional recovery after stroke, which was mediated by BDNF [46]; however this effect was markedly reduced in older animals, possibly due to age-related deficits of BDNF. By administering CX1837 intravenously alongside local delivery of BDNF in a hyaluronan/heparan sulfate proteoglycan hydrogel, they observed improved motor recovery in the aged mice compared to CX1837 treatment alone, highlighting critical signalling pathways that must be stimulated synchronously to elicit optimal repair [147]. Instead of using scaffolds to release biologics, others have used scaffolds functionalized with trophic factors to promote repair, or antibodies to sequester harmful or overly abundant molecules from the stroke microenvironment. For example, Ma et al. immobilized a polyclonal antibody to the Nogo-66 receptor onto a HA hydrogel to decrease Nogo-A’s inhibitory effects to neurite outgrowth, which enabled axonal growth into the gel, although functional recovery was not significant compared to the vehicle control [148]. Zhang et al. adsorbed VEGF onto a TEOS-PDMS scaffold for local implantation,
which enabled increased proliferation of both endothelial cells and astrocytes, as well as infiltration into the scaffold compared to vehicle only controls [149].

1.7 Assessing the Bioactivity of Exogenous BDNF

BDNF is a delicate protein, prone to denaturation and subsequent loss of activity [150]. As such, assessing the bioactivity of this molecule in vitro is crucial to ensure that potential BDNF delivery strategies will be effective in vivo. Cultured dorsal root ganglion (DRG) cell bodies have been heavily investigated in vitro to determine the activity of neurotrophins. Incubation of these cell bodies with bioactive NT-3 or BDNF has been shown to enhance neurite outgrowth [151], which can be quantified and compared to controls in the absence of neurotrophins. The bioactivity of PLGA-encapsulated NT-3 was previously tested using a DRG assay using E17 Sprague-Dawley rat embryos [152]. This protocol was used in the current work to evaluate the bioactivity of encapsulated and un-encapsulated BDNF released from the PLGA-HAMC composite.

1.8 Rodent Models for Stroke Injury

Mice and rats are commonly used models for stroke injury, as their relative cost is low compared to other models and multiple behavioural assays have been established to assess recovery of various functions. With a higher degree of intelligence than mice, rats can be trained to perform various tasks that generate more meaningful data for comparison with human studies [153]. Sprague-Dawley rats in particular exhibit characteristics similar to the human population most prone to stroke in that they are fairly sedentary and less excitable than other strains, such as Long-Evans. To create a stroke injury in the rat model, middle cerebral artery occlusion (MCAO), photothrombosis with Rose Bengal dye or transient vasoconstriction with endothelin-1 (Et-1) are commonly used methods [154].

The Corbett lab has done extensive work with the Et-1 model of stroke in rats, and has developed a robust protocol to generate focal ischemia [155]–[157]. Due to the transient nature of the vasoconstricting peptide, this stroke injury closely resembles the ischemic events that transpire in the human brain. Thus, the resulting injury size in the Et-1 model is generally a better clinical representation than models like the MCAO that tend to destroy a very large portion of the affected hemisphere [158]. Et-1 can be injected at any depth, provided a hole is
drilled through the skull to allow for needle insertion, and provides a great degree of control over infarct size and location. It offers an advantage over photothrombosis, in that it is not limited to the cortex by the depth that a light source is able to penetrate into the tissue through the skull [159]. This is an important distinction, as cortical-only injury has been shown to allow a significant degree of spontaneous recovery [160], and the addition of a deeper striatal injection coordinate creates a deficit that is maintained in non-treated animal groups (Appendix, Section 7.1). The cortical coordinates for Et-1 injection developed by the Corbett lab correspond to the forelimb representation in the primary motor cortex for most animals. Slight differences in vasculature among individuals contribute to the variation in forelimb deficit observed and also generate more of a deficit in the hindlimb than forelimb in some animals due to the proximity of this motor map to the targeted area.

1.8.1 Behavioural Assays

The rat model of stroke lends itself well to behavioural assessment following injury and experimental treatment owing to the intelligence, size and easy handling of the animal [153]. Among the most common rat behavioural assays for stroke studies are the Montoya staircase task for forelimb function, the tapered beam task for hindlimb function, the cylinder task for spontaneous forelimb use and the adhesive removal test for tactile responses [161]. The Montoya staircase task is the gold standard for assessing forelimb function and dexterity, requiring the animal to reach, grasp, and bring a food pellet to its mouth. The animal must first be trained to reach for these pellets, located to either side of the platform on which the animal’s body rests. The pellets are distributed on staircase inserts that vary the degree of difficulty associated with retrieval and isolate the forelimbs, allowing the assessment of performance on each side independently. Though time-consuming in terms of training, this assay offers the advantage of immediate data collection upon completion of the testing as the number of pellets is used to score the animal’s performance. With the tapered beam task, animals are trained to walk along the top platform of a beam that narrows at the far end. Hindlimb slips off of the top platform, onto the ledges that line the sides of the beam are counted as the animal runs in a series of trials. This task only requires 2-3 days of training to acclimatize the animal to the task, but scoring the videos of the trials after the fact is time-intensive. Forelimb exploration inside of a Plexiglas cylinder gives another option for measuring forelimb function, as an uninjured animal will tend to rear up and press against the cylinder with both forelimbs equally. Preferring one forelimb to the other is
therefore indicative of a deficit and is used as alternative or additional forelimb function assessment. With the adhesive removal task, the animal’s ability to sense a piece of tape on it’s forepaw, as well as it’s ability to remove the adhesive are measured and provide a means of scoring sensorimotor function.

1.9 Hypothesis and Objectives

1.9.1 Hypothesis

Sustained local delivery of brain-derived neurotrophic factor to the stroke-injured rat brain from a PLGA-HAMC composite will promote synaptic plasticity and functional recovery.

1.9.2 Objectives

1. Design a PLGA-HAMC formulation to achieve sustained delivery of bioactive BDNF

   1.1 Investigate the mechanism of release for BDNF loaded into a PLGA-HAMC composite.

   1.2 Tune the release of BDNF from the PLGA-HAMC composite such that it is sustained out to 28 days in vitro.

   1.3 Assess the bioactivity of released BDNF using a DRG neurite outgrowth bioassay.

2. Investigate the effect of locally delivered BDNF on the functional recovery and synaptic plasticity of stroke-injured rats.

   2.1 Test the diffusion of BDNF into the stroke-injured rat brain from the PLGA-HAMC composite

   2.2 Assess the functional recovery and synaptic plasticity of rats treated with the sustained local delivery of BDNF.
2 Encapsulation-Free Controlled Release

This chapter has been peer-reviewed and published as:


This paper is also included in the PhD theses “Combined Delivery of Chondroitinase ABC (chABC) and Stromal-Derived Factor 1α (SDF1α) for Spinal Cord Regeneration” by Malgosia Pakulska and “Controlled Delivery of Therapeutic Proteins to the Injured Spinal Cord” by Irja Elliott Donaghue.

Reprinted with permission from AAAS. J.M.O., M.P, I.E.D. and M.S.S. wrote and edited the manuscript. J.M.O performed all experimentation involving BDNF including formulation and synthesis of composite components, M.P. performed all experimentation involving SDF, I.E.D. performed all experimentation involving NT-3. A.T. performed experimentation and wrote methods for EPO release from the composite. C.K.M. performed experimentation and wrote methods for TEM imaging of nanoparticles.

2.1 Abstract

Encapsulation of therapeutic molecules within polymer particles is a well-established method for achieving controlled release, yet challenges such as low loading, poor encapsulation efficiency, and loss of protein activity limit clinical translation. Despite this, the paradigm for the use of polymer particles in drug delivery has remained essentially unchanged for several decades. By taking advantage of the adsorption of protein therapeutics to poly(lactic-co-glycolic acid) (PLGA) nanoparticles, we demonstrate controlled release without encapsulation. In fact, we obtain identical, burst-free, extended-release profiles for three different protein therapeutics with and without encapsulation in PLGA nanoparticles embedded within a hydrogel. Using both positively and negatively charged proteins, we show that short-range electrostatic interactions between the proteins and the PLGA nanoparticles are the underlying mechanism for controlled release. Moreover, we demonstrate tunable release by modifying nanoparticle concentration, nanoparticle size, or environmental pH. These new insights obviate the need for encapsulation
and offer promising, translatable strategies for a more effective delivery of therapeutic biomolecules.

2.2 Introduction

Controlled biomolecule release is a key strategy for reducing both the systemic side effects associated with high drug concentrations and the frequency of drug administration. One of the most widely studied methods for controlling biomolecule release is encapsulation within a polymeric matrix, which slows diffusion. PLGA is widely used for encapsulation because of its biocompatibility, biodegradability, wide range of degradation rates, and long clinical history [162][163]. PLGA is typically formulated into injectable, protein-containing microparticles or nanoparticles by double-emulsion solvent evaporation, phase separation, or spray-drying techniques [164]. All of these methods involve organic solvents, high shear forces, and/or high temperatures that result in protein denaturation and aggregation [165]. In addition, PLGA protein formulations often suffer from low encapsulation efficiency and low protein loading [166][167]. The modification of process parameters—such as solvent type, volume [168][169], or co-encapsulation with various excipients such as poly(ethylene glycol) [170], sugars [152], or bases [171]—can improve encapsulation efficiency and protein stability, yet, the requirement for encapsulation to achieve sustained release remains unchanged. Embedding PLGA nanoparticles (PLGA np) within a hydrogel enables their localization at the site of hydrogel injection [172][173], and is known to reduce the initial burst and to extend the release of encapsulated proteins [152] [174].

While studying the release of three growth factors relevant to CNS repair strategies [stromal cell–derived factor 1a (SDF1a; hereafter referred to as simply SDF), neurotrophin-3 (NT-3), and brain-derived neurotrophic factor (BDNF)], we found that their release profiles were independent of encapsulation. Sustained, burst-free release profiles were obtained whether the proteins were encapsulated in PLGA np that were then dispersed within a hydrogel (Fig. 3A) or simply mixed into the identical hydrogel with blank PLGA np (Fig. 3B). To the best of our knowledge, this is the first study of PLGA np being used for long-term controlled release without encapsulation. We hypothesize that adsorption due to short-range electrostatic interactions between the proteins and the PLGA np is the governing factor for release in this system, and we
tested this hypothesis by controlling the release through nanoparticle concentration, nanoparticle size, and pH.

**Figure 3.** Two different PLGA np systems are compared for controlled protein release. (A) Protein encapsulated in PLGA np dispersed in a hydrogel. (B) Protein and blank PLGA np dispersed in a hydrogel. For the latter, protein adsorbs to the PLGA np but is not encapsulated with-in them.

### 2.3 Materials and Methods

PLGA was purchased from Sigma-Aldrich (50:50 lactide/glycolide, carboxy-terminated, molecular mass of 7 to 17 kD), and LACTEL Absorbable Polymers were purchased from Durect Corp. (50:50 lactide/glycolide, carboxy-terminated, 0.15 to 0.25 dl/g in hexafluorisopropanol). Proteins were sourced as follows: SDF (R&D Systems), NT-3 (PeproTech), BDNF (PeproTech), and EPO (Janssen). Enzyme-linked immunosorbent assay (ELISA) kits for SDF and NT-3 were purchased from R&D Systems, and those for BDNF were purchased from Promega. All other materials were obtained as indicated.

#### 2.3.1 PLGA np formation

PLGA np were formed by water/oil/water double-emulsion solvent evaporation, as previously described by Baumann et al. [175]. Briefly, 120 mg of PLGA and 0.05% Pluronic NF-127 (BASF) were dissolved in 900 ml of dichloromethane (DCM; Caledon Laboratories) and
vortexed with 100 ml of bovine serum albumin (BSA; 120 mg/ml; Sigma Aldrich) in aCSF [149 mM NaCl, 3 mM KCl, 0.8 mM MgCl2, 1.4 mM CaCl2, 1.5m M Na2HPO4, and 0.2 mM NaH2PO4 (pH7.4)] to achieve 10% w/w BSA/PLGA in the final formulation. The mixture was then sonicated for 2 min on ice. For encapsulation of SDF, NT-3, BDNF, or EPO, these proteins were dissolved in the aqueous phase before sonication. The primary emulsion was then added to 3ml of a 2.5% (w/v) **solution of poly(vinyl alcohol) (30 to 70 kg/mol; Sigma Aldrich), vortexed, and sonicated for 2 min on ice. This secondary emulsion was added to a hardening bath of poly(vinyl alcohol) (PVA) and stirred for a minimum of 4 hours to allow the DCM to evaporate. The resultant nanoparticles were washed four times by ultracentrifugation, lyophilized, and stored at −20°C. Nanoparticles were sized by DLS (Malvern Instruments). For 1000-nm nanoparticles, the volume of PVA added in the secondary emulsion was increased to 5 ml.

2.3.2 Measurement of encapsulation efficiency

2.3.2.1 Stromal cell–derived factor 1α.

Encapsulation efficiency was measured by dissolving 10 mg of dry protein-loaded PLGA np in 1 ml of dimethyl sulfoxide for 1 hour at 37°C. Ten milliliters of 0.05MNaOH containing 0.5% (w/v) sodium dodecyl sulfate was then added, and the sample was left for another hour at room temperature on a rocker. SDF concentration was then measured by ELISA at a 1:10 dilution as directed.

2.3.2.2 BDNF/NT-3.

Encapsulation efficiency was measured by dissolving 5 mg of dry protein-loaded PLGA np in 5 ml of 0.05 M NaOH on a shaker at room temperature for 30 min. The protein concentration was determined by ELISA.

2.3.3 Preparation of PLGA np/hydrogel DDS

2.3.3.1 Composite HAMC.

To form the HAMC hydrogel, we dissolved methylcellulose (300 kg/mol; Shin-Etsu) and sodium hyaluronate (HA; 1.4 to 1.8 × 106 g/mol; NovaMatrix) in aCSF, using a dual asymmetric centrifugal mixer (FlackTek), to a final concentration of 2.8% (w/v) HA and 6% (w/v)
methylcellulose. Nanoparticles were dispersed in aCSF at 20% (w/w) by 5 min of bath sonication, and protein (NT-3, BDNF, or EPO) was then added. Composite HAMC was formed by blending the nanoparticle dispersion and HAMC at a 1:1 ratio using a dual asymmetric centrifugal mixer.

### 2.3.3.2 Composite XMC.

XMC was prepared as previously described [176], except that empty PLGA np dispersed in aCSF and SDF were added before mixing in the cross-linker.

### 2.3.3.3 Composite agarose.

Ultralow gelling temperature SeaPrep Agarose (Lonza) was dissolved in aCSF to a concentration of 3% (w/v) by microwaving on high power in 4-s bursts and vortexing until clear. The solution was allowed to cool to room temperature before further handling. Nanoparticles were dispersed in aCSF at 20% (w/w) by 5 min of bath sonication, and BDNF was then added. Room temperature agarose was added to the nanoparticle dispersion at a 1:1 volume ratio, and a dual asymmetric centrifugal mixer was used to create the composite agarose. One hundred microliters of composite agarose was pipetted into a 2-ml centrifuge tube and allowed to gel at 4°C for 1 hour.

