Neurotrophin-3 as a promoter of spinal cord plasticity in a distal middle cerebral artery occlusion model of stroke

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Abstract

Ischaemic stroke is a cause of locomotor disability and, in combination with other cardiovascular diseases, is the 3rd most common cause of death worldwide. It occurs when a region of the brain is deprived of oxygen and metabolites through occlusion of a blood vessel in the brain and as a result, the affected area of the brain is unable to perform normal functions. Stroke often leads to disability and neglect of the opposite side of the body, which requires effective, efficient treatments.

This project used a model of large, permanent, ischaemic stroke to measure functional recovery in adult and elderly rats following treatment with neurotrophin-3. To do this, blood flow to the sensorimotor cortex was reduced through the use of a distal, permanent occlusion of the middle cerebral artery with the addition of common carotid artery occlusions, for a three-vessel occlusion model. The model was reproducible, permitting assessment of forelimb sensory or motor function. Additionally, the model was well tolerated in elderly rats, and indeed, produced more sustained deficits from which to measure functional repair. However, in most cases, there was substantial spontaneous recovery in adult rats, and NT3 treatment did not lead to any detectable benefit against this background of recovery.

Neurotrophin-3 is essential during development, particularly for spinal reflexes involved in movement. Clinical trials using NT3 in other conditions have shown that systemic, high doses are well tolerated. The studies in this thesis show that neurotrophin-3 produces modest effects on behavioural recovery, in addition to reducing hyper-reflexia when treatment is initiated subcutaneously 24 hours after stroke, which is a feasible time-frame for delayed treatment in human beings.

One objective of this project was to also identify recovery mechanisms behind improvement observed with or without neurotrophin-3 treatment. One way to do this was with the use of functional MRI techniques and measurement of spontaneous recovery networks of activity. From these data it was possible to identify brain regions important in spontaneous recovery. In addition, mRNA regulation was assessed with RNASeq of cervical dorsal root ganglia to determine changes in gene expression associated with neurotrophin-3 treatment after stroke. Genes were identified that could be involved in this mechanism, and could be important for future work in this field.

This work shows that subcutaneous delivery of neurotrophin-3 improves function in elderly rats, when treatment is initiated 24 hours after a large ischaemic stroke. This adds to the existing literature on the use of this therapy following an ischaemic stroke.

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Table of Abbreviations

Abbreviation	Definition
5HT	Serotonin
AAV	Adeno-associated virus
AIC	Akaike's Information Criterion
AR1	First order autoregressive model of covariance
ARRIVE	Animal Research: Reporting of *In Vivo* Experiments
BBB	Blood-brain barrier
BDA	Biotinylated dextran amine
BDNF	Brain Derived Neurotrophic Factor
bFGF	Basic fibroblast growth factor
BOLD	Blood Oxygen Level Dependent
BST	Bulbospinal tract
C	Cervical
CBF	Cerebral blood flow
CCA	Common Carotid Artery
chABC	Chondroitinase ABC
CNS	Central nervous system
CPu	Caudate putamen
CS	Compound symmetric model of covariance
CSF	Cerebro-spinal fluid
CSPG	Chondroitin sulphate proteoglycans
CST	Corticospinal tract
CT	Computed tomography
DNA	Deoxyribonucleic Acid
DRG	Dorsal Root Ganglion
ECA	External carotid artery

Abbreviation	Definition
EEG	Electroencephalography
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked Immunosorbent Assay
EMG	Electromyogram
Emr1	Gene associated with macrophage accumulation
EPI	Echo planar imaging
ERC	European Research Council
fMRI	Functional magnetic resonance imaging
FOV	Field of view
FSE	Fast spin echo
GABA	Gamma aminobutyric acid
GAG	Glycosaminoglycan
GAL	α-galactosidase gene
GAP43	Growth associated protein 43
H-reflex	Hoffman's reflex
HBB	Beta globin gene
Ia	Primary
ICA	Internal carotid artery
Ig	Immunoglobulin
IL-6	Interleukin-6
IN1	Anti-Nogo1 antibody
M1	Primary motor cortex
MAPK	Mitogen-activated protein kinase
MCA	Middle cerebral artery
MCAO	Middle cerebral artery occlusion
MCS	Multiple cloning site

Abbreviation	Definition
MEG	Magnetoencephalography
MR	Magnetic resonance
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MTHFR	Methylenete-tetrahydrofolate reductase
NACWO	Named Animal Care and Welfare Officer
NC3R	National Centre for the Replacement, Refinement and Reduction of Animals in Research
NGF	Nerve Growth Factor
NHS	National health service
NICE	The National Institute for Health and Care Excellence
NINDS	National institute of neurological diseases
NMDA	N-methyl D-aspartate
NO	Nitric oxide
NT3	Neurotrophin 3
$p75^{NTR}$	p75 neurotrophin receptor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PET	Positron emission tomography
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-kinases
PKCγ	Protein kinase C gamma
PLCγ	Phospholipase C gamma
PMd	Premotor cortex
PNS	Peripheral nervous system
RNA	Ribonucleic acid