### 2.3.4 In vitro release assays

#### 2.3.4.1 Composite HAMC/XMC.

One hundred microliters of HAMC, XMC, composite HAMC, or composite XMC, with or without dispersed protein, was injected into a 2-ml centrifuge tube. After 10 min of gelation at 37°C, 900 ml (for release of BDNF and NT-3) or 400 ml (for release of SDF) of pre-warmed aCSF was added. For BDNF releases, the aCSF also contained 0.1% (w/v) BSA to ensure sustained bioactivity. The supernatant was completely replaced at designated time points for the duration of the release study, and the protein concentration was assessed by the relevant ELISA. At the conclusion of the study, the remaining gel was processed as described above for encapsulation efficiency, and the remaining protein was quantified by ELISA. Releases were performed in triplicate. For release of SDF at different pH values, the pH of the aCSF was adjusted to pH 5 or pH 3 and used in both the gel formation and the supernatant.
2.3.4.2 Agarose/agarose with high salt.

Agarose or agarose composite with or without dispersed BDNF, previously prepared in a 2-ml centrifuge tube, was placed in an incubator at 37°C to warm for 10 min. Nine hundred microliters of pre-warmed aCSF (0.1% w/v BSA) was added to the composite, and this supernatant was completely replaced at designated time points for the duration of the release study. For the high-salt agarose composite, aCSF with 0.5 M NaCl was used to create the gel and the release supernatant. At the end of the study, 0.5 ml of 0.5 M NaCl was added to the gel to release any remaining BDNF. ELISA was used to determine BDNF concentration at each time point and the BDNF remaining in the gel. Releases were performed in triplicate.

2.3.5 Adsorption study

Two micrograms of BDNF was mixed with 20mg of PLGA np in 200 ml of aCSF and incubated for 0 min (as fast as possible), 10 min, and 60 min at 37°C. At each designated time point, the mixture was centrifuged at 12,000 rpm for 2 min to pellet the nanoparticles. The supernatant was carefully removed and analyzed by ELISA. Controls of soluble BDNF in aCSF with no nanoparticles were processed in the same way at each time point to take into account any losses resulting from centrifugation or adsorption to the tube. Samples of PLGA np in aCSF with no BDNF were used as blanks for the ELISA. The amount of BDNF adsorbed to the nanoparticles was calculated as 2 mg minus the amount detected in the supernatant. Each time point was performed in triplicate.

2.3.6 Bioactivity assays

All animal procedures were performed in accordance with the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care), and protocols were approved by the Animal Care Committee at the University of Toronto.

2.3.6.1 NT-3: Dorsal root ganglia explant assay.

The bioactivity of released NT-3 was quantified as previously described by Stanwick et al. [152]. Sprague-Dawley rat embryo dorsal root ganglia (DRG; E17) were harvested and pooled in neural basal media containing 1 volume% fetal bovine serum, 2 volume % B-27 serum-free supplement, 1 volume % penicillin-streptomycin, and 1 volume % L-glutamine. The DRG were placed on glass coverslips (12 mm in diameter) coated with poly-D-lysine (50 mg/ml
in sterile water) and laminin (5 mg/ml in phosphate-buffered saline) in a 24-well plate. Media with aCSF alone (control media), media with soluble (standard) NT-3, or media with released NT-3 were added to each well. The DRG were fixed after a 48-hour incubation in 4% paraformaldehyde and stained with NF200 and 4′,6-diamidino-2-phenylindole (DAPI; Sigma Aldrich). DRG were imaged on an Olympus FV1000 confocal microscope. Image analysis was performed with ImageJ (National Institutes of Health). For each DRG, the DAPI-positive area of the multicellular DRG body was subtracted from the total NF200-positive area (center + extended neurites) to yield the area of neurite outgrowth. A minimum of three DRGs were assayed per group.

2.3.6.2 SDF: Neurosphere migration assay.

In vitro activity of SDF was quantified as previously described [176]. Briefly, 125 ml of SDF release samples or controls was diluted to 2000 ng/ml (or as high as possible) in serum-free media and adjusted to pH 7. Each was then added to 125 ml of adult rat spinal cord neurosphere suspension in a fibronectin-coated 48-well tissue culture plate. Neurospheres were allowed to adhere for 1 hour in a tissue culture incubator at 37°C and 5% CO2. Images of four neurospheres per well were collected using an inverted Olympus laser scanning confocal microscope with automated stage at ×10 magnification. The coordinates of each image were saved. The plate was then returned to the incubator. Using the saved coordinates, we again imaged the same neurospheres 24 hours later. Images were analyzed using ImageJ (National Institutes of Health). The initial area of the neurosphere (A1) and the final area of cells migrated out from the neurosphere (A2) were measured and subtracted to yield the total area of migration (A2 - A1).

2.3.6.3 BDNF: DRG neurite outgrowth assay.

In vitro activity of BDNF was determined using a DRG bioassay. Rat embryo DRG (E17 female Sprague-Dawley rats, n = 3) were removed and pooled in media composed of neural basal media supplemented with 2% B-27 serum-free supplement, 1% penicillin-streptomycin, and 1% L-glutamine (Life Technologies). The DRG were then placed on 12-mm-diameter glass coverslips coated with poly-D-lysine (50 mg/ml) and laminin (5 mg/ml) in a 24-well plate. Each release sample replicate was tested on a separate plate. All wells contained three DRG and were treated with 0.5 ml of media and 0.5 ml of the BDNF release study supernatant (7, 28, 35, and 42 days). For the controls, 0.5 ml of media and 0.5 ml of aCSF (without BDNF) were added to the
wells. The DRG were grown for 48 hours at 37°C and 5% CO2, fixed with 4% paraformaldehyde, and processed for immunocytochemistry. The DRG were imaged using an inverted confocal laser scanning microscope (Olympus FV1000). Neurite outgrowth area was calculated by subtracting the cell body area from the total area of the DRG neurite outgrowth. To account for any differences in cell body size, we standardized the neurite outgrowth area to the cell body area for statistical analysis. Neurite outgrowth of DRG treated with release samples was compared to controls to assess bioactivity.

2.3.7 Transmission electron microscopy

PLGA np were suspended in water at 2 mg/ml with sonication for 5 min. A sample (5 ml) was deposited onto a freshly glow-discharged 400-mesh carbon-coated copper TEM grid (Ted Pella Inc.) and allowed to adhere for 4 min. Excess liquid was removed with filter paper, and nanoparticles were then stained with 2% uranyl acetate (UA, w/v, 5 mL, pH 4.3, 15 seconds). Stain was wicked away with filter paper, and samples were immediately imaged using a Hitachi H-7000 microscope operating at 75 kV. Images were captured using an Advanced Microscopy Techniques XR-60 CCD camera with typical magnifications between ×30,000 and ×75,000.

2.3.8 ζ potential measurements

PLGA np were suspended at 0.1 mg/ml in distilled water with 1 mM KCl. ζ potential was measured in a disposable folded capillary cell using a Zetasizer Nano ZS (Malvern Instruments).

2.3.9 Swelling study

An in vitro swelling study was performed as previously described [176]. Briefly, 180 ml of HAMC or composite HAMC was injected into a 2-ml centrifuge tube. After incubation at 37°C for 10 min, 1620 ml of prewarmed aCSF was added. At each time point, the tube was weighed after complete removal of the aCSF. The normalized swelling ratio, Q, was determined by the equation

\[ Q = \frac{m_t - m_{tube}}{m_0 - m_{tube}} \]

where \( m_t \) is the total mass at each time point, \( m_0 \) is the total mass at time 0, and \( m_{tube} \) is the mass of the centrifuge tube.
2.3.10 Statistical analysis

Statistical analysis was performed using GraphPad Prism software. For all tests, *P < 0.05, **P < 0.01, and ***P < 0.001. Releases were analyzed using two-way analysis of variance (ANOVA), followed by the Sidak test for multiple comparisons if there were two groups or Tukey’s test for multiple comparisons if there were more than two groups. Activities of NT-3, SDF, and BDNF were analyzed using one-way ANOVA followed by Dunnett’s post hoc test comparing them to controls.

2.4 Results

2.4.1 Sustained release without encapsulation

We tested the release of three proteins from two hydrogels with and without embedded PLGA np: SDF from a click cross-linked methyl cellulose (XMC) hydrogel [176] (Fig. 4A), and NT-3 (Fig. 4B) and BDNF (Fig. 4C) from a physical blend of hyaluronan and methylcellulose (HAMC) [177]. The diffusional release of SDF, NT-3, and BDNF from the hydrogel alone is rapid, generally reaching completion by day 2. By encapsulating the proteins in PLGA np before mixing them into the hydrogel (composite XMC and composite HAMC), we achieved sustained release for at least 28 days with no burst; however, we observed a 4- to 7-day delay in the release. Encapsulation efficiencies in PLGA np of 49 ± 4%, 47 ± 2%, and 47 ± 7% were achieved for SDF, NT-3, and BDNF, respectively. In an effort to overcome this delay, we mixed additional proteins directly into the hydrogel with the protein-loaded PLGA np. We anticipated an initial burst release followed by a sustained release, reflecting fast diffusion from the hydrogel and slower diffusion from the nanoparticles. Instead, we observed an identical release profile, with only the total amount of released proteins increased (Appendix, Section 7.2) [178]. We also observed a similar release profile when all of the proteins were simply mixed into the hydrogel with blank PLGA np (Fig. 4, A to C). All proteins remained bioactive when released using this encapsulation-free method (Appendix, Section 7.3), with all of the proteins incorporated into the system being available for release. This overcomes the <50% efficiency obtained with PLGA np encapsulation.
Figure 4. Controlled sustained release of positively charged proteins from PLGA np does not require encapsulation. (A to C) Three different proteins show nearly identical release profiles whether encapsulated within PLGA np or simply mixed with blank PLGA np in a hydrogel: (A) SDF (pI 10.9, molecular mass of 8 kD, 200 ng/release), (B) NT-3 (pI 9.5, molecular mass of 27 kD, 1000 ng/release), and (C) BDNF (pI 10.9, molecular mass of 27 kD, 300 ng/release). The cumulative percentage released is significantly greater for soluble proteins with no PLGA np compared to soluble proteins with blank PLGA np or proteins encapsulated in PLGA np at all time points (P <0.05, n = 3 for all releases, mean ± SD plotted). Asterisks indicate significant differences between release of soluble protein with PLGA np and release of protein encapsulated in PLGA np (*P <0.05, **P < 0.01, ***P < 0.001). Curves for soluble SDF with no PLGA np and SDF encapsulated in PLGA np were taken with permission (15). (D) EPO (pI ~4, molecular mass of 30 kD, 84 ng/release) shows no attenuated release when mixed into HAMC with PLGA np versus just HAMC alone (without PLGA np), indicating that the phenomena observed with SDF, NT-3, and BDNF are based on short-range electrostatic interactions between positively charged proteins and negatively charged PLGA (n =3, mean ± SD plotted).

2.4.2 Adsorption governed by electrostatic interactions

To understand the release mechanism, we examined the physical characteristics of this encapsulation-free release system. Nanoparticle morphology and size were characterized by transmission electron microscopy (TEM) and dynamic light scattering (DLS). Nanoparticles
were spherical, with some visible surface morphology, an average diameter of $293 \pm 19$ nm, and a polydispersity index of $0.21 \pm 0.02$ by DLS (Appendix, Section 7.4). $\zeta$ potential measurements showed that the surface of the PLGA np was negatively charged ($-14.0 \pm 0.7$ mV), in agreement with previous reports [179][180]. The swelling ratio of HAMC was compared to that of composite HAMC (that is, PLGA np dispersed in HAMC). Composite HAMC had a significantly higher swelling ratio after 3 days compared to HAMC alone (Appendix, Section 7.5).

SDF, NT-3, and BDNF all have a net positive charge at physiological pH, with an isoelectric point (pI) of 10.9 for SDF, 9.5 for NT-3, and 10.1 for BDNF [181]. We postulated that the delayed release that we observed was primarily caused by adsorption due to short-range electrostatic interactions between the protein and the PLGA np. To test this hypothesis, we examined the release of erythropoietin (EPO)—another protein that has shown benefits for CNS repair strategies [143] and has a pI of 3.3 to 4.3 [182], making it negatively charged at physiological pH. Soluble EPO was released just as rapidly from composite HAMC as from HAMC alone, with complete release observed after 2 days (Fig. 4D). This is consistent with our hypothesis that adsorption is mediated by electrostatic interactions.

The hydrogel itself seems to have a minimal role in controlling release because the release of SDF from XMC and that of NT-3 and BDNF from HAMC show similar profiles. We examined this further by replacing both XMC and HAMC with another common gel, agarose. Release of soluble BDNF from agarose alone was the same as that from HAMC alone, whereas release of soluble BDNF from agarose containing PLGA np exhibited the same delayed-release profile that we observed with HAMC containing PLGA np (Fig. 5A). Incubation of BDNF with free PLGA np in artificial cerebrospinal fluid (aCSF) at the same protein/PLGA ratio as in the hydrogels resulted in almost complete adsorption within minutes (Fig. 5B), suggesting that the mechanism for controlled release is mediated by the nanoparticles, not the hydrogel.

Adsorption mediated by electrostatic interactions can be disrupted by raising the level of competing ions in solution. When the salt concentration of the release media was raised from 149 mM (physiologically relevant) to 0.5 M, the release of BDNF from the agarose PLGA np composite was fast and almost identical to the release profile observed in the absence of the nanoparticles (Fig. 5C).
Figure 5. Sustained release of BDNF from agarose containing PLGA np is disrupted by increased salt. (A) BDNF shows the same delayed- and sustained-release profile from agarose containing PLGA np as from HAMC with PLGA np while diffusional release from agarose alone is still fast. (B) Almost all soluble BDNF is adsorbed to PLGA np after incubation, even at short times. (C) NaCl (0.5M) completely disrupts the interaction of BDNF with PLGA np, resulting in purely diffusional release (P >0.05 for all timepoints, n = 3 for all releases, mean ± SD plotted).

2.4.3 Tuning release by controlling the surface area of PLGA np

To further investigate the role of PLGA np in controlling release, we examined the role of PLGA np surface area because the amount of protein adsorbed is proportional to the available surface area. We investigated the release of BDNF from composite HAMC containing either 300-nm- or 1000-nm-diameter nanoparticles. Assuming similar densities and spherical nanoparticles, the 1000-nm nanoparticles would have three times less available surface area than the 300-nm nanoparticles for the same mass (see calculations in the Appendix, Section 7.6). Consistent with our hypothesis that the interaction between PLGA np and positively charged proteins controls release, the larger nanoparticles (with the overall lower surface area) resulted in
faster release (Fig. 6A). The available PLGA np surface area can similarly be controlled by the concentration of nanoparticles within the gel. We observed a direct correlation between the number of PLGA np dispersed in the hydrogel and the release rate of NT-3 from composite HAMC. With the lowest concentration of nanoparticles (0.1 wt %; Fig. 6B, i), there was a faster initial release rate that approached the diffusion-controlled release profile of NT-3 from HAMC alone (0 wt %; Fig. 6B, i). As the concentration of nanoparticles increased, the release rate slowed. We were able to increase the concentration of NT-3 by 20-fold (from 0.5 to 10 mg) in the 10 wt % PLGA np composite while maintaining a virtually identical release profile (Fig. 6B, ii). Thus, we have a facile method to control both the release rate and the amount of protein released.

**Figure 6.** Release of positively charged proteins from composite HAMC can be tuned by changing the available nanoparticle surface area. (A) Cumulative percentage release of BDNF from composite HAMC is higher with 1000-nm-diameter PLGA np than with 300-nm-diameter PLGA np while keeping PLGA np mass constant. (B) (i) Release of NT-3 from composite HAMC with 0, 0.1, 0.5, 1, and 10 wt % PLGA np. Cumulative percentage of NT-3 released is significantly lower for 10 wt % PLGA np than for all other curves at t >1day (P < 0.05). Cumulative percentage of NT-3 released is significantly higher with 0 wt % PLGA np than with
0.1 wt % (t = 1 day, P < 0.05), 0.5 wt % (1 day ≤ t ≤ 3 days, P < 0.05), and 1 wt % (t > 3 hours, P < 0.05). (ii) Release of 0.5, 1, or 10 mg of NT-3 from composite HAMC with 10 wt % PLGA np. The concentration of NT-3 incorporated can be increased up to 20 times with virtually no change in release profile. Cumulative percentage of NT-3 released is significantly higher for 10 mg than for 1 and 0.5 mg at 10 and 14 days (P < 0.05) and is significantly lower for 0.5 mg than for 1 and 10 mg at 21 and 28 days (P < 0.05) (n = 3 for all releases, mean ± SD plotted).

2.4.4 Tuning release by modifying adsorption affinity

Because adsorption can be controlled by modifying the interaction strength between the proteins and the PLGA np, we next examined the role of supernatant pH in protein release from our drug delivery system (DDS), as supernatant pH would affect both the charge of the protein and that of the PLGA np surface. SDF was released from composite XMC into media at pH 3, 5, and 7. The release rate of SDF from composite XMC (that is, in the presence of nanoparticles) significantly increased with decreasing pH (Fig. 7A); yet, in the absence of nanoparticles, the release rate of SDF was unaffected by decreasing pH (Appendix, Section 7.7). Moreover, the difference in release cannot be explained by destabilization of the gel at lower pH, because there was no significant mass loss of PLGA from the hydrogel over the first 7 days of release at either pH 3 or pH 7 (Appendix, Section 7.8). Thus, these data indicate that pH can be used to control release and that, at lower pH (where the carboxylate anions of PLGA are protonated), the electrostatic interaction between PLGA anions and protein cations is diminished. To investigate the effects of local pH on protein release, we compared the release of BDNF from composite HAMC containing PLGA np with and without encapsulated MgCO₃. Because PLGA is known to degrade into acidic products by bulk degradation, the encapsulation of basic salts has been used to delay pH changes within PLGA np [171]. Release of soluble BDNF from composite HAMC had a delay of approximately 7 to 10 days with encapsulated MgCO₃ (Fig. 7B). In contrast, in the absence of MgCO₃, the delay was shortened to just 4 days. This suggests that the mechanism for protein release is related to a change in PLGA charge from negative carboxylate anions to neutral carboxylic acids, which is slowed in the presence of MgCO₃.
Figure 7. Release of positively charged proteins can be tuned by changing the environmental pH. (A) Release of SDF from composite XMC into media at pH 3, pH 5, or pH 7. Cumulative percentage release of SDF is significantly greater at pH 3 than at pH 5 (t <10 days, P <0.01) and pH 7 (t > 0 days, P < 0.0001), likely because PLGA carboxylate anions are protonated to carboxylic acids, thereby reducing electrostatic interactions with positively charged proteins. (B) Release of BDNF from HAMC with PLGA np with or without encapsulated magnesium carbonate (MgCO₃). Release is delayed with encapsulated MgCO₃ (a basic salt), which neutralizes acidic degradation products and thereby maintains a higher/neutral local pH (n = 3 for all releases, mean ± SD plotted).

### 2.5 Discussion

Models of drug release from PLGA np usually consider polymer degradation, erosion, and diffusion of the drugs through the resulting water-filled pores as rate-determining [162] [183]. However, our results show very similar release profiles for three different proteins whether they are encapsulated in PLGA np or simply mixed with them, indicating that another mechanism must be involved. We postulate that initially the protein is fully adsorbed to the negatively charged nanoparticle surface (Fig. 8, i). As the nanoparticle begins to degrade, acidic components build up and decrease the local pH (Fig. 8, ii). At a certain threshold, the original negative carboxylate anion on the nanoparticle surface becomes protonated to carboxylic acid, thereby weakening the electrostatic interactions with the positively charged proteins and initiating release (Fig. 8, iii). Release can then be governed by nanoparticle degradation and protein diffusion and/or adsorption/desorption, depending on the system.
Figure 8. Adsorption may be rate-limiting for the release of positively charged proteins from PLGA np. (i) Initially, the protein is fully adsorbed to the negatively charged nanoparticle surface. (ii) As the nanoparticle begins to degrade, acidic components build up and decrease the local pH. (iii) At a certain threshold, the nanoparticle surface becomes neutral, weakening the electrostatic interactions with the positively charged proteins and initiating release.

It is possible that the presence of the PLGA np affects the release of proteins simply by changing the gel mechanics. Methylcellulose forms a gel via hydrophobic interactions, and its shear modulus increases upon incorporation of PLGA np, suggesting additional hydrophobic interactions between methylcellulose and PLGA np [172]. These hydrophobic interactions have been postulated to affect the release rate of proteins from PLGA np [152]. However, our results show that the addition of PLGA np actually increases the hydrogel swelling ratio, likely as a result of the presence of carboxylate anions on the PLGA np surface. Increased swelling of poly(acrylic acid) gels with the addition of polystyrene nanoparticles has been attributed to the sulfonic acid groups on the nanoparticle surface [184], and carboxylate anions have previously been found to affect the swelling of hydrogels. Increased swelling should lead to an increased mesh size and subsequently increased release rate of a protein [185]; however, our data show the opposite effect, indicating an interaction between PLGA np and the protein. In addition, changes in mesh size would be expected to affect all proteins, depending on their size; however, EPO,
which has a molar mass larger than that of SDF and similar to those of NT-3 and BDNF, does not show sustained release in the presence of PLGA np. Thus, gel mechanics are unlikely the mechanism controlling the release of proteins from composite PLGA np hydrogels.

Adsorption is recognized as one of the factors affecting the release of biomolecules encapsulated in PLGA particles [186] and is a major cause of incomplete release [187] [188]. Adsorption of proteins at the surface of PLGA particles is also often cited as the cause of the initial burst observed in the release from PLGA np formulations [189] and is attributed to a combination of electrostatic and hydrophobic interactions [179] [190]. The release of proteins adsorbed to the surface of PLGA particles is often fast (within the first 1 to 8 hours of release) and has not been fully studied [179] [191].

To examine the possibility of a hydrogel effect, we tested release from agarose, an uncharged polymer with a mechanism of gelation [192] different from that of either XMC [176] or HAMC [177] [193]. The release profiles of BDNF were the same whether from composite HAMC or composite agarose, suggesting a specific interaction between the protein and the PLGA np, not the hydrogel. When we incubated BDNF with PLGA np alone at the same ratio used in the release studies, almost all the BDNF was removed from the supernatant within minutes, demonstrating a strong interaction with the PLGA np. Adsorption is governed by noncovalent interactions, including electrostatic, hydrophobic, and van der Waals forces. Given that SDF, NT-3, and BDNF are all positively charged at neutral pH, and a negatively charged protein (EPO) did not show the same release behavior, we hypothesized that electrostatic interactions were governing protein adsorption. The range of electrostatic interactions is governed by the Debye length. For our release system, the Debye length is very small (<1 nm), so long-range electrostatic interactions are not important; however, short-range interactions likely still exist. Electrostatic interactions such as these can be disrupted with an excess of ions. In fact, BDNF is often purified using ion exchange chromatography, and a salt concentration of 0.5 M is within the range used for elution [194] [195]. When we examined the release of BDNF from composite agarose in the presence of 0.5 M NaCl, the release rate approached diffusional values. This supports a hypothesis of predominantly electrostatically controlled adsorption to the PLGA np, with little contribution from hydrophobic and van der Waals forces [196].
Control over the release rate is essential for the success of sustained delivery formulations. We explored different parameters of our DDS and their effects on the release rate of soluble proteins. Models of drug release controlled by adsorption/desorption demonstrate that release is governed by the number of available binding sites and the strength of the interaction [179] [197]. Nanoparticle size can be readily varied by modifying the formulation conditions [165] and is therefore a facile method to control release. Assuming a similar density, smaller nanoparticles provide an overall larger surface area and would therefore increase the extent of adsorption. We show that BDNF released from HAMC with 300-nm nanoparticles showed a longer delay and slower release than that from HAMC with 1000-nm nanoparticles. Because protein loading and nanoparticle concentration are decoupled in our DDS, changing the nanoparticle concentration provides a simple method to tune release. In general, release of NT-3 is faster with lower nanoparticle concentrations. When the nanoparticle concentration is decreased from 10 to 1 wt % with a constant amount of protein, a significant burst release is seen. On the other hand, increasing the protein concentration 10-fold while maintaining the nanoparticle concentration at 10 wt % does not change the release profile of NT-3 despite an identical nanoparticle/protein mass ratio as the 1 wt % case above. A similar effect is seen in affinity-controlled release systems: as long as the available binding sites are in excess, the release rate depends on the concentration of binding ligand (here the PLGA np), not on the binding ligand/protein ratio [198].

Theoretically, complete surface coverage of our PLGA np occurs at 1.41 wt % protein loading (Appendix, Section 7.6). When increasing protein concentration, we never exceeded 0.1 wt % protein loading by mass because of the significant costs of these proteins; however, on the basis of our calculations, we expect that a 1–wt % loading could be achieved without significantly altering the release profile. This suggests that our DDS could deliver substantially higher loadings than previously reported for PLGA encapsulation of therapeutic proteins such as NT-3 [199], BDNF [140], [200], [201] or nerve growth factor [202][203]. PLGA np lose their negative charge at or below pH 5 [180], thus decreasing their charge attraction to positively charged proteins. We observed increased release rates of SDF at pH 3 and 5 compared to pH 7. This is not attributable to gel destabilization at low pH because there is no significant mass loss of PLGA np over 7 days at pH 3 or pH 7. The ability to increase the release rate at acidic pH may have interesting implications for in vivo delivery at acidic sites, such as the tumor
microenvironment [204]. In all of the release profiles, a constant, slow release phase was observed after the initial delay or burst. PLGA degrades by bulk degradation into acidic components, which are often trapped within the nanoparticles before their dissolution, decreasing the pH inside the nanoparticles [171] [188]. We postulate that this reduction in local pH reduces the magnitude of the protein-polymer interaction (as shown in our pH study) and initiates protein release as a certain pH threshold is reached. Thus, by controlling the pH change within the PLGA np, it should be possible to eliminate or extend this delay. Indeed, encapsulation of a basic salt, MgCO$_3$, slowed the release of BDNF from PLGA np dispersed in HAMC, likely by decreasing the rate of PLGA degradation and pH change within the nanoparticles. Similarly, PLGA polymers with different properties (molecular weight, end group, and lactide/glycolide ratio) could also be used to formulate nanoparticles with various degradation rates to achieve different release profiles. Although we cannot rule out the possibility that the polymer degradation, irrespective of the pH change, decreases the affinity of the protein for the nanoparticles, previous studies have shown that the available surface area for adsorption actually increases as the nanoparticles degrade, which would likely result in increased adsorption and slower release [187]. Therefore, it seems more likely that polymer degradation indirectly affects protein release through pH change.

### 2.6 Conclusion

Using PLGA for the release of bioactive proteins without encapsulation is a paradigm shift in controlled delivery, and the use of the well-established PLGA facilitates clinical translation. Furthermore, controlled release is obtained without any modifications to the protein that may affect activity. Tunable release is achieved by varying simple formulation parameters, and stimuli-responsive release is demonstrated by modifying environmental pH. The next step is the extension of these concepts to additional proteins. Although we show similar release profiles for three different proteins, not all proteins can be expected to interact in the same way. A simple adsorption experiment could quickly ascertain whether this method is suitable for a given protein. Polymer composition (for example, end group and lactide/glycolide ratio) might also be tailored to suit a particular protein. Although not explored herein, for controlled release of negatively charged proteins, PLGA np could be coated with positively charged polymers, such as chitosan [205], or positively charged polymeric nanoparticles that degrade into basic components.
could be used instead. The results presented here provide the basis for a fundamental change in the way we use PLGA for protein delivery.
3  BDNF Delivery for Enhanced Plasticity and Recovery Following Stroke

This chapter was submitted for publication:


J.M.O. and M.S.S wrote and edited the manuscript. J.M.O. performed all experimentation and analysis of data. A.T. and S.L.P. contributed to experimental design and surgery execution. E.H. contributed to behavioural task training, testing and scoring. C.M.M. contributed to experimental design.

3.1 Abstract

We induced synaptic plasticity following ischemic stroke in rats by delivering brain-derived neurotropic factor (BDNF) directly to the brain. This protein is a potent modulator of plasticity in the developing and adult central nervous system; however, the therapeutic potential of BDNF has been largely thwarted by its inability to cross the blood brain barrier at an effective concentration. Herein, we demonstrate enhanced acute recovery of forepaw dexterity and enhanced hindlimb function at 7 weeks post-injury by delivering BDNF locally, with sustained release in vivo for up to 21 days. Using an encapsulation-free methodology, BDNF was dispersed in a hydrogel composed of hyaluronan and methyl cellulose (HAMC) with poly(lactic-co-glycolic acid) (PLGA) nanoparticles and this composite was deposited epi-cortically, directly above the stroke lesion. BDNF delivery augmented plasticity, as evidenced by synaptophysin staining in the contralesional hemisphere of BDNF-treated rats, and presence of the vehicle reduced the lesion volume and prevented neuron loss in peri-lesional tissue. With local, sustained delivery directly to the brain, we demonstrate the benefit of BDNF in the treatment of stroke injury in a rodent model.

3.2 Introduction

Traumatic neurological events, such as stroke, activate plastic processes in the brain to promote limited recovery [206]; yet, neurological deficits persist in the majority of stroke
survivors [27], [37], [207] due to the insufficiency of endogenous plasticity. It is hypothesized that enhancing neural plasticity would lead to improved patient recovery as a result of improved neural networks. BDNF is a key regulator of plasticity in the healthy and injured brain [7], [208], [209], initiating plasticity, cell survival and differentiation [61] upon binding to tyrosine receptor kinase B (TrkB). Studies linking increased BDNF expression to physical activity [69] and rehabilitation practices [210], [211] have established the importance of its presence during the recovery of motor function after stroke. Immediately following stroke, BDNF expression is upregulated both in the ipsilesional and contralesional brain hemispheres; however, this increase in expression is short-lived [212], suggesting an opportunity for delivery of exogenous BDNF for brain repair.