Abbreviation	Definition
RNASeq	RNA Sequencing
ROI	Region of interest
rsMRI	Resting state functional magnetic resonance imaging
RST	Reticulospinal tract
s. cut	Subcutaneously
S1	Primary sensory cortex
S1FL	Primary sensory cortex of the forelimb area
S2	Secondary sensory cortex
SCI	Spinal cord injury
SD	Standard deviation
SEM	Standard error of the mean
sMRI	Structural magnetic resonance imaging
SN	Substantia nigra
STAIR	Stroke Therapy Academic Industry Roundtable
T	Tesla
T	Thoracic
TE	Echo time
TENS	Transcutaneous Electrical Nerve Stimulation
TIA	Transient Ischaemic Attack
TMS	Transcranial magnetic stimulation
TNFα	Tumour necrosis factor alpha
tPA	Tissue plasminogen activator
TR	Repetition time
Trk	Tropomyosin-related kinase
UN	Unstructured model of covariance
WHO	World Health Organisation

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Publications

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Wayman, C, Duricki, DA, Mesquita, M, Wood, T, Cash, D, Moon, L.D.F (2015) Spontaneous changes in sensorimotor function and BOLD fMRI maps in adult rats after cortical stroke Brain Conference Abstract, Vancouver, Canada

Duricki D.A., Wayman C., Cash, D., Westphal, R.R., Mesquita, M.B., Wood T.C. and Moon L.D.F (2014). Exploring the correlation of behavioural scores and resting state functional MRI connectivity in young adult and aged rats, in a permanent distal middle cerebral artery occlusion model of stroke. *Society for Neuroscience* Conference Abstract, Washington DC, USA

Chapter 1 Introduction

1.1 Stroke

1.1.1 Rationale for Stroke Research

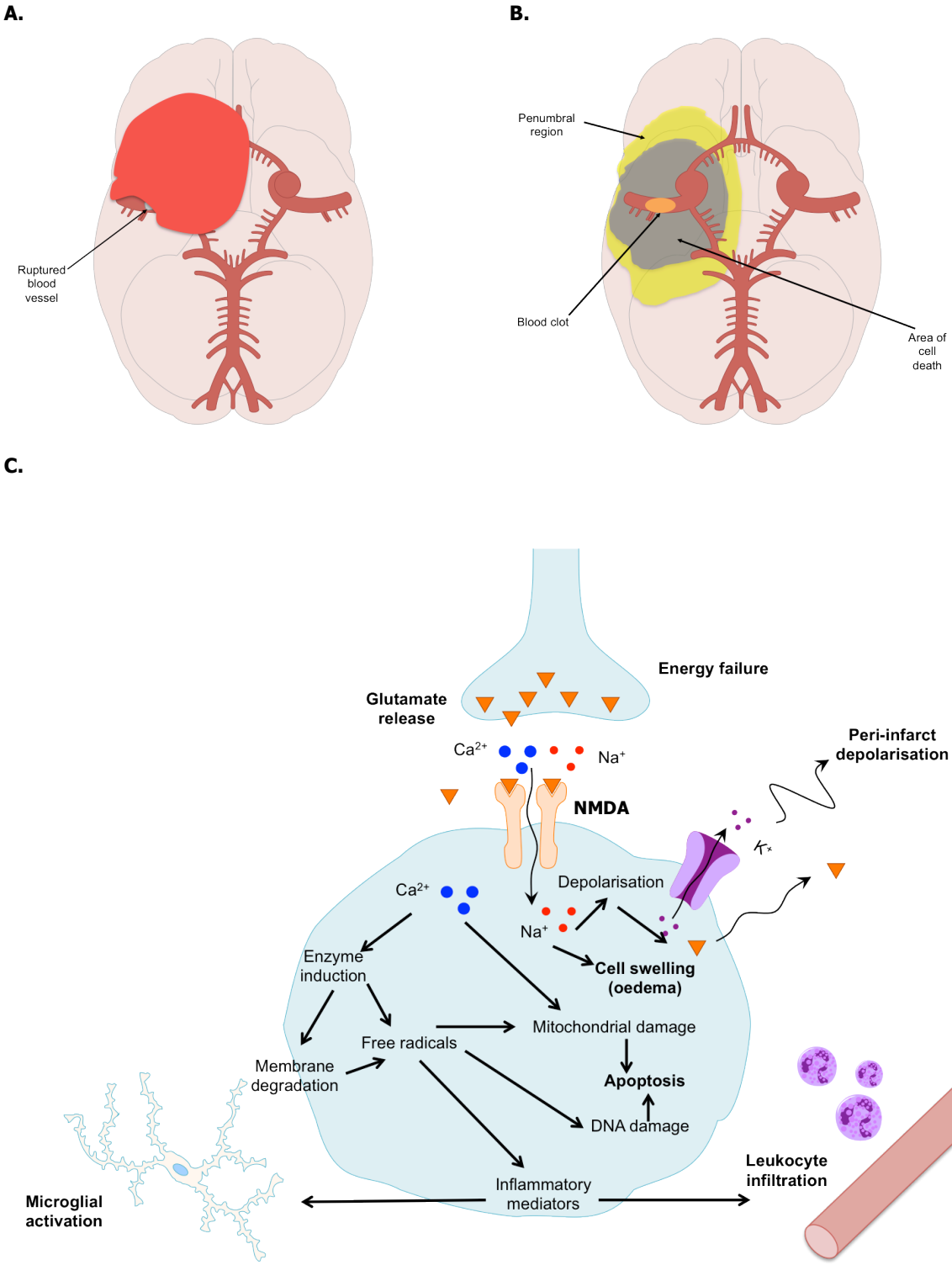
A stroke occurs once every 2 seconds worldwide (Feigin et al., 2014) and is a devastating disease for those who suffer from it. CNS infarction is brain, spinal cord, or retinal cell death attributable to ischaemia, based on pathological, imaging, or other objective evidence of ischaemic injury of these regions in a defined vascular distribution; or clinical evidence of cerebral, spinal cord, or retinal focal ischaemic injury based on symptoms persisting ≥24 hours or until death, and other causes excluded (Sacco et al., 2013). In the UK, 152,000 people suffer a stroke annually (Townsend et al., 2012) and a quarter of these events are fatal within one year (Office of National Statistics, 2015). Of the survivors, half of patients are left permanently disabled; indeed, stroke is the biggest cause of locomotor disability in the United Kingdom and places a huge burden on the healthcare system (Adamson et al., 2004, Mackay and Mensah, 2004).