Ploughman et al. substantiated the role of BDNF in functional recovery by blocking BDNF expression following ischemic injury. Delivery of a BDNF antisense oligonucleotide to the contralesional hemisphere via infusion pump for 28 days, beginning 4 days post-injury, negated the beneficial outcomes of rehabilitation therapy that are normally observed [213]. A similar effect was observed when BDNF was blocked after injury with a TrkB-Fc-loaded hydrogel that sequestered the BDNF present at the lesion site [214]. In the context of learning and memory, there is mounting evidence that BDNF is responsible for the formation and maintenance of long-term potentiation (LTP), a strengthening of synapses that contributes heavily to memory formation and storage [59], [62]. Patterson et al. perfused recombinant BDNF in BDNF+/− mouse hippocampal slices and rescued LTP [215]. Taken together, these experiments show that BDNF is necessary for recovery and that exogenous BDNF can influence synaptic plasticity.

BDNF is unable to cross the blood-brain barrier (BBB) due to its large size and short half-life [216], [79]. This presents a significant challenge for the delivery of effective concentrations of BDNF to the peri-infarct tissue. Some groups have modified this protein with BBB targeting vectors [217], [218] while others have used intraventricular injections or infusions to bypass the BBB [84], [85]. Direct injection of BDNF into the lateral ventricle demonstrated some beneficial effects; however, multiple injections, high dosing or neural implants are usually required to maintain effective concentrations in the parenchyma and are highly invasive [219]. Transplantation of cells that express BDNF is another option [220], but cell delivery to the CNS is rife with challenges, including cell survival and/or uncontrolled proliferation, and requires
direct tissue injection [5]. Off-target side effects associated with systemic delivery, increased risk of infection and invasive procedures associated with tissue injection greatly limit the therapeutic efficacy of these approaches and call for a more refined methodology [221].

Controlled release strategies obviate the need for repeated bolus injections and local delivery to the brain circumvents the BBB. For BDNF delivery, polymeric nano/microparticles and hydrogels are most common and have yielded some notable outcomes, as reviewed by Geral et al. [208]. For example, Cook et al. injected a hyaluronan poly(ethylene glycol) crosslinked hydrogel into stroke-injured brain tissue to sustain the release of BDNF in mice over a period of 3 weeks. At 9 weeks post-injury, there was an increase in axonal sprouting in the contralesional striatum of BDNF-treated animals and some motor map reorganization [138]. These studies were performed in mice, making functional recovery challenging to assess [153]; however, the positive outcomes observed with BDNF delivery highlight the importance of developing a less invasive paradigm for delivery.

We developed a biomaterials-based strategy for local delivery of BDNF that is minimally invasive and allows sustained delivery of un-encapsulated, bioactive protein to the brain. Our delivery vehicle consists of a HAMC hydrogel in which we disperse PLGA nanoparticles. Instead of encapsulating BDNF in the nanoparticles to control its release, we simply mixed BDNF into the PLGA-HAMC composite, thereby formulating an easy and efficient system for in vivo application [142]. We deposited this BDNF-loaded composite on the surface of the cortex above the cortical stroke lesion, and tested the efficacy of this epi-cortical delivery strategy in a rat model of stroke in terms of synaptic plasticity, behavioural recovery and BDNF diffusion in stroke-injured rat brain tissue.

3.3 Materials and Methods

All reagents were acquired from Sigma-Aldrich (Oakville, ON, Canada), unless otherwise indicated.

3.3.1 PLGA nanoparticle fabrication

PLGA nanoparticles were synthesized by water/oil/water double emulsion-solvent evaporation, as previously described [142], in a laminar flow biosafety cabinet with sterile technique and materials. Briefly, 120 mg of PLGA (Lactel Absorbable Polymers, Durect
Corporation, 50:50 lactide:glycolide, carboxy terminated, 0.15-0.25 dL/g in HFIP), 4 mg of MgCO$_3$ and 0.05% Pluronic® NF-127 (BASF, Mississauga Canada) were dissolved in 900 µl dichloromethane (DCM) (Caledon Labs, Georgetown Canada). 100 µl of 120 mg/mL bovine serum albumin (BSA) in artificial cerebrospinal fluid (aCSF) (149 mM NaCl, 3 mM KCl, 0.8 mM MgCl$_2$, 1.4 mM CaCl$_2$, 1.5 mM Na$_2$HPO$_4$, 0.2 mM NaH$_2$PO$_4$, pH 7.4) was added and vortexed for 10 s to achieve 10% w/w BSA/PLGA in the final formulation. The mixture was then sonicated on ice for 10 min. This primary emulsion was added to 3 mL of a 2.5% w/v solution of poly(vinyl alcohol) (PVA) (30-70 kg/mol), vortexed, and sonicated on ice for 10 min. The secondary emulsion was added to a hardening bath of PVA and stirred overnight to allow the DCM to evaporate. The nanoparticles were washed four times by ultracentrifugation, lyophilized, and stored at -20 °C.

3.3.2 Preparation of BDNF-loaded PLGA-HAMC composite

To prepare the BDNF-loaded PLGA-HAMC composite, PLGA nanoparticles were first dispersed in aCSF (0.1% BSA) to achieve 10% w/v in the final formulation. The nanoparticle solution was vortexed and bath-sonicated for 5 min to ensure proper dispersion of the particles. Sterile methyl cellulose (MC, 300 kg/mol, Shin-Etsu, Tokyo Japan) and sterile sodium hyaluronate (HA, 1.4–1.8 × 10$^6$ g/mol, NovaMatrix, Sandvika Norway) were dissolved in the nanoparticle solution using a dual asymmetric centrifugal mixer (Flacktek, Landrum USA) to a final concentration of 1.4% w/v HA and 3% w/v MC. Complete mixing was achieved by alternating vertical and horizontal speed mixing until composite appeared homogenous and was then stored overnight at 4 °C. BDNF was added to the top of the gel to give a final concentration of 0.33 µg/µL in the composite and mixed using a protocol optimized to maintain bioactivity; after the addition of BDNF, the composite was speedmixed vertically for 10 s, horizontally for 10 s, centrifuged at 4 °C for 10 s and speedmixed vertically again for 10 s.

3.3.3 Animal approval

All animal work was approved by the University of Toronto Animal Care Committee and was conducted in accordance with the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care). Male Sprague Dawley rats, 8-10 weeks old and weighing between 225–350 g were used (Charles River, QC, Canada).
3.3.4 Endothelin-1 stroke and drug delivery

Rats were first anesthetized with isoflurane, shaved and secured in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA). An incision was made in the scalp and a retractor was inserted to hold the incision open. Sham animals were fitted with two curved 5.9 mm diameter polycarbonate disks on the surface of the skull, secured with bone glue (Loctite 454, Henkel Corporation, Rocky Hill, CT, USA), and the wound sutured closed. In all other groups a burr hole with a diameter of 2.7 mm was drilled using a trephine drill bit (Cat. #18004-27, Fine Science Tools Inc., Vancouver, BC, Canada), centered at AP +1.15 mm and ML +3.0 mm. Endothelin-1 (Et-1) (human/porcine, 1 µg/µL in ddH₂O, Cat. #ab120471, Abcam, Cambridge, MA, USA) was loaded into a 10 µL Hamilton syringe with a 26 G, 45° bevel needle (Model 1701 RN, Hamilton) and injected using an automated pump (Pump 11 Elite Nanomite, Harvard Apparatus, Saint-Laurent, QC, Canada). Two injections were made in the primary motor cortex and one in the striatum for a total of three Et-1 injections:

AP + 2.3 mm, ML +/- 3.0 mm, DV - 2.2 mm

AP 0 mm, ML +/- 3.0 mm, DV - 2.2 mm

AP + 0.7 mm, ML +/- 3.8 mm, DV – 6.9 mm

Mediolateral (ML) and anteroposterior (AP) coordinates are relative to bregma and dorsoventral coordinates are relative to the brain surface. The needle was lowered an additional 0.1 mm DV at each injection site and then raised DV + 0.1 mm for the final DV coordinate. Once in position, the needle was left for one minute to equilibrate. Following equilibration, Et-1 was injected at 0.5 µL/min until a volume of 1 µL had been injected. Following a pause of 1-min, an additional 1 µL was injected at the same rate. Once the injection was complete, needles were allowed to equilibrate for 3 min and then slowly withdrawn to prevent Et-1 backflow. A duroectomy was performed using a surgical microscope to expose the cortical surface. For the injury group, a 2.3 mm diameter circle of medical grade silicone sheeting (Cat. #CUST-20001-005, BioPlexus, Ventura, CA, USA) was placed on the tissue and a small piece of Surgifoam® gelatin sponge (Cat. #1972, Ethicon, Markham, ON, Canada) was soaked in saline and placed in the trephine hole on top of the silicone sheeting. This was done to prevent soft tissue adhesion and swelling into the space created by removing the skull. Two 5.9 mm diameter polycarbonate
disks were secured over the burr hole with bone glue and the entire assembly covered with Ortho-Jet™ BCA dental cement (Cat. #1334CLR, Lang Dental, Wheeling, IL, USA).

Surgeries for the vehicle and BDNF groups followed the same procedure, with the only difference being the addition of BDNF to the composite formulation for the BDNF animals (2 µg/animal). Following duroctomy, these groups were fitted with a 5.9 mm polycarbonate disk with a concentric 2.7 mm hole, secured over the burr hole with bone glue. They then received 6 µL of PLGA-HAMC composite, deposited directly onto the surface of the cortex in the space created by skull and disk. A second 5.9 mm polycarbonate disk with no opening was secured over top of the first disk with bone glue, the entire assembly was covered with dental cement (Ortho-Jet™, Lang Dental Manufacturing Company, Wheeling, IL, USA), and the skin was sutured closed.

3.3.5 Behavioral task training and testing

To assess forelimb dexterity, the Montoya staircase task was used. Animals were trained twice per day for 14 days prior to surgery to retrieve pellets with both paws from the staircase apparatus, as described by Ploughmann et al. [154]. Of the 21 pellets accessible to each forelimb, each animal needed to reach, grasp and eat 15 with at least one forelimb to be considered fully trained and included in the study. The last 4 trials, 2 days prior to surgery, were averaged to generate baseline performance values expressed as a percentage of the maximum number of pellets that could be reached (i.e. 21). Handedness was determined based on the best-performing forelimb and the injury was thus given on the dominant side. Animals were sorted into the treatment groups and the investigators blinded.

Staircase testing was conducted bi-weekly for 3 consecutive days, twice per day, with the last 4 trials averaged to give the animals’ score at each timepoint. The first testing period began 4 days post-stroke (Week 1) to allow a deficit to be observed in injured animals before BDNF was expected to begin releasing from the composite. This injury baseline was expressed as a percentage of the uninjured baseline performance and animals were included in further analysis if they had a performance between 20-70% of uninjured baseline at week 1 testing as this indicated a sufficient deficit in the staircase task [155].
For the tapered beam task, animals were trained to cross a 1 m tapered beam elevated off the floor to reach a darkened goal box at the far end containing food pellets. Foot slips onto the ledge on either side were counted as faults if the limb appeared to be weight-bearing. Runs were recorded using a video camera and later analyzed in a blinded fashion. Animals were required to traverse the beam such that the number of steps in the runs totaled greater than 40 steps, usually taking between 4 and 6 runs. Animals were trained for two days prior to surgery, with the last day serving as the baseline uninjured performance. Animals were tested on the tapered beam task bi-weekly. Animals were included in behavioural analysis for the tapered beam task if they exhibited at least a 10% hindlimb slippage at 1 week post-injury and did not experience major complications during or after surgery.

3.3.6 Brain tissue preparation for histological analysis

Animals were sacrificed after the week 7 behavioural testing and brains were extracted and fixed in 4% paraformaldehyde at 4 ºC for 10 days, followed by cryoprotection in 15% and then 30% sucrose. Brains were snap frozen and cryosectioned coronally (30 µm).

3.3.7 Immunohistochemistry

Sections stained for synaptophysin (syn) and NeuN were first permeabilized for 15 min (1% Triton X-100 in PBS) at room temperature, blocked for 30 min (0.1% Triton X-100 and 5% BSA in PBS) and incubated with rabbit anti-synaptophysin (1/400 dilution in blocking solution, ab23754, Abcam, Cambridge, MA, USA) primary antibody, and mouse anti-NeuN (1/500 dilution in blocking solution, MAB377, Millipore Inc., Billerica, MA, USA) primary antibody at room temperature for 2 h. Sections were then washed 3 times for 5 min in PBS before incubation with Hoechst 33342 nuclei stain (1 µg/mL in blocking solution, Cat. # 62249, Invitrogen Inc., Burlington, ON, Canada) and the highly cross-adsorbed secondary antibodies AlexaFluor 488 goat anti-rabbit IgG (1/500 dilution in blocking solution, A11034, Invitrogen Inc., Burlington, ON, Canada) and AlexaFluor 546 goat anti-mouse (1/500 dilution in blocking solution, A11030, Invitrogen Inc., Burlington, ON) for 1 h at room temperature. Sections were washed 3 times for 5 min in PBS, and mounted with ProLong® Gold Antifade Mounting Medium (Cat. #P36934, Thermo Fisher Scientific, Mississauga, ON, Canada).
3.3.8 Imaging and quantification

Coronal sections spaced 300-600 µm apart were chosen between AP +4.0 mm and AP -1.0 mm relative to bregma to assess the effects of local BDNF delivery on the tissue. Images were taken with a 20x objective on an Axio Scan.Z1 Slidescanner (Zeiss, Oberkochen, Germany), using consistent microscope settings for each section. Lesion area was defined as the area lacking NeuN\(^+\) staining, using the contralesional hemisphere as a reference, and the tracing of the lesion enabled the selection of regions of interest (ROI) for syn and NeuN staining.

NeuN staining was employed to assess the number of mature neurons in the peri-infarct tissue, using the same ROI as for syn quantification. The average size of a neuron in NeuN\(^+\) pixels was attained by sampling 100 randomly chosen neurons across 10 sections from different animals, which were traced and analyzed in ImageJ. The NeuN\(^+\) pixels in R1 and R2 were divided by this average to estimate the number of neurons present. To determine the lesion size, ImageJ software was used to measure the traced lesion area for 10 sections per animal. These areas were first multiplied by the inter-section AP distance to determine the average volume of damage between sections and then added to determine total lesion volumes. This was done for both cortical and striatal lesions separately, and these volumes added to give total lesion volume for each animal.

As a measure of synaptic plasticity, syn\(^+\) pixels were quantified in 6 sections per animal using Fiji ImageJ Software. Images were first converted to 8-bit and thresholded using the “Moments” thresholding algorithm, then four 500 µm x 500 µm cortical ROI were chosen in both hemispheres. For the ipsilesional hemisphere, two ROI were defined directly adjacent to the lesion on either side of the cavity and were averaged to give the R1 values reported. An additional two ROI, 500 µm away from the lesion on either side of the cavity were averaged and reported as R2 values (Appendix, Section 7.9). Homotopic regions in the contralesional hemisphere were quantified in the same manner.

3.3.9 Analysis of BDNF diffusion in stroke-injured brain tissue

To determine BDNF concentrations in the brain after local delivery, surgeries were performed as described above. Animals were sacrificed after 4, 7 and 21 days post-injury and the brain tissue was harvested, snap frozen and stored at -80 \(^\circ\)C. Tissue sections were prepared for
diffusion analysis, as previously described [6]. Briefly, six serial 1-mm thick coronal sections surrounding the stroke lesion were cut using a McIlwain tissue chopper (Mickle Laboratory Engineering Company, Surrey, UK). A Leica CM3050S cryostat system was then used to section these coronal slices dorsoventrally, each 0.3 mm thick. Tissue from each coronal section at the same depth was collected together in pre-weighed 2 mL polystyrene microtubes and homogenized to give average BDNF concentration at each depth spanning 300 \( \mu m \), down to 3 mm below the surface of the tissue.

To extract BDNF from the tissue 200 \( \mu L \) of homogenization buffer (20 mM Tris-HCl buffer pH 8, 137 mM NaCl, 1% vol/vol Triton-X 100, 10% vol/vol glycerol) and 3-5, 1.0 mm zirconia beads (Cat.#11079110zx, Biospec Products, Bartlesville, OK, USA) were added to each microtube. Tissue was homogenized for 30 s with a Mini-beadbeater 16 (Biospec Products), centrifuged at 4 °C for 5 min at max speed, homogenized for another 30 s and centrifuged a final time at 4 °C for 15 min at max speed. Supernatant from the tubes was collected and an ELISA (BDNF E_\text{max}® ImmunoAssay Systems, Promega, WI, USA) was used to quantify BDNF in each segment of the tissue. Tissue from ipsilesional vehicle control brains was spiked with BDNF to generate the ELISA standard curve so as to account for endogenous BDNF levels and any effects of processing.

### 3.3.10 Statistics

Data is reported as mean ± standard deviation unless otherwise specified. Statistical analyses were performed using Prism 6.0 (GraphPad Software Inc.). For behavioural task data analysis, multiple comparisons were performed using a one-way ANOVA within groups and the Tukey’s post-hoc correction for multiple comparisons. For immunohistochemical analysis, multiple comparisons were performed using a one-way ANOVA between groups with the Holm-Sidak post-hoc correction for multiple comparisons. Reported p-values are adjusted for multiple comparisons.
3.4 Results

3.4.1 Short-term recovery of affected forepaw is enhanced with local BDNF delivery

The stroke lesion targeted the forelimb area of the primary motor cortex and thus the Montoya staircase task was used to evaluate the performance of the injured forelimb in stroke-lesioned rats (Figure 9). Only BDNF-treated animals showed a significant improvement in staircase performance at week 3 relative to the week 1 injured timepoint, demonstrating a beneficial effect. No significant improvements in performance were observed for the vehicle or injury groups in this task. Sham animals remained at baseline performance throughout the testing period.
Figure 9. Local delivery of BDNF enhances early forepaw recovery following stroke injury. Data plotted as individual animal staircase task scores with mean ± SD shown, *p≤0.05. (A) Paradigm for staircase task training and testing relative to stroke injury and composite delivery. (B) BDNF-treated rats demonstrated significantly improved forelimb performance at week 3 relative to the week 1 injured timepoint (n = 10). (C) Vehicle treated rats (n = 11) and (D) injury
only rats (n = 9) did not exhibit significant recovery at any time examined. (E) Sham group staircase performance remained consistent throughout (n = 8).

3.4.2 Recovery of affected hindlimb is enhanced with BDNF local delivery at 7 weeks

To assess hindlimb function, we used the tapered beam task (Figure 10A). BDNF-treated animals exhibited recovery at both week 3 (p<0.1, p = 0.0879) and week 7 (p<0.05) relative to week 1 (Figure 10B) in the affected hindlimb post-injury, suggesting that the effects of BDNF take some time to manifest in this task. Vehicle treated animals exhibited a significant improvement in performance at week 3 relative to week 1, suggesting a transient benefit associated with HAMC, but this was not maintained to 7 weeks (Figure 10C). Injury group animals did not show any significant improvement in performance at any testing point (Figure 10D), and sham animals remained at their baseline level of performance (Figure 10E).
Figure 10. Local delivery of BDNF and vehicle enhances hindlimb recovery following stroke injury. Data plotted as individual animal beam task scores with mean ± SD shown, #p<0.1, *p<0.05, **p<0.01. (A) Behavioural training and testing paradigm for tapered beam task. (B) BDNF-treated rats show some hindlimb recovery at week 3 with a #p<0.1 (p-value = 0.0879) relative to week 1 and significantly (*p<0.05) improved hindlimb performance at week 7 relative to the week 1 injured timepoint (n = 9). (C) Vehicle-treated rats exhibited a deficit at week 1 and
a significant improvement in hindlimb function at week 3 compared to week 1 that was not maintained to week 7 (n = 10). (D) Injury-only rats did not exhibit any significant recovery relative to the week 1 injured timepoint (n = 4). (E) Sham group staircase performance remained unchanged throughout the testing period (n = 11).

3.4.3 Stroke lesion volume is decreased by the presence of vehicle

NeuN staining was used to investigate the effect of local BDNF delivery on the stroke lesion volume. The lesion was defined as NeuN$^-$ and was traced using ImageJ software to quantify the area in each section (Figure 11A). The total lesion volume (cortical and striatal combined) in BDNF and vehicle groups was significantly less than that of the injury group, indicating that vehicle itself was enough to reduce tissue loss. The cortical lesion volume in BDNF and vehicle groups was significantly decreased compared to the injury group, while the striatal lesion volumes were similar across all groups (Figure 11B-D). As no significant difference was seen between the BDNF- and vehicle-treated groups, BDNF is unlikely to have contributed to this reduction of lesion volume.
Figure 11. Lesion volume, a proxy for tissue degeneration, is decreased in animals that received the vehicle with or without BDNF. Data plotted as individual mean ± SD, *p ≤ 0.05, **p ≤ 0.01.

(A) Lesioned tissue was defined as NeuN⁺ and traced to quantify the area it occupied and regions of interest, R1 and R2, were defined on either side of the lesion for additional immunohistochemical quantification. (B) Cortical lesion (CL) volume was significantly decreased in BDNF- and vehicle-treated animals relative to injury only animals. (C) Striatal lesion (SL) volume was unaffected by treatment with vehicle or BDNF. (D) Cortical and striatal lesion volumes were combined to give a total lesion volume, where a significant decrease was observed in BDNF- and vehicle-treated animals relative to injury animals.

3.4.4 Loss of mature neurons surrounding the lesion without vehicle

In addition to influencing plasticity, BDNF is known to have a pro-survival effect, acting mainly through the PI3K/Akt pathway [222], [223]. To determine whether our BDNF delivery strategy promoted neuronal survival in peri-infarct tissue, the number of NeuN⁺ cells was quantified in ROI (500 μm²) directly adjacent to the lesion (R1) and 500 μm away from the
lesion (R2). Two regions for R1 and two regions for R2 were chosen on either side of the lesion and averaged together to give a representative sample of the tissue. Injury-only animals showed a significant loss of NeuN in R1 while the BDNF and vehicle treated animals showed no significant loss (Figure 12).

**Figure 12.** Injured animals experience loss of mature neurons in peri-lesional ROI. Data plotted as individual mean + SD, *p<0.05, **p<0.01. (A, B) No differences in the number of neurons were observed between groups in the homotopic regions of interest in the contralesional hemisphere. (C) In the ipsilesional regions of interest, injured animals experienced a significant loss of neurons in the peri-infarct area while animals that received the vehicle either alone or with BDNF did not exhibit this loss. (D) No differences were observed between the groups in ipsilesional region of interest R2.

### 3.4.5 Local delivery of BDNF increases synaptophysin expression in homotopic contralesional hemisphere

Given the reported role of BDNF as an agent of plasticity, we investigated synaptic plasticity with synaptophysin, a synaptic vesicle protein expressed pre-synaptically [224]. We
observed significant increases in synaptophysin expression in the BDNF-treated animals in the contralesional hemisphere ROI R1 and R2 (Figure 13). These increases were significant compared to vehicle and injury groups in region R1 and significant compared to vehicle, injury and sham groups for region R2 (Figure 13A,B). The regions of interest on either side of the lesion (in the ipsilesional hemisphere) did not display a significant upregulation of synaptophysin expression in any of the injured groups (Figure 13C,D).

**Figure 13.** Local BDNF delivery results in significantly increased synaptophysin expression in the homotopic contralesional hemisphere. Data plotted as individual mean + SD, *p<0.05, **p<0.01. (A) Contralesional homotopic region of interest R1 in BDNF-treated animals exhibited significantly increased synaptophysin expression compared to vehicle and injury groups. (B) Contralesional homotopic region of interest R2 in BDNF-treated animals exhibited significantly increased synaptophysin expression compared to vehicle, injury and sham groups. (C,D) No significant differences between groups were observed in ipsilesional regions of interest.
3.4.6 Epi-cortical PLGA-HAMC composite allows sustained delivery of BDNF into the stroke-injured brain without encapsulation

To gain greater insight into the role of BDNF in repair, we characterized its tissue penetration in the brain at the behavioural testing timepoints (Figure 14). At 1 day post-delivery, BDNF was not detectable in the tissue above endogenous levels at any depth. This is consistent with in vitro release profiles that exhibit a delay period before BDNF is released from the composite [142]. Diffusion into the tissue was first observed at 4 days post-delivery, with detectable concentrations throughout the collected depths. The highest concentration in the tissue occurred at 7 days post-delivery, and detectable concentrations were maintained out to 21 days. Thus, while the BDNF composite was applied at the time of injury, BDNF tissue diffusion, and hence treatment, was not immediate and allowed a statistically significant deficit to be created in the animals. When we delivered the BDNF composite 4-days post-injury to more closely simulate a clinical scenario, BDNF was detected in the tissue at 13 and 21 days post-delivery to a depth of 3 mm. Additional depth analysis past 3 mm revealed the presence of BDNF at 4.5 and 5 mm below the surface of the brain (Appendix, Section 7.10).
Figure 14. BDNF diffuses into stroke-injured rat brain tissue from the PLGA-HAMC composite deposited on the surface of the cortex, superficial to the lesion. (A) Total mass of BDNF detected in stroke-injured rat tissue at 1, 4, 7 and 21 days post-delivery, as measured by BDNF tissue ELISA. (B) Depth diffusion profiles demonstrate that BDNF accumulated in the stroke-injured rat brain between 4 and 21 days. (C) No BDNF was detected above endogenous levels at 1 day post-delivery. Spatial distribution analyses at 4, 7 and 21 d timepoints show that BDNF diffuses to a depth of at least 3 mm from the brain surface. (Mean + SD reported, n = 4).

3.5 Discussion

In the first few hours following ischemia, the BDNF gene is upregulated in both peri-infarct and homotopic contralesional tissue, but this spike in expression quickly diminishes to baseline levels by 24 h post-injury [212]. We demonstrated that local, sustained delivery of exogenous BDNF to the stroke-injured rat brain has beneficial behavioural and tissue effects, and that this can be achieved using our novel, minimally invasive delivery strategy. As functional recovery is the overall goal of any stroke treatment strategy, behavioural testing was an integral part of our investigation. With our Et-1 stroke injury, we targeted the primary motor cortex with coordinates specific to the forelimb and thus used the staircase test. These coordinates are also
known to affect hindlimb function due to the close proximity of the areas [154], and so we included the tapered beam tasks in our assessments as well.

We observed an early benefit of local BDNF delivery in the staircase task at week 3. This task requires a fine degree of motor control of the affected limb and since animals were not exposed to fine motor use or training scenarios outside of staircase task testing, it is not surprising that these circuits were not further enhanced throughout the course of the testing period. In contrast, performance in the tapered beam task reflected necessary daily movement. Relative to week 1, BDNF-treated animals recovered function at week 3 (with p<0.10) and at week 7 (with p<0.05). Interestingly, vehicle-treated animals showed functional recovery at week 3 as well (with p<0.01), yet this effect was not sustained. Immunohistochemical staining at the week 7 timepoint revealed tissue effects that gave additional insight into these behavioural outcomes.

In BDNF- and vehicle-treated animals, we observed preservation of tissue in the cortical lesion site and the absence of mature neuron loss in the peri-lesional tissue. These effects can be attributed to the epi-cortical placement of the composite, directly superficial to the cortical lesion, as high molecular weight HA is known to be anti-inflammatory [131] and our HAMC hydrogel has similar anti-inflammatory properties in the CNS [143]. These findings are consistent with previous studies that demonstrated the ability of the PLGA-HAMC composite to reduce lesion volume when administered epi-cortically above the lesion site in a mouse model of stroke [143]. By modulating the immune response, the HAMC hydrogel limits the extent of damage, thereby creating a more permissive environment for plasticity. This immune modulation may have allowed more endogenous plasticity to occur in the short term, resulting in the observed recovery of both BDNF and vehicle treated animals in the beam task at week 3; however, long-term effects observed at week 7 in the BDNF-treated animals indicate a benefit from the sustained presence of exogenous BDNF, possibly because this provided a greater window of increased plasticity.

Unexpectedly, we observed greater plasticity in the contralesional vs. ipsilesional hemisphere as determined by synaptophysin staining. Increases in synaptophysin expression are associated with synaptogenesis and synaptic strengthening, and used to quantify synaptic plasticity [225], [222]. Consistent with others, we observed increased synaptophysin expression
in BDNF treated brains following stroke, though these differences were in the contralesional, and not the ipsilesional, hemisphere. Given that the glial scar contributes to the inhibitory environment, it may have prevented observable plasticity in ipsilesional tissue at 7 weeks post-delivery. The absence of glial scar in the contralesional hemisphere results in a more permissive environment for plasticity and this may explain why effects were only observed here.

Of the studies that have used synaptophysin as an indicator of synaptic plasticity in ischemic injury, few have investigated it in the contralesional hemisphere; however, the contralesional hemisphere is known to provide support in a compensatory manner [226], and increases in the number of synapses and the volume of dendritic processes have been reported in layer V of the contralesional forelimb motor cortex following ischemia [227]. Reorganization of motor circuits takes time, even when rehabilitation therapy is used to promote cortical plasticity and recovery [35], and thus, examination at different timepoints would reveal varying tissue effects. Studies in both humans [44], [228] and rats [229] that have investigated the role of contralesional activity in relation to functional outcome have found that contralesional activity is transiently increased in the short-term, and that greater recovery requires a subsequent increase in the ipsilesional activity at chronic timepoints. Eventually, balanced hemispheric activity may be the key to optimal recovery [230].