1.1.1.1 Stroke subtypes and Pathophysiology

Stroke is a disease where blood flow to a focal region of the brain is diminished due to the rupture of a blood vessel or a blockage in the arteries, preventing oxygen and glucose from reaching regions of the brain. The management of these subtypes is different and, therefore, the clinical distinction of these events in a time-effective manner is crucial for treatment and long-term management (Donnan et al., 2008).

1.1.1.1.1 Haemorrhagic Stroke

Haemorrhagic stroke occurs in approximately 20% of all stroke patients (Auer and Sutherland, 2005); it tends to be more severe and have increased mortality compared to ischaemic strokes (Royal College of Physicians Sentinal Stroke National Audit Programme, 2014). 75% of haemorrhagic strokes occur when the walls of blood vessels dilate and weaken, typically in arterioles leading from the basilar arteries or anterior, middle, or posterior cerebral arteries. This aneurysm can then rupture (Figure 1-1A), causing blood from the vessels to leak into the parenchyma (Qureshi et al., 2001).


A.
Ruptured blood vessel
B.
Penumbral region
Blood clot
Area of cell death
C.
Energy failure
Glutamate release
Ca2+
Na+
NMDA
Peri-infarct depolarisation
K+
Depolarisation
Enzyme induction
Cell swelling (oedema)
Mitochondrial damage
Free radicals
Membrane degradation
Apoptosis
DNA damage
Leukocyte infiltration
Inflammatory mediators
Microglial activation