The contralesional plasticity we observed in BDNF-treated animals is likely a product of multiple pathways, including: interhemispheric axonal sprouting, diffusion of exogenous BDNF across the corpus callosum, and/or re-distribution of exogenous BDNF via CSF flow (Figure 15).
Figure 15. Potential pathways of BDNF influence. Contralesional effects could be governed by interhemispheric axonal sprouting initiated by BDNF treatment in the ipsilesional hemisphere, anisotropic diffusion guidance of delivered BDNF along the corpus callosum, and/or redistribution of the delivered BDNF via CSF flow in the subarachnoid space.

The epi-cortical placement of the composite directly above the cortical lesion allows BDNF to reach the peri-infarct tissue, as demonstrated by tissue penetration data. As we were targeting plastic processes in the peri-infarct tissue, the observed tissue penetration was sufficient. Even without modification or additional diffusion facilitators, BDNF diffused to a depth of at least 3 mm from the epi-cortical composite. This diffusion depth comprises the cortical layer of tissue and includes the interface between cortex and corpus callosum. With BDNF diffusing to this depth in the tissue, it is possible that the anisotropic orientation of the white matter tracts of the corpus callosum facilitated protein diffusion to the contralateral hemisphere. Additionally, BDNF diffusion to the contralesional hemisphere may have been facilitated by CSF flow in the subarachnoid space and the lateral ventricles [231]. Though effects from these pathways of diffusion are possible, we postulate that the most likely route of BDNF influence was via the MEK-ERK-CREB cascade. Due to the feed-forward nature of this mechanism, BDNF delivered to the ipsilesional hemisphere would have upregulated endogenous
BDNF expression in the ipsilesional hemisphere that, in turn, led to BDNF expression upregulation in both hemispheres. Upregulation of BDNF results in the strengthening and remodeling of existing axonal sprouting and sprouting of new interhemispheric connections, as was demonstrated in the recent study by Cook et al., in which stroke-injured mice received a BDNF-loaded hydrogel, injected into the stroke cavity tissue. They showed that BDNF-treated animals exhibited less loss of cortical interhemispheric projections and increased interhemispheric axonal sprouting within the cortico-striatal systems [138]. It should also be noted that the observed plasticity would likely not have been possible without the survival of mature neurons surrounding the lesion site, suggesting a neuroprotective role for the BDNF-loaded composite. As the timeline for the study paradigm was relatively short, the preservation and strengthening of existing axonal connections would have contributed to the observed tissue and behavioural effects more than those that were newly formed.

Though the focus of our work has been stroke, the applicability of this delivery system for BDNF is evident for other neurotraumatic events and degenerative diseases. BDNF dysregulation has been implicated in many CNS pathologies, including Alzheimer’s disease [209], and Parkinson’s disease [232], and effective delivery of this therapeutic in a minimally invasive manner could unlock new therapeutic options for these hard-to-treat conditions.

3.6 Conclusions

We validate a novel strategy for eliciting synaptic plasticity and recovery of function following ischemic stroke in rats. Local delivery of un-encapsulated BDNF with our PLGA-HAMC composite results in short-term recovery in forelimb function, as well as hindlimb recovery at week 7 testing. The PLGA-HAMC vehicle demonstrated beneficial effects when delivered at the time of injury, preserving mature neurons in the peri-lesional tissue and decreasing lesion volume, likely through the anti-inflammatory properties of the HA. At 7 weeks post-delivery, synaptic plasticity in the homotopic contralesional hemisphere was increased, as indicated by a substantial increase in synaptophysin expression. Our PLGA-HAMC formulation, loaded with un-encapsulated BDNF, resulted in detectable concentrations of BDNF in the tissue to a depth of 3 mm below the surface for 21 days. We demonstrate the benefits of epi-cortical, minimally-invasive strategies for the treatment of stroke and suggest its applicability to neurodegenerative diseases.
4 Thesis Discussion

Despite major advances in our understanding of the brain, it remains largely a mystery. Studies of developmental biology can give us some clues as to how reparative processes might unfold [233], but the dynamics of the injured CNS are undoubtedly more complex. In infancy, the brain is highly plastic, allowing the formation of connections to establish circuits necessary for survival. [234] As we age, cues from our environment, including the people we are surrounded with and the experiences we have, dictate which of these formed connections will stay and which will be subject to pruning [235]. Following a traumatic neurological injury, such as stroke, a window of increased plasticity arises once again to allow some degree of recovery, and raises the question: how do we harness this injury-induced plasticity most effectively, and is there any way to increase that window of time? Examining the endogenous repair mechanisms the brain employs and correlating them to observed behavioural outcomes can help us to identify pathways crucial to recovery, as well as factors that limit spontaneous recovery. BDNF has emerged as a modulator of these crucial pathways [62] and dysregulation of its expression continues to be implicated in increasingly more neuropathologies [236],[237]. This work aimed to support the plasticity contributing to recovery by delivering exogenous BDNF to the stroke-injured brain using a minimally invasive biomaterial-based strategy.

With an investigation into delivery strategies for protein therapeutics, we first developed a solid rationale for the use of a PLGA nanoparticle and HAMC hydrogel composite in this application. The discovery of an electrostatic-mediated release from the composite, discussed in Chapter 2, provided us with an exciting new avenue to explore that has the potential to revolutionize the delivery of protein therapeutics. Through rigorous experimentation, we teased apart the nuances of this improved, un-encapsulated composite formulation, which allowed us to tune the system to suit the delivery of BDNF and other similar proteins. We demonstrated that this new configuration did not sacrifice the integrity of the protein – on the contrary, BDNF bioactivity was retained and we could achieve higher loadings in the composite. When we applied this system in vivo, shown in Chapter 3, it enabled minimally invasive delivery of BDNF into the brain tissue for a sustained period of time and elicited beneficial behavioural and tissue effects that we attribute to enhanced plasticity. Here, we highlight the importance of these findings for the fields of protein delivery, neuroplasticity and stroke recovery, and discuss some of the challenges that arose in the course of this work.
4.1 Addressing Challenges Associated with Protein Delivery

4.1.1 Protein Stability

Therapeutic proteins, such as BDNF, other neurotrophins and similar molecules, are notoriously difficult to deliver to the human body [238]. The complex conformation required for these proteins to function properly imbues a delicate nature to them, calling for extra precautions to be taken when packaging them into delivery vehicles. For this reason, the great successes reported with small molecule encapsulation in polymer nanoparticles via emulsification do not translate to proteins; small molecule drugs boast fantastic encapsulation efficiencies, typically more than 90% [239], while those of protein therapeutics rarely achieve 50% [166],[168]. The reason for this is well documented: adsorption of the proteins to the solvent/water interface and to PLGA in the primary emulsion results in unfolding and aggregation [240],[168]. The sonication used to create the emulsions also exerts shear stress and increases the temperature of the mixture over time, both of which are usually detrimental to protein stability as well. The use of different organic or aqueous solvents, co-encapsulation of excipients, such as BSA, and reducing sonication time or temperature are all strategies that have been employed to address these issues, but these have only modestly improved protein encapsulation [168]. Using a solid-in oil-in water (s/o/w) method removes the interface between water and organic solvent present with the w/o/w method, but does not significantly improve encapsulation efficiency compared to w/o/w double emulsion [241]. Other methods of protein encapsulation that have offered some improvement of encapsulation efficiency include nanoprecipitation, ionic gelation with natural polymers and the use of supercritical fluids [163].

Aside from the nanoparticle fabrication process, protein stability can be compromised at other points during in vitro and in vivo experimentation. In our PLGA-HAMC system, the PLGA degrades into acidic byproducts, decreasing the local pH in the gel and negatively affecting protein activity [242]. Our inclusion MgCO$_3$ in the nanoparticle formulation to buffer this shift was effective in both encapsulated and un-encapsulated protein configurations. Additional BSA in the hydrogel and the release media also helped to mitigate the effects of storage at physiological temperature during release and after samples were collected and frozen. It should be noted that depending on the quantification method used, reported efficiencies might not be values that reflect the amount of bioactive molecule present. For example, ELISA does not
necessarily detect only bioactive therapeutic if the specific receptor for the protein is not employed and usually requires consubstantiation with a specific bioassay to ensure activity [152]. Our un-encapsulated system eliminates the protein losses associated with these encapsulation processes and bioactivity testing with the DRG bioassay confirmed the retention of neurotrophin activity following release.

4.1.2 Avoiding Encapsulation to Improve Protein Stability

As we discussed in Chapter 2, the fortuitous delay in release we observed with encapsulated BDNF, NT-3 and SDF led us to question the mechanism we originally thought to be responsible for sustained release - diffusion through pores created in the nanoparticles as they degrade. Expecting the inclusion of additional un-encapsulated protein to cause a burst release at the earlier timepoints, we were surprised that release rate was unaffected, with the only difference to the profile being that it continued past those of previous experiments. Mixing soluble protein with blank nanoparticles in hydrogels composed of varying materials produced the same effect and pointed to the nanoparticles as the source of this phenomenon. Subsequent experimentation verified this hypothesis and demonstrated that we did not need to encapsulate the proteins in order to control their release. While the diffusion of the protein through the hydrogel and from the hydrogel to the supernatant contributes to the overall release profile, we have shown that the main governing process is the reversible charge attraction between the protein and the nanoparticle surface.

Diffusion of the protein out of the nanoparticle as it degrades is not a contributing factor in the case of the un-encapsulated formulation; however, degradation of the polymer still contributes largely to the release rate. PLGA has been shown in previous investigations to begin degrading approximately 4 days after wetting [243], which is in agreement with the delay we observed. Following this delay, linear release ensued for the next 7-20 days, which we attribute to the change in local pH and resulting neutralization of the carboxylic end groups as the PLGA begins to degrade [242]. Neutralization of the end groups facilitates protein diffusion away from the particles, but this hydrolytic cleavage also increases the number of exposed carboxylic end groups. Thus, these opposing forces provide sustained release of the protein as the PLGA degrades. Through understanding this mechanism behind release, we were able to determine different parameters that would allow us to tune the release profile of our proteins of interest. We
found that PLGA molecular weight and composition, particle size and concentration in the hydrogel and pH could all be easily manipulated to give the desired release characteristics while maintaining protein activity.

Obviating the need for encapsulation has important implications for clinical translation of these protein-based therapies. Simpler systems that are robust and reproducible will encounter less resistance when being developed for clinical use, and removing protein encapsulation from our composite formulation process does a great deal towards this. We are able to prepare the composite vehicle a day in advance of its intended use and mix in BDNF immediately before application. An optimized speed mixing protocol and less time exposed to ambient temperatures has allowed us to retain ~70% of the theoretical loading in the composite, where previous formulations that encapsulated the protein could only achieve ~30%. This is partly due to the different limiting factors in the two formulations. With the un-encapsulated composite, the possible protein loading becomes dependent on solubility in the composite and the nanoparticle surface area, as opposed to the encapsulation efficiency in the nanoparticles and the particle concentration. Increasing this upper limit of loading lends itself to the development of minimally invasive delivery strategies, as less material is required to stimulate the desired effects.

4.1.3 Protein Detection in Tissue

In order to determine the efficacy of a CNS drug delivery strategy, it is necessary to be able to detect the delivered therapeutic in the targeted tissue. BDNF is an endogenously produced protein, and the recombinant BDNF we delivered displayed the same ELISA reactivity as the endogenous rat BDNF. As such, the concentration of exogenous BDNF in the tissue at the time of tissue collection needed to exceed endogenous levels in order to be detected. Tissue collected from injured animals that received a vehicle control composite (no BDNF) was used to create the standard curve for this ELISA quantification, spiking known concentrations of the recombinant BDNF into the tissue and processing it alongside the BDNF composite-treated tissue. While we were able to detect delivered BDNF in the tissue above endogenous levels at all depths at 4, 7 and 21 days following delivery, these concentrations were small; however, it is important to acknowledge the many mechanisms of transport and clearance that exist in vivo and that we were detecting BDNF at specific points in time, thereby likely missing any accumulated protein. The epi-cortical placement of the PLGA-HAMC composite exposes it to 90 μL of CSF,
circulating at a rate of 2 \( \mu L/min \) in the rat [231], [244], which likely distributed some BDNF to other areas of the brain. The protein that diffuses into the tissue immediately is subjected to extracellular degradation, receptor-mediated cell uptake and intracellular degradation [245]. The rapid nature of these processes combined with the dynamic environment make it difficult to ascertain the release profile characteristics in vivo, as only snapshots of tissue concentration are available. When the animals are sacrificed, retrieval and dissolution of the composite would theoretically allow a mass balance to be performed. Complete retrieval is unreliable though, as it is hard to distinguish between composite and host tissue, especially at later timepoints. These challenges were mediated by running side-by-side in vitro releases with all in vivo experiments to provide estimates of BDNF concentrations at specific time points.

4.2 Evaluating the Therapeutic Effects of BDNF Local Delivery

4.2.1 Behavioural Measures of Functional Recovery

The aim of any therapeutic strategy is to provide some benefit to the patient. In stroke, meaningful benefit generally refers to some degree of enhanced functional recovery following the injury. Therefore, in the absence of behavioural measures it becomes difficult to draw conclusions about the efficacy of an intervention strategy or understand any observed tissue effects. It is for this reason that it was necessary to include behavioural measures in our evaluation of the BDNF-loaded PLGA-HAMC composite. The two tasks employed for this evaluation were the staircase task and the tapered beam task, for assessment of forelimb and hindlimb performance, respectively [161]. Accounting for the differences in neurovasculature in individual animals, we anticipated that some variability would exist in terms of where the deficit was most evident. To address this, we used different exclusion criteria for the two behavioural assays to ensure that only animals with a sufficient deficit were included in analysis. With the staircase task, BDNF-treated animals exhibited early, short-term recovery of their affected forelimb at week 3 compared to their injury baseline, whereas the tapered beam task revealed delayed recovery in BDNF-treated animals at week 7 post-injury. Vehicle-treated animals also exhibited some short-term recovery in the tapered beam task, echoed to a slightly lesser degree by the BDNF-treated animals at a 90% confidence interval, but this effect in the vehicle-treated animals was not sustained.
We designed the study such that animals would receive staircase training for two weeks prior to injury, allowing their uninjured forelimb performance to be ascertained and used to distribute animals into treatment groups. Though sorting would have ideally occurred 4 days post-stroke and before the administration of any therapeutic, this was not feasible logistically. Delivery at the time of injury, though not as clinically relevant, also provided us with the best opportunity to affect positive change due to the known anti-inflammatory effect of the HAMC hydrogel, and minimizing the number of surgical procedures. Sorting the animals before knowing the degree of injury they received potentially influenced the recovery effects we observed. The severity of the injury has been shown to affect plastic pathways, as the contralesional hemisphere may be relied upon more in the case of a larger volume of affected tissue in the ipsilesional hemisphere. This provided rationale for intra-group comparisons as a function of time when performing statistical analyses.

The differences in recovery observed in the tapered beam vs. staircase assays may have been influenced by daily use. For example, the tapered beam requires coordination of the hindlimbs and these were used daily by the animals whereas the staircase assay requires fine motor control of the forelimbs, which was only exercised during testing. Housing the animals in an enriched environment and adding rehabilitation therapy to their daily activities would have encouraged increased use of the affected forelimb and may have sustained the effects seen in the short-term staircase performance [246]. We performed a pilot study with the enriched environment and rehabilitation protocol developed by the Corbett lab for eventual combination with the BDNF local delivery strategy, but unfortunately we were not able to reproduce the benefits observed by the Corbett lab in our facilities (Appendix, Section 7.11).

4.2.2 Tissue Analysis and Quantification of Plasticity

In an effort to diversify tissue quantification methods and aid definitive conclusions, brain tissue collected from the behavioural study was randomly assigned to either immunohistochemistry or western blot analysis groups. This ultimately diminished the power of both analysis methods, once exclusion criteria were applied, warranting a second behavioural study to boost group numbers for one analysis method. With interesting results observed in the preliminary IHC data, this method was chosen. Results from the second behavioural study were in agreement with the preliminary data, strengthening the case for BDNF local delivery. We
stained the tissue with antibodies specific to NeuN, a marker of mature neurons, and synaptophysin, a synaptic vesicle protein, to assess any neuroprotective or plastic effects of the BDNF-loaded PLGA-HAMC composite.

We defined the lesion as NeuN- stained, tracing the border of this area on serial sections to quantify the lesion volume [6]. This staining also allowed us to identify regions of interest in peri-lesional tissue in which to quantify mature neurons and synaptophysin pixel density. The PLGA-HAMC composite demonstrated an obvious neuroprotective effect, in agreement with previous work [143]. Vehicle-treated animals exhibiting significantly decreased cortical lesion volume and no substantial decrease in mature neurons surrounding the infarct. Delivering the composite at the time of injury, regardless of the inclusion of additional therapeutic factors, has the potential to limit injury progression, and may diminish the time required for the injury to stabilize. Faster stabilization could subsequently enable earlier commencement of rehabilitation therapy, which has been implicated in greater degrees of recovery [247].

Synaptophysin is widely used as a marker of synaptic plasticity, as many associate increases in its expression with the generation of new synapses [248]. This protein is found pre-synaptically within the membranes of synaptic vesicles that traffic neurotransmitters to the synapse. Studies that have investigated endogenous BDNF [69], as well as delivered BDNF activity [47], [209] have reported a positive influence of this factor on synaptophysin expression. Our observations are consistent with these studies in that delivery of BDNF to the stroke-injured brain resulted in increased synaptophysin staining; however, the location of the observed increase was unexpected. Most groups have restricted their investigation of synaptophysin expression to the peri-lesional tissue with few looking to the contralesional hemisphere for effects. In Chapter 3, we show that the regions of interest in the peri-lesional tissue do not exhibit significantly increased synaptophysin expression at 7 weeks post-injury. The contralesional homotopic regions, on the other hand, demonstrated significant upregulation of this protein. With BDNF delivered to the ipsilesional hemisphere, this is counter-intuitive, and we postulate that several pathways facilitated this effect.

As mentioned previously, the epi-cortical placement of the PLGA-HAMC composite subjected the biomaterial to CSF, which flows within the subarachnoid space. This could contribute to widespread dispersion of any BDNF that did not diffuse in the parenchyma. The
rationale for this composite placement was two-fold: 1) previous investigations indicated that peri-lesional tissue could potentially be salvaged and re-wired with exogenous BDNF application [138], and 2) drilling at this site was already necessary for the injection of Et-1 to create the injury. While the target of our delivery strategy was the peri-lesional tissue, widespread delivery of this therapeutic may be more beneficial to plasticity-based recovery from stroke. BDNF involvement in the growth of axons [249] may also explain the contralesional synaptogenesis observed. With support from BDNF, surviving neurons in the peri-lesional tissue may have developed new interhemispheric connections to replace the local connections lost with injury. Other groups have used biotinylated dextran amine (BDA) axonal tracers to track the growth of these axons following ipsilesional BDNF delivery, demonstrating an increased occurrence of this interhemispheric connection in BDNF-treated animals [138]. There is also the possibility that interhemispheric diffusion of BDNF was facilitated by the anisotropic corpus callosum [231], as diffusion depth analysis demonstrated that the protein was able to reach this structure in the rat brain. The feed-forward effect that BDNF exerts on its own expression [222] may also lower the required dose for therapeutic efficacy, as this would produce an amplification of expression.

While we have reported our observed plastic effects in terms of synaptogenesis, a more thorough understanding of the role of synaptophysin reveals that an increase in the expression of this protein is not necessarily due to the formation of new synapses. BDNF has been reported to increase the number of synaptic vesicles that are docked in the active zone, prepared for release into the synapse, while the reserve pool of vesicles remains unaffected. This action increases the overall number of synaptic vesicles and thus, the expression of synaptophysin. By increasing the active zone docking of vesicles, BDNF strengthens the synapse, as more neurotransmitter is released with the depolarization of the pre-synaptic neuron. Both synaptogenesis and synaptic strengthening (LTP) are considered plastic processes, and both may be occurring simultaneously with the addition of exogenous BDNF, making it difficult to isolate the effects of each with only synaptophysin staining. Additional immunohistochemistry for the dendritic marker, microtubule-associated protein 2 (MAP-2), was performed as another measure of tissue plasticity; however, these data were inconclusive potentially due to high variability arising from section thickness and large background signal.
5 Conclusions

In this thesis, we developed a minimally invasive strategy for local delivery of BDNF to the stroke-injured brain. This strategy harnessed the electrostatic interaction between positively charged protein and negatively charged particles to overcome limitations associated with protein stability in nanoparticle delivery formulations. Enhanced behavioural recovery was demonstrated in a rat model of stroke injury with epi-cortical placement of the BDNF-loaded PLGA-HAMC composite. Tissue effects revealed that this was mediated via a synaptic plasticity mechanism in the contralesional hemisphere through either synaptogenesis or synaptic strengthening. This validated the primary hypothesis that “Sustained local delivery of brain-derived neurotrophic factor to the stroke-injured rat brain from a PLGA-HAMC composite will promote synaptic plasticity and functional recovery”.

5.1 Achievement of Objectives

Herein, we describe a minimally invasive strategy for local delivery of BDNF to the stroke-injured rat brain that provides sustained release using a PLGA-HAMC composite that enhances recovery through contralesional synaptic plasticity. Achievement of the objectives originally stated in Chapter 1 are summarized below:

1. Design a PLGA-HAMC formulation to achieve sustained delivery of bioactive BDNF

1.1 Investigate the mechanism of release for BDNF loaded into a PLGA-HAMC composite.

Electrostatic interaction between the PLGA nanoparticles and BDNF was found to be responsible for the sustained release profile of the system. The experiments used to elucidate this mechanism are discussed in chapter 2.

1.2 Tune the release of BDNF from the PLGA-HAMC composite such that it is sustained out to 28 days in vitro.

By investigating the effects of nanoparticle size, concentration, and pH on the release profile, formulation parameters were chosen to provide a 5-7 day delay before sustained release was achieved out to 28 days, as shown in Chapter 2.

1.3 Assess the bioactivity of released BDNF using a DRG neurite outgrowth bioassay.
DRG cell bodies were cultured for two days with BDNF released from the PLGA-HAMC composite in vitro and demonstrated increased neurite outgrowth relative to controls. Quantification of the neurite outgrowth as a measure of bioactivity is described in Chapter 2.

2. Investigate the effect of locally delivered BDNF on the functional recovery and synaptic plasticity of stroke-injured rats.

2.1 Test the diffusion of BDNF into the stroke-injured rat brain from the PLGA-HAMC composite

BDNF was found to diffuse into the brain of stroke-injured rats from the epi-cortically placed PLGA-HAMC composite over a period of 21 days. The protein diffused to a depth of at least 3 mm, as demonstrated by a depth analysis, shown in Chapter 3.

2.2 Assess the functional recovery and synaptic plasticity of rats treated with the sustained local delivery of BDNF.

Rats treated with the BDNF-loaded PLGA-HAMC composite demonstrated early improved forelimb performance, as well as a delayed improvement in hindlimb function. Synaptophysin expression increases in the contralesional hemisphere of these animals were observed, indicating enhanced synaptic plasticity induced by BDNF delivery, discussed in Chapter 3.
6 Recommendations for Future Work

Recommendations for future investigation that builds off of this work falls into three main categories: (1) nanoparticles for negatively charged protein delivery, (2) modification of the BDNF delivery paradigm, (3) BDNF combinatorial strategies and (4) additional recommendations.

6.1 Modifications to the BDNF Delivery Paradigm

Time is a critical aspect of stroke injury progression and many questions still exist surrounding the best time to deliver therapeutics for optimal efficacy and to avoid causing additional harm. The tunable nature of the PLGA-HAMC composite should enable the design of a formulation that begins to release the protein immediately upon administration, but still sustains delivery for a period of weeks. Adjusting the molecular weight of the PLGA to be above that used in this work would mean less carboxylic acid end groups would be exposed, decreasing the negative charge of the particles and allowing for faster release, as shown in Chapter 2. Additionally, by foregoing the inclusion of MgCO$_3$, acidic degradation byproducts would not be buffered and the auto-catalytic nature of the system would increase the rate of degradation, which in turn would speed release. Developing this system for immediate release would also provide an opportunity to investigate delivering the BDNF-loaded composite 3-4 days post-injury. This would more closely adhere to a clinical timeline, allowing the injury to stabilize [48] and become more permissive for plastic changes. In Chapter 1, we discussed the inflection point at which AMPA receptor activation changes from detrimental to beneficial, owing to its relationship with excitatory glutamate that is overabundant in acute stroke injury [214]. While this strategy would pass up the opportunity to influence neuroprotection with BDNF or vehicle, it would answer important questions concerning therapeutic efficacy in a realistic setting.

Placement of the PLGA-HAMC composite presents another interesting modification that could be made to the existing paradigm. As we mention in Chapter 5, the placement of the composite for this work was directed by the peri-lesional tissue target as well as the drilling necessary for the administration of Et-1 to create the injury. With the observed effects on the contralesional hemisphere, it may be beneficial to target this uninjured tissue, though in a clinical setting, risk of infection to this healthy hemisphere might dissuade the use of this tactic. Some groups have demonstrated benefit in stroke treatment by delivering a biomaterial depot of drug
directly into the lesion site [138]. For a superficial injury, such as the Et-1 stoke used throughout this thesis, this presents little risk of causing additional damage, and may even provide some neuroprotection with the anti-inflammatory properties of the high molecular weight HA used in the HAMC hydrogel. The presence of PLGA nanoparticles in this scenario may pose a problem, however, as delivery directly into the lesion site would restrict diffusion of acidic degradation products, potentially exacerbating the hostile conditions of the stroke microenvironment.

6.2 BDNF Combinatorial Strategies

The endogenous pathways that govern healthy brain function and reparative processes alike are complex and still poorly understood. What we do know is that no one pathway operates in isolation; each has their specific role to play, but they do so in tandem with multiple others. This gestalt effect suggests that the delivery of just one molecule may be insufficient to affect the kind of recovery we hope to see in stroke therapy. Although there has been substantial evidence demonstrating the potential of BDNF as a therapeutic agent for stroke, the complexity of the brain may call for combinatorial approaches to plasticity modulation.

6.2.1 BDNF Delivery with Additional Exogenous Factors

The PLGA-HAMC composite has been formulated for use with other factors previously, and been shown to be suitable for the delivery of multiple factors simultaneously or in a sequential fashion [143]. There are many molecules that are appealing candidates for delivery with BDNF, and here we highlight two that would act synergistically with BDNF to promote plasticity and recovery.

6.2.1.1 Immune Modulation with PACAP

Microglia are the resident immune cells of the CNS, phagocytosing debris and detecting pathogenic bacteria and molecular signals of injury with Toll-like receptors (TLR) and NOD-like receptors (NLR). The pathological cascade of events initiated by stroke result in the activation of these cells, from M2 to M1 phenotype, and initiates the transcription of pro-inflammatory genes. While this activation is necessary in the acute phase of injury, when dead cells and the apoptotic factors they secrete must be cleared away, the continued presence of the M1 phenotype in chronic stages of injury may limit recovery. With this polarity, microglia have the potential to secrete both pro- and anti-inflammatory cytokines and can contribute to either destructive or
reparative processes [250]. Pituitary adenylate cyclase-activating polypeptide (PACAP) is an endogenously produced molecule that is known to reverse the activation of microglia, facilitating their polarization back to the M2 phenotype. Experimentation both in vitro and in vivo has demonstrated multiple beneficial effects of this neuropeptide. When administered either just before or a few hours following an MCAO stroke in mice, lesion volume and neurological deficits were found to be diminished [251].

Modulating the immune system may create a more permissive environment for plastic changes to occur following ischemic injury, and make a combinatorial strategy with PACAP and BDNF an exciting avenue to explore. Understanding the roles of both microglial phenotypes would be helpful to determine the appropriate timing of PACAP administration, though the aforementioned study indicates that early or even pre-emptive administration would be beneficial. Delivering PACAP and BDNF immediately following the injury might enhance neuroprotection [252],[253], though it may also be beneficial to delay the administration of BDNF by 3-4 days until the injury has stabilized to avoid exacerbating excitotoxicity. Most studies that have investigated the use of PACAP in stroke have not harnessed the ability of biomaterials to provide sustained release of this compound, opting to use systemic, intravenous or intraventricular delivery methods instead. This presents an opportunity to deliver PACAP in a novel way that could be beneficial to the effects already observed with this molecule.

6.2.1.2 Glial Scar Degradation with ChABC

CSPGs are an integral component of the regular ECM in a mature brain, forming perineuronal nets to stabilize connections. In a healthy brain, these are crucial structures, as they enable long-term memory formation and the preservation of circuits required for normal function [254]. With stroke injury, this architecture is damaged along with cells, inducing a state of heightened plasticity. As the brain hastens to limit the extent of the damage, CSPGs are used to wall off the site of injury, forming the glial scar [104]. A crucial process in the acute phase, the glial scar becomes more of a barrier to axon regeneration in chronic stages, diminishing the plastic potential that is required for recovery [53]. Sequential delivery of chondroitinase ABC (ChABC), followed by BDNF would act to first remove this barrier to plasticity and then encourage and support plastic processes.
The main challenge with this combination of molecules is that the PLGA-HAMC composite would not be suitable for the delivery of ChABC, as this enzyme would quickly degrade the hyaluronan component of the hydrogel. Successful delivery of bioactive ChABC to the spinal cord has been achieved using a crosslinked methylcellulose (XMC) hydrogel [255] that could potentially be used in combination with a BDNF-loaded PLGA-HAMC composite. Injection of the ChABC-loaded XMC gel into the lesion site and situating the BDNF-loaded composite above may offer a feasible solution to this problem, though this combined factor strategy might require the creation of a novel biomaterial system that can accommodate both molecules. The glial scar in stroke is still an area that requires more investigation, as most studies have focused on the effects of degrading this barrier to regeneration in spinal cord injury models [50]. As with immune modulation, timing is important to consider, and by furthering our understanding of the glial scar in stroke, we may be better equipped to determine the optimal timing for sequential delivery of these factors.