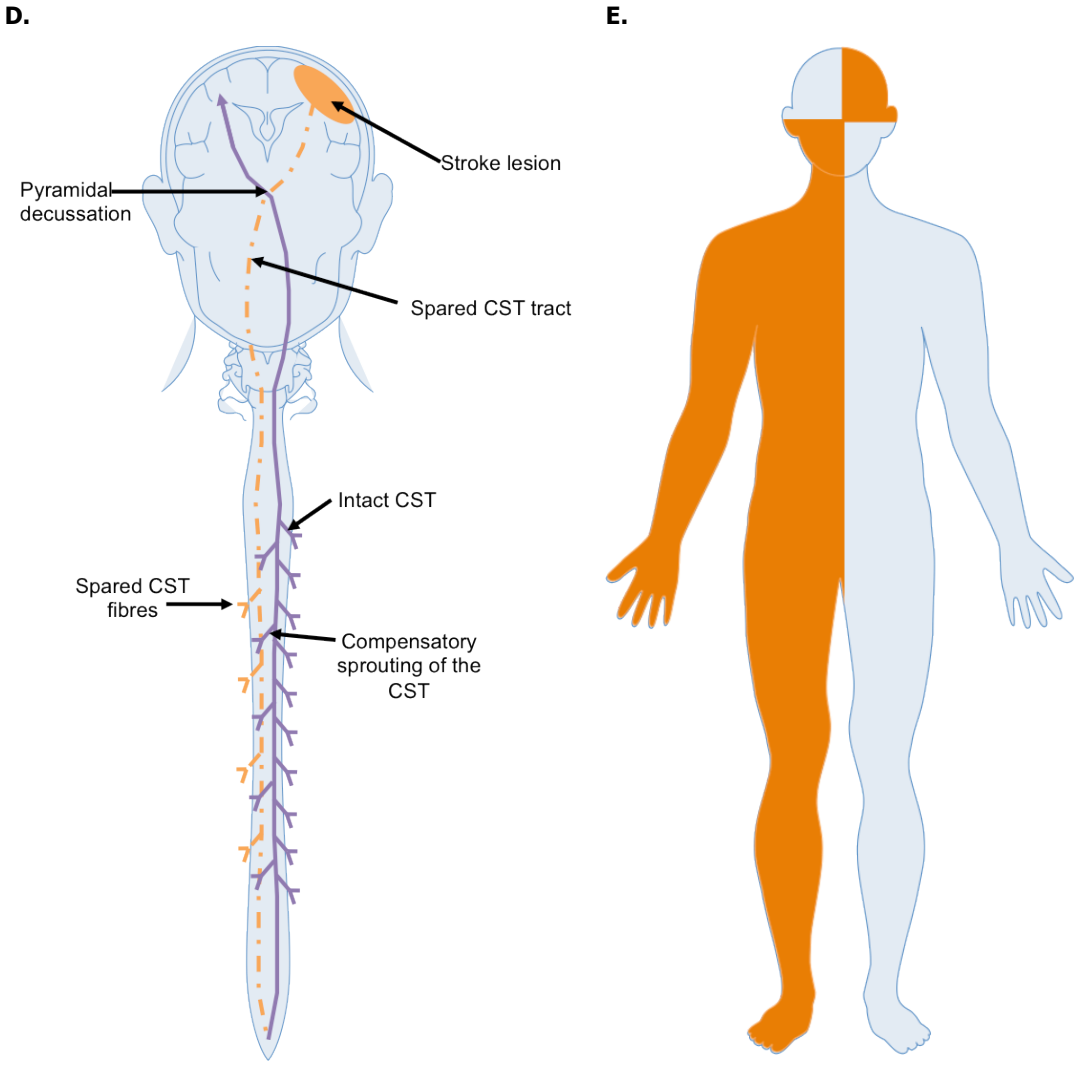


Figure 1-1 : Stroke subtypes, the ischaemic cascade and connectivity after stroke

A. Schematic of a haemorrhagic stroke where a blood vessel has ruptured leading to a bleed within the brain. B. Schematic representation of an ischaemic stroke, where a blood clot (orange) has occluded an artery and resulted in restricted blood flow to an area of brain. This leads to a lesion core (grey), which is surrounded by an area of ischaemic damage, known as the penumbra (yellow). An overview of the ischaemic cascade is shown in C. Energy failure leads to the depolarisation of neurons and the release of glutamate. Increased glutamate receptor activity results in Ca^{2+} and Na^{+} entry, and K^{+} release, leading to peri-infarct depolarisations. Oedema is caused by water entry into the intracellular spaces. Finally, free radicals are generated, resulting in an increase in inflammatory mediators, activating microglia, inflammatory cell recruitment, and ultimately apoptosis. Adapted from Dirnagl et al., 1999. After a stroke lesion in the territory of a middle cerebral artery (D.), the main sensory and motor symptoms that are observed are caused by the degeneration of the corticospinal tract (CST). The recruitment of spared fibres and the promotion of compensatory sprouting of the CST are key targets for recovery. In E., a schematic demonstrates that hemiparesis affects the contralateral side of the body to the lesion side.

Subarachnoid haemorrhages form the remaining 25% of haemorrhagic strokes and are even more devastating than intracerebral haemorrhages with mortality in 25% of patients within 24 hours (Intercollegiate Stroke Working Party, 2012). This subcategory of haemorrhage results from blood leaking into the area between the brain and the skull.

The accumulation of blood within the skull results in high intracranial pressure leading to compression of brain tissue, which can distort and therefore damage areas of the affected brain (Auer and Sutherland, 2005).

1.1.1.1.2 Ischaemic Stroke

Ischaemic strokes make up the remaining 80% of all strokes and can have cardioembolic, thrombotic or lacunar origin (Donnan et al., 2008). An embolic event occurs when a clot or part of a clot is dislodged from another organ and is relocated *via* the bloodstream to the brain, where the clot lodges in the narrowing arteries, arterioles, or capillaries of the brain. Alternatively, a thrombus can form within the artery, usually around an atherosclerotic plaque, restricting blood flow in strokes of thrombotic origin. Both of these events result in the prevention of oxygenated blood reaching a region of the brain. The area immediately surrounding the occluded blood vessel, the so-called ischaemic core, loses the ability to exchange metabolites within minutes. The oxygen-deprived tissue surrounding the lesion is known as the penumbra (Figure 1-1B), and many current treatments for stroke rely on improving outcome in this area, as neurons have been shown to be able to survive for many hours (or even up to 10 days) in the penumbra (Dereski et al., 1993). Subsequent reperfusion of this tissue with oxygenated blood can salvage the tissue and result in an improved functional outcome.

Reperfusion of the tissues is not without its dangers. The inflammatory response occurring after the re-establishment of circulation has a role in producing reperfusion injury, which can be deadly for patients (Warach and Latour, 2004). Although not a definitive cause of reperfusion injury, inflammatory components such as cytokine release and leukocyte adhesion play a key role, and are components of the ischaemic cascade.