### 6.2.2 BDNF Delivery with Cells

In Chapter 1, we discussed the potential of exogenous cell sources to replace lost or damaged tissue and to contribute to the reparative process by the secretion of various factors, including BDNF. In collaboration with the Nagy lab, we have explored the use of cortically specified neuroepithelial stem cells for stroke therapy, using the HAMC hydrogel to improve survival during the transplantation process [256]. In order to culture these cells effectively, BDNF is included in the media as a pro-survival factor, and inclusion of this protein in the hydrogel during transplantation would likely have similar benefits. In this application BDNF would not only support the cells in the hostile stroke microenvironment, but would also be able to diffuse into surrounding tissue and influence plastic changes in host circuitry. This combination may also facilitate integration of the transplanted cells by encouraging axon growth and synaptic strengthening between exogenous and endogenous cells. BDNF is also known to direct differentiation of stem cells toward the neuronal fate [257], and this may contribute to the replenishment of neurons in the infarct core.

### 6.2.3 Environmental Enrichment and Rehabilitation Therapy

There are a multitude of studies demonstrating a link between BDNF expression and physical activity. Our collaborators in the Corbett lab have been very interested in BDNF and its
involvement in plastic processes as they relate to rehabilitation therapy. A study performed by their lab looked at the effect of blocking the expression of BDNF in the contralesional hemisphere on rehabilitation therapy. With this, they demonstrated that BDNF expression is required in order to accrue the benefits they normally observed with rehab therapy [213]. With this knowledge, we can postulate that delivery of exogenous BDNF to the stroke-injured brain in conjunction with rehabilitation therapy may facilitate an enhanced effect over the two strategies alone. BDNF may be capable of initiating plasticity, but without the guidance of repetitive movement or specific tasks, it may only lead to the generation of aberrant synapses that could actually detract from the recovery efforts [235]. Environmental enrichment is an essential component of the Corbett lab rehabilitation protocol, providing animals with larger, multi-level cages and different stimulatory objects that are changed weekly to incite animal interest [246]. In the course of this thesis, steps were taken towards the eventual combination of these therapies; however, rehabilitation studies are non-trivial to perform with even greater logistical challenges than a regular behavioural study as rehabilitation therapy must be performed twice daily for the duration of the testing period following injury/therapeutic delivery. Unfortunately, a pilot study we performed in our animal facilities was unable to reproduce the rates of recovery the Corbett lab has been able to achieve (Appendix 11), and so was not pursued further. Though challenging to execute, a study that combined both of these treatments is still worthy of consideration and may be facilitated through greater collaboration.

6.3 Nanoparticles for Negatively Charged Protein Delivery

In Chapter 2, we described the mechanism responsible for our controlled release system without the need for protein encapsulation in polymer nanoparticles. The proteins of interest that this phenomenon was originally discovered with shared the characteristic of being positively charged at physiological pH, all with isoelectric points above 10. As the negatively charged PLGA nanoparticles degraded, they released acidic byproducts that protonated the carboxylate anions, neutralizing the interaction between protein and particle and allowing protein release from the composite. While many therapeutic proteins are positively charged, there are also a host of negatively charged proteins and the delivery of these could potentially benefit from a similar encapsulation-free formulation. EPO is one such protein that has been used in the treatment of stroke [258] and the similarly low pIs of DNA [259] and siRNA make them potential candidates for this strategy as well. Formulation of a nanoparticle with a net positive charge that experiences
similar self-catalyzing bulk degradation in an aqueous environment would be a challenge.

Chitosan is a natural polymer that has been formulated into cationic nanoparticles for drug delivery by other groups [260] and may provide a good starting point. Cationic coatings such as polyethyleneimine (PEI) [261] and poly(L-Lysine) [259] are also frequently used to produce particles with a positive surface charge and could be applied to the surface of PLGA particles, with the degradation of the coating enabling release of the negatively charged molecule. Another potential material option is a poly-glutamine peptide, though this peptide has been linked to a number of progressive neurodegenerative diseases [262]. Many cationic particles have also demonstrated cytotoxic effects [263], rendering this recommendation more of a proof of concept unless this toxicity is addressed.

6.4 Additional Recommendations

6.4.1 Variability in Animal Behavioural Studies

Animal studies that include behavioural assays require a great deal of familiarity with the protocols in order to collect meaningful results. Even when performed by experienced personnel, the inherent variability of these studies make large group numbers a necessity in order to observe any significant effects between groups or even within groups. It is for this reason that we recommend having dedicated animal handlers or technicians for these studies that would be responsible for all training, testing and surgical procedures.

As there is already inherent variability between individual animal neurovasculature, it is impossible to avoid slight differences in the location and degree of injury produced with the Et-1 peptide. This variability is exacerbated by the use of a stereotaxic apparatus that is not properly calibrated and fitted with the appropriate arms for drilling and injection procedures. Maintaining equipment used for these surgeries would serve as an easy way to decrease variability in these studies.

6.4.2 X-Clarity™ System for Full-Brain Imaging

In order to quantify the amount of BDNF diffusing into the tissue, the tissue was snap-frozen upon sacrifice, sectioned and homogenized for use with a tissue ELISA. Using a fluorescently labeled BDNF protein would allow a system such as X-Clarity™ to be used to image the full brain without sectioning and would provide a better representation of the diffusion
profile over time [264]. Using a transgenic mouse model that expresses fluorescently-tagged BDNF, such as the firefly luciferase BDNF transgenic mouse created by Fukuchi et al. in combination with X-Clarity™ would also allow investigation of the effect of exogenously delivered BDNF on the activation of endogenous BDNF expression [265]. This would help to visualize the feed-forward cascade of BDNF expression and compare in vitro and in vivo release profiles. Additionally, this system could be used to image various tissue markers, such as synaptophysin, using genetic strategies to express fluorescently tagged proteins [266] to better elucidate tissue effects of this treatment strategy.
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7 Appendices

7.1 Stroke Model Development

In order to observe any significant benefit of delivered BDNF, we required a deficit that would not succumb to spontaneous recovery during the course of the treatment and testing periods. Initially using only two Et-1 coordinates in the motor cortex to create the injury, we saw many animals recovering to levels that would not allow a therapeutic window to be maintained. On the recommendation of the Corbett lab, we included an additional coordinate in the striatum, as this structure receives multiple afferent inputs from the cortex and is largely responsible for integrating and coordinating motor processes [267] [268]. With this additional lesion site, we achieved a level of behavioural deficit that was sustained throughout our experimentation (Figure A1), and as such, this was the model used throughout this thesis work.

Figure A1: Additional Et-1 injection site in the striatum produces a deficit that is maintained throughout the required experimental timeline as measured by the staircase reaching task. Data plotted as mean ± standard deviation, n = 5 per group.
7.2 Release of NT-3 from a PLGA np/hydrogel DDS containing encapsulated and soluble NT-3

Figure A2. (A) Addition of soluble NT-3 increases the cumulative mass of NT-3 released from the PLGA np/hydrogel DDS, but not (B) the cumulative percent release. The release profile remains largely unchanged (n = 3, mean ± standard deviation plotted). Data for “NT-3 encapsulated in PLGA np + soluble NT-3” was reproduced from (17) with permission from the Royal Society of Chemistry.
7.3 Bioactivity of proteins released from hydrogels with embedded PLGA np

Figure A3. (A) Activity of released NT-3 was assessed using a DRG neurite outgrowth assay. Released NT-3 elicits significantly increased neurite outgrowth for up to 10 days compared to a 0 ng/mL control, with a trend towards increased outgrowth for 21 days (n = 3, mean ± standard deviation plotted). (B) SDF activity was assessed using a neurosphere migration assay. Released SDF caused significantly higher neural stem/progenitor cell migration for up to 7 days compared to a 0 ng/mL control, with a trend towards increased migration for 28 days (n = 5 independent releases, mean ± standard deviation plotted). (C) Activity of released BDNF was assessed using a DRG neurite outgrowth assay. Released BDNF elicits significantly increased neurite outgrowth for 42 days compared to a 0 ng/mL control (n = 3, mean ± standard deviation plotted). (* p < 0.05, ** p < 0.01, *** p < 0.001).
7.4 Characteristics of PLGA np

Figure A4: (A) Representative TEM images of PLGA np stained with uranyl acetate. Scale bar is 100 nm. (B) Representative dynamic light scattering trace. Nanoparticles have an average diameter of ~300 nm with a PDI of ~0.2.
7.5 Swelling of HAMC hydrogel with and without PLGA np

Figure A5: HAMC with 10 wt% PLGA np has a significantly higher swelling ratio than HAMC alone between 3 and 28 days (p < 0.05, n = 3, mean ± standard deviation plotted).
7.6 Calculation of the relative surface area for PLGA np of different sizes

Assuming spherical nanoparticles, the mass of nanoparticles within the gel is given by

\[ m = \rho N \frac{4}{3} \pi r^3 \]

where \( N \) is the total number of nanoparticles within the gel.

Given equal masses of two different nanoparticle populations where \( r_2 = 3r_1 \) and assuming equal nanoparticle densities

\[ \rho N_1 \frac{4}{3} \pi r_1^3 = \rho N_2 \frac{4}{3} \pi (3r_1)^3 \]

So

\[ N_1 = 27N_2 \]

The total nanoparticle surface area within the gel is

\[ N4\pi r^2 \]

Therefore

\[ S_1 = N_1 4\pi r_1^2 \]

\[ S_2 = \left( \frac{N_1}{27} \right) 4\pi (3r_1)^2 \]

\[ S_2 = \left( \frac{9}{27} \right) S_1 = \frac{S_1}{3} \]

So for a nanoparticle 3 times the radius, we obtain three times less surface area for the same mass.
Calculation for maximum surface coverage

These calculations are done using SDF (MW ~ 8000 Da) as an example. Assuming a spherical (globular) protein with hydrodynamic radius of approximately 2 nm and a nanoparticle radius of approximately 150 nm (based on our DLS measurements in Appendix 4), we have

\[ A_{\text{protein}} = \pi (2)^2 = 12.57 \text{ nm}^2 \]
\[ S.A_{\text{particle}} = 4\pi (150\text{nm})^2 = 2.83 \times 10^5 \text{nm}^2 \]

This means there would be approximately

\[ \frac{2.83 \times 10^5 \text{nm}^2}{12.57 \text{nm}^2} = 22513 \]

proteins per nanoparticle given complete surface coverage.

The mass of this number of proteins is

\[ m_{\text{protein}} = \frac{22513 \times \text{MW}}{N_A} = \frac{22513 \times 8000 \text{g/mol}}{6.02 \times 10^{23}} = 2.99 \times 10^{-16} \text{g} \]

The mass of a nanoparticle is

\[ m_{\text{particle}} = \frac{4}{3} \pi r^3 \rho = \frac{4}{3} \pi (0.000015 \text{cm})^3 \left( 1.5 \frac{\text{g}}{\text{cm}^3} \right) = 2.12 \times 10^{-14} \text{g} \]

Assuming a density of 1.5 g/cm³. So the ratio of protein to polymer by mass that gives 100% coverage is

\[ \frac{2.99 \times 10^{-16} \text{g}}{2.12 \times 10^{-14} \text{g}} \times 100\% = 1.41\% \]
7.7 Effect of pH on the Release of SDF

Figure A6: Release of soluble SDF from XMC alone into aCSF at pH 3, pH 5, or pH 7. Release results indicate no effect of varying pH on protein detection by ELISA ($n = 3$, mean ± standard deviation plotted).
7.8 Mass loss of PLGA from the release system at pH 3 and 7

Figure A7. There is no significant mass loss of PLGA from the hydrogel over the first 7 days of release, regardless of pH (p > 0.05 at all timepoints, n = 3, mean ± standard deviation plotted).
7.9 Example image for quantification of syn$^+$ pixels in ROIs R1 and R2.

Figure A9. The same ROIs were used as for the quantification of the number of neurons surrounding the stroke lesion. R1 boxes and R2 boxes were averaged to give values for synaptophysin pixel density quantification. Scale bar represents 500 $\mu m$. 
7.10 Additional In Vivo BDNF Diffusion Study

Male, Long-Evans rats received a cortical-only stroke injury and were administered the BDNF-loaded after a period of 4 days. At 13 and 20 days post-delivery, animals were sacrificed and tissue was prepared in the same manner as previously described. BDNF was quantified in the tissue using an ELISA, and was detectable above endogenous levels to a depth of 3 mm. Additional depths were included in this experiment, extending the depth to 5 mm and thus including the tissue from the lateral ventricles. In this deeper tissue, we detected an increase above endogenous levels again, suggesting potential circulation of BDNF through the ventricles via the CSF.

Figure A10. BDNF diffuses into stroke-injured rat brain tissue from PLGA-HAMC composite deposited epi-cortically, 4 days post-stroke (n = 4). Data presented as mean ± standard deviation.
7.11 Rehabilitation and Enriched Environment Pilot Study

With the hypothesized synergy between BDNF and enriched rehab (enriched housing environment with rehabilitation therapy) to guide plastic changes, we performed a pilot study to determine if the enriched rehab paradigm could be recapitulated in our hands. Female Sprague-Dawley rats were used as they offered three potential advantages over males: 1) females are less aggressive and would fight less amongst cage-mates, 2) females hit a growth plateau earlier in development than males and would not have to be switched to larger staircase task boxes at weeks 5 and 7, decreasing potentially confounding factors, and 3) the Corbett lab has experienced shorter learning curves for the staircase task with females, resulting in faster training and more animals meeting criteria. The study was designed to Corbett lab specifications [160] and was taken out to 5 weeks post-stroke (Figure A11). We used the coordinates developed by the Corbett lab specifically for the female Sprague-Dawley forelimb deficit to create the Et-1 injury in this study:

\[
\text{AP} + 2.3 \text{ mm, ML +/- 2.5 mm, DV - 2.2 mm}
\]

\[
\text{AP} 0 \text{ mm, ML +/- 2.5 mm, DV - 2.2 mm}
\]

\[
\text{AP} + 0.7 \text{ mm, ML +/- 3.8 mm, DV – 6.9 mm}
\]

Enriched rehab rats were housed in enriched environment cages and post-stroke and received rehabilitation therapy (forelimb reaching with modified staircase inserts) twice daily for the duration of the study, where non-rehab animals were housed in standard rat cages and did not receive rehab therapy sessions. The staircase task, the tapered beam and the cylinder task were used to assess behaviour and efficacy of the rehab protocol. Unfortunately, the significantly improved behavioural function with enriched rehabilitation observed by the Corbett lab previously was not seen in the staircase task results of this study. Therefore, this avenue of investigation was not pursued further.
Figure A11: Failure to demonstrate benefit of rehabilitation therapy in study performed at the University of Toronto under the guidance of the Corbett lab. (A) Study paradigm, indicating when training, testing and rehabilitation was performed. (B) Standard care animals were housed in standard rat cages and did not receive any rehabilitation sessions, n = 7. (C) Enriched rehabilitation animals were housed in larger cages with extra environmental stimulation, n = 11. Data presented as mean ± standard deviation.