1.1.1.1.2.1 Ischaemic chain reaction

An ischaemic event initiates a cascade in the penumbra (Figure 1-1C), where insufficient glucose results in energy failure, depolarisation, excitotoxicity, and peri-infarct depolarisations in the minutes following stroke, with inflammation affecting the brain within 4–6 hours after the event, and apoptosis only occurring days later (Dirnagl et al., 1999). Excitotoxicity, resulting from glutamate overactivity, changes the ionic gradients within the neurons resulting in oedema, which can not only reduce the perfusion of the surrounding areas but also increase intracranial pressure, vascular compression, and herniation of the brain. Calcium influx from overactive NMDA receptors also increases

the synthesis of nitric oxide (NO), which increases local tissue damage by release of the free radical peroxynitrate through the nitric oxide synthase (NOS) pathway. Free radicals such as these then disrupt mitochondrial function and release cytochrome C, a marker for apoptosis (Chamorro et al., 2016).

Simultaneously, activity-dependent glutamate receptors (from the release of glutamate from dying cells in the ischaemic core) result in repetitive depolarisations in the penumbra and calcium uptake into the neuron. Calcium is also crucial for calcium-regulated activation of second messenger systems and, ultimately, the expression of transcription factors for inflammation mediators such as chemokines and tumour necrosis factor alpha (TNFα). These factors cause the activation of microglia and hypertrophy of astrocytes within 4–6 hours and promote the infiltration of monocytes and macrophages into the brain. Finally, apoptosis in the ischaemic penumbra occurs within 1–3 days following cytochrome C and caspase production (Dirnagl et al., 1999).

The initial neurological deficits after ischaemic injury directly correlate with the core injury and the penumbra, with saved penumbral areas showing normal function after 2 weeks in the clinic (Sigler et al., 2009); with effective thrombolysis or other neuroprotective treatment, function can be restored (See Section 1.2 on page 27), as long as treatment is administered within a timeframe to not cause reperfusion injury. The focus of this thesis will be permanent ischaemic stroke, intervening not at acute times, but after the initial ischaemic cascade has passed.

1.1.2 Incidence and Risk Factors

1.1.2.1 Epidemiology

In the UK, stroke is the 4th leading cause of mortality (Office of National Statistics, 2015). There are factors that make an individual more or less likely to suffer a stroke event. Stroke incidence is most strongly associated with age. The risk of stroke doubles every decade after the age of 55 (Wolf et al., 1992, Brown et al., 1996); indeed, 91% of strokes occur in people over the age of 65 (Kelly-Hayes et al., 2003). Stroke occurrence also appears to be affected by ethnicity and race. Black populations have been shown to have double the likelihood of having a stroke compared to their Caucasian counterparts and at younger ages (Wang et al., 2013), largely due to higher incidence of risk factors such as increased blood pressure, diabetes and sickle cell disease (Hajat et al., 2004, Scarborough et al., 2009, Rees et al., 2010). Other epidemiology studies have also shown an increased incidence of stroke in Asian (He et al., 1995) and Hispanic populations (Sheinart et al., 1998).

There is also evidence that gender affects stroke prevalence. Traditionally, it was thought that women were more at risk of stroke compared to men due to oestrogen levels, causing smaller strokes with more oestrogen (Kelly-Hayes et al., 2003). This is supported by the data that show that menopausal women, who have lower concentrations of this hormone, are more at risk of having a stroke than men of similar ages (Lisabeth et al., 2009). However, recent studies show that men are at a 25% higher risk of having a stroke event compared to women, although this is sometimes not recognised as women have a longer life expectancy and therefore collectively have more strokes in total compared with men, who are more likely to die from other causes (Townsend et al., 2012, Royal College of Physicians Sentinal Stroke National Audit Programme, 2014). This discrepancy might be because of previous studies neglecting that both men and women over 65 have similar levels of oestrogen (Khosla et al., 1998). This is supported by the fact that hormone replacement therapy has been associated with increased risk of ischaemic stroke compared with patients without hormonal therapy (Bath and Gray, 2005).

Studies in twins, families, and animal models have suggested a role of genetic risk for ischaemic stroke (Jeffs et al., 1997, Floßmann et al., 2004). Although there has not been any individual gene that has been shown to definitively increase risk of ischaemic stroke, genes that have been shown to convey risk include mutations in NOTCH3; beta-globin (HBB), which conveys sickle cell disease; and GAL, which produces α-galactosidase A. Other genes include homocysteine metabolism enzymes such as methylenete-tetrahydrofolate reductase (MTHFR), or other enzymes such as angiotensin converting enzyme, plasminogen activator inhibitor 1 and phosphodiesterase 4D (Dichgans, 2007).

Although non-modifiable risk factors contribute some ischaemic stroke risk, modifiable risk factors account for most of the risk (Goldstein et al., 2006, Feigin et al., 2014).

1.1.2.2 Stroke Prevention and Modifiable Risk Factors

Given the disease burden of stroke, prevention is a key target for treatment and in 80% of cases strokes could be prevented (Chiuve et al., 2008, Gorelick, 2008), saving costs for the National Health Service (NHS). It has been shown that neglecting to treat these risk factors contributes to increased stroke recurrence, and therefore treating and modifying lifestyle to account for these risk factors is important for both prevention and care of stroke (Xu et al., 2007). After age, the biggest risk factor is hypertension, and treating this could reduce incidence of stroke by up to 40%, with reduction of tobacco intake the other significant modifiable factor (Mackay and Mensah, 2004).

1.1.2.2.1 Hypertension

The most modifiable risk factor is hypertension with evidence showing that reducing blood pressure by a minimum of 6 mmHg can result in a 40% decrease in stroke incidence (Collins et al., 1990, Barone et al., 1992, Chobanian et al., 2003). The number of people with high blood pressure has increased consistently since 2005 and this is a large contributing factor for up to 54% of strokes (Stroke Association, 2015).

With 9.2 million people in the UK registered as hypertensive and many more (up to 6.8 million) undiagnosed (Stroke Association, 2015), there is a clear need to manage these symptoms as they are not only a risk factor for stroke, but also a major factor in ischaemic heart disease.

1.1.2.2.2 Obesity

Obesity is a severe health risk factor for a number of reasons including increased risk of diseases such as arthritis, heart disease, diabetes, and stroke. Being obese increases risk of stroke by 64%; even being slightly overweight can increase the risk by 22% (Strazzullo et al., 2010). However, the stroke risk contributed by obesity is in part mediated through hypertension and diabetes, therefore, risk contributed by each of these risk factors individually is difficult to calculate as they often occur concurrently (Malik et al., 2004). There are, however, direct effects of obesity that result in increased stroke prevalence. Obesity has been shown to cause a prothrombotic state in the body and increases in inflammatory markers (Hotamisligil et al., 1995, Kern et al., 1995), which can cause adverse events such as endothelial wall damage and vascular hypertrophy (Lundgren et al., 1996, Steppan et al., 2001); all of these factors increase risk of ischaemic stroke. A body mass index of more than 30 resulted in significantly higher occurrence of stroke compared to a control group of patients aged 25–30 years (Mathew et al., 2008); patients should be encouraged to reduce their weight and pursue a healthy lifestyle to reduce stroke risk.

Exercise alone can reduce stroke incidence by 27% (Lee et al., 2003a) and, with incorporation of a healthy nutritional programme, it could be predicted that there could be even more benefits. Furthermore, a negative dose-dependent relationship has been observed between the consumption of fruit and vegetables and stroke risk (Dauchet et al., 2005).

1.1.2.2.3 Diabetes

5% of people in the UK, approximately 3.3 million, are registered with diabetes; having diabetes doubles the risk of stroke and is a contributing factor to 20% of strokes (Stroke Association, 2015). It is sometimes unclear if diabetes confers an excess risk of stroke that is independent of blood pressure, as hypertension itself is a risk factor for both stroke and diabetes. Increased stroke rates have been shown in diabetic patients, even after stratifying for systolic blood pressure, indicating that diabetes can confer risk of stroke independent of blood pressure (Barrett-Connor and Khaw, 1988). Indeed, diabetes has been shown to confer a doubled risk for a wide range of vascular diseases including stroke, independently from other risk factors. In people without diabetes, fasting blood glucose concentration is modestly linked to risk of vascular disease (The Emerging Risk Factors Collaboration, 2010).

1.1.2.2.4 Cholesterol

Reducing cholesterol by as little as 1 mmol/L reduces stroke risk by more than 21% (Amarenco and Labreuche, 2009). Easily modifiable factors such as reducing saturated fat in the diet, smoking, alcohol intake, and increasing exercise would help to reduce the amount of cholesterol circulating in the bloodstream and therefore reduce the build-up of atherosclerotic plaques. Cholesterol levels should be a maximum of 4.0 mmol/L in order to have a reduced risk of stroke onset. The use of statins to control cholesterol levels has been shown to concurrently reduce the risk of stroke by 29%, even in patients that have not been shown to have high cholesterol levels (Hebert et al., 1997, Heart Protection Study Collaborative Group, 2004). However, the inverse is true when considering risk of haemorrhagic stroke (Iso et al., 1989). In order to reduce cholesterol levels in the blood, it is recommended to modify cholesterol intake to less than 200 mg/day (Pearson et al., 2002).

1.1.2.2.5 Smoking and Alcohol Consumption

The regular consumption of alcohol also increases risk of stroke (Guiraud et al., 2010). In the UK, the culture for binge drinking contributes to a higher stroke risk compared with other European countries, with 31% of adults drinking more than the recommended amount at least 1 day in a week (Stroke Association, 2015). In particular, binge drinking (consuming more than 7.5 units of alcohol on a single occasion) can increase the

likelihood of stroke onset among other diseases such as gastrointestinal tract cancers, diabetes, and ischaemic heart disease (Lim et al., 2013).

Smoking doubles both the risk of stroke onset and the risk of a stroke event being fatal (Peters et al., 2013, Thun et al., 2013); 20% of the UK population confess to being active smokers. Smoking also increases risk of stroke in the younger population (de los Ríos et al., 2012). Even passive smoking confers a higher risk of developing stroke (Bonita et al., 1999).

1.1.3 Outcomes after stroke

17 million people worldwide were known to suffer a stroke in 2010, increased from 15 million in 2004, and this rise was due to a rise in population and adverse risk factor trends (Mackay and Mensah, 2004, Bennett et al., 2014, Feigin et al., 2014). Care of stroke survivors costs the EU €38 billion a year with total stroke care costing €64.1 billion (Truelsen et al., 2005, Olesen et al., 2012). This estimate is set to rise with the increase in the ageing population (Pendlebury et al., 2004). These factors reveal that there is an urgent need for research in this field. Long-term disability is a serious problem (Lloyd-Jones et al., 2010). Depending on the location of the stroke, victims can suffer a number of symptoms including hemiparesis, visual impairments, aphasia, and loss of bladder and bowel control. Stroke is also the second most common cause of dementia, a frequent cause of depression, the most common cause of epilepsy in elderly people, and a major cause of secondary medical problems, such as falls and fractures (Rothwell, 2001).

77% of stroke survivors suffer from hemiparesis of the contralateral upper limb, but only 40% suffer hemiparesis chronically (Lawrence et al., 2001, Langhorne et al., 2009). Ischaemic stroke results in sensorimotor losses, including a decreased grasp ability, and impairments in fine motor manipulation (Carey et al., 1992). Deficits in sensory sensations such as touch, proprioception and inattention to the affected side, are frequently reported (Doyle et al., 2010); inattention or neglect is seen in almost one third of cases (Royal College of Physicians Sentinal Stroke National Audit Programme, 2014). Recovery of motor and sensory function is, therefore, a key target and this is the focus of the studies in this thesis.

1.1.4 Economic burden of stroke and underfunding of research

The amount of money spent in the UK on stroke patients is about £9 billion per annum, accounting for approximately 5% of NHS costs (Saka et al., 2009). That number is rising as stroke treatment improves; fatal stroke cases fell 46% from 1990 to 2010 (Feigin et al., 2014) and has resulted in more stroke survivors requiring post-stroke care. The occurrence of disabilities has continued to affect half of the surviving populations, forcing the net number of patients with disabilities to rise. The two largest causes of death worldwide, ischaemic heart disease and stroke, when combined, are the largest sources of this burden, particularly in the low-income and middle-income countries of Europe and Central Asia, where they account for more than a quarter of the total disease burden; in higher income countries they account for about one in five cases (Lopez et al., 2006).

With the ongoing care of disabilities being the largest burden to the NHS, the amount required to spend on patients will increase in coming years from the £23,315 per patient that is spent currently (Stroke Association, 2015). With 1.2 million current stroke survivors in the UK, assessment of the increase in the ageing population and the increase in comorbidities relating to stroke results in the conclusion that the burden of stroke will have doubled by 2030 (Feigin et al., 2014).

Stroke is the 2nd most common cause of death worldwide after coronary heart disease and yet is severely less funded than cancer and ischaemic heart disease (World Health Organisation, 2014). The amount of research spent per stroke patient is £48 compared to £241 spent per cancer patient (Stroke Association, 2014). Moreover, stroke kills twice as many women as breast cancer, and 1.5 times more than men with testicular and prostate cancer combined (Office of National Statistics, 2015). A study by Pendlebury (2007) collected information from studies in the USA, Europe, and the UK, showing a consistent underfunding of stroke research in all countries studied. There is clearly a great need for increased funding opportunities within the field of stroke research.

1.2 Current Treatments

Every minute a stroke victim goes untreated, 1.9 million neurons are starved of oxygen and are lost (Saver, 2006). With stroke incidence high, there is an urgent need for effective and rapid treatment following a stroke. Reducing patient time in hospital and, therefore, reducing exposure to infection, deep vein thrombosis, and urinary tract infections are clearly of benefit to the patient if treatment can be delivered quickly. The shorter the length of a hospital stay will also be a cost benefit to the NHS.

1.2.1.1 Tissue plasminogen activator (tPA)

Tissue type plasminogen activator (tPA) is a compound that was first used in the treatment of coronary heart thrombosis and animal studies began in 1985 to determine the efficacy of this treatment in stroke; particularly as tPA had the potential to treat both embolic and thrombotic strokes (Zivin et al., 1985). tPA is now used as the gold standard for treatment of ischaemic stroke. Thrombolysis disrupts fibrin in the clot enabling restoration of blood to ischaemic areas and has been shown to improve outcome by 30%, increasing the number of patients with little or no disability at three months (if administrated within 3–4.5 hours after onset compared to stroke patients not receiving treatment) without an increase in mortality (NINDS Stroke rt-PA Trial, 1995, Hacke et al., 2008, Davis and Donnan, 2009, Del Zoppo et al., 2009). It also shows a 20% increase in recanalisation with the use of ultrasound (Alexandrov et al., 2004, Tsivgoulis et al., 2010). Failures in early multi-centre trials were observed, largely because of recruitment of patients after 4–5 hours of stroke onset (Hacke et al., 2004), which could be linked to reperfusion injury (Jean et al., 1998), indicating that the correct selection criteria are of the utmost importance in stroke treatment with tPA. There is evidence that tPA can be used beyond the permitted 4.5 hours, if salvageable tissue can be identified using diffusion-weighted/perfusion-weighted mismatch MRI (Neumann-Haefelin et al., 1999), up to a maximal 9 hours after stroke, although this has not yet been approved (Röther et al., 2002, Ribo et al., 2005). Adverse haemorrhagic events occur in 6–7% of patients following tPA treatment within 7 days (Donnan and Davis, 2008) and the treatment cannot be given to patients with haemorrhagic stroke as clotting factors will fail and the bleed will continue to expand. Additionally, tPA is neurotoxic if not confined within the vasculature (Yepes et al., 2009), and lethal if administered to a haemorrhagic stroke patient. This highlights the need for rapid diagnosis of the type of stroke before administering treatment to ensure that the therapy

is correct for the type of cerebral ischaemia within the effective treatment window. This is usually done using computerised topography (CT) scanning, as the equipment is widely available and provides a relatively quick diagnosis compared to other imaging methods.

Because of the narrow treatment window and limitations in administering tPA, only up to 6–12% of stroke patients receive treatment after an ischaemic event (Chiu et al., 1998, Gilligan et al., 2005), since only approximately 15% are eligible for this treatment (Royal College of Physicians Sentinal Stroke National Audit Programme, 2014). Additionally, even if patients are selected carefully for the best possible outcome (as in the CASES trial), the likelihood of a positive outcome after treatment is only 37% (Hill et al., 2005). As a result, it is imperative to implement new treatments that require less stringent selection criteria.

1.2.1.2 Acute Stroke Units

One key reason why patients fail to receive treatment is that the time from stroke onset to treatment falls outside the 4.5 hour treatment window: waiting times for patients to see a specialist often cause a delay in treatment beyond the recommended time to treat (Warlow et al., 2003). Specialised, dedicated stroke units have been introduced with the concept that with specialist care, focus on diagnostic tests including brain imaging, more physiological monitoring, high staff-to-patient ratio, and rapid treatment interventions within the critical timescale could improve the proportion of patients to receive treatment. 83% of all stroke patients are eligible for treatment at a stroke unit (Gilligan et al., 2005). Patient access to a stroke unit in England, Wales, Scotland and Northern Ireland has reached 29, 21, 41 and 52% respectively (Stroke Unit Triallists' Collaboration, 2007). However, in 1999, only 12% of UK consultants admitted patients directly to a dedicated stroke unit, although this number is increasing as stroke expertise and allocation of appropriate resources improves (Ebrahim and Redfern, 1999). The use of stroke units improves the mortality of patients by a third, with 30-day fatality rates decreasing from 27% to 15% (Stone, 2002, Candelise et al., 2007, Chiu et al., 2007). Additionally, the use of stroke units has been reported to reduce complications and the length of time patients spend in hospital (Moodie et al., 2006, Chiu et al., 2007, Stroke Unit Triallists' Collaboration, 2007). The National Institute for Health and Care Excellence (NICE) recommendations now include the admission to an acute stroke unit (National Collaborating Centre for Chronic Conditions (UK), 2008) despite the increased cost of treatment (Patel et al., 2004).

1.2.1.3 Aspirin

Anti-platelet therapy and anticoagulant therapy are also useful early treatments after stroke, preventing further clots from occurring. Aspirin is preferred over heparin anti-platelet therapy as it results in fewer haemorrhagic events, which carry higher risk of death, although only a 1% reduction in mortality is seen (International Stroke Trial Collaborative Group, 1997). Aspirin is recommended in the early stages after diagnosis of an ischaemic stroke (Warlow et al., 2003) and immediate treatment decreases the risk of early recurrent stroke and increases the chance of survival (Gubitz and Sandercock, 2000). Up to 69% of stroke patients are eligible to receive aspirin treatment within 48 hours; treatment reduces the risk of recurrent stroke and increases the chance of survival free from disability, albeit only marginally (Gilligan et al., 2005). More recent studies have shown that using antiplatelet therapy prior to tPA treatment results in better functional outcomes (Xian et al., 2015). However, the poor efficacy of these treatments highlights the need to discover more effective therapies.

1.2.1.4 Decompressive Surgery

One therapy that has been shown to have good clinical outcome in younger patients following malignant middle cerebral artery occlusion (MCAO) with space-occupying oedema is decompressive surgery (Donnan et al., 2008). Space-occupying oedema occurs in up to 10% of patients and results in an increase of intra-cranial pressure. This can occur up to 5 days after a stroke, although a third of patients can have debilitating symptoms within 24 hours (Shaw et al., 1959, Qureshi et al., 2003). The use of decompressive surgery to relieve the buildup of pressure in the skull has had excellent results so far; trials of hemicraniectomy and duraplasty after malignant strokes of this type have shown a reduction of mortality with no increase in the numbers of patients with disabilities (Hofmeijer et al., 2006, Vahedi et al., 2007). This is of particular benefit in patients if the surgery is commenced within 48 hours of stroke onset; indeed, without intervention, up to 80% of patients with this type of stroke are likely to die (Hacke et al., 1996). This procedure has now been approved by NICE after middle cerebral artery infarctions (Pathways, 2016). However, this process is invasive, and only useful in a subset of patients, rendering the need for more research into other potential therapies.

1.2.1.5 Hypothermia

Hypothermia has been suggested as a potential therapy in randomised clinical trials for patients with conditions such as global cerebral ischaemia after cardiac arrest (Bernard et al., 2002), infants with hypoxic-ischaemic encephalopathy (Shankaran et al., 2005), and in an experimental cohort of cervical spinal cord injury patients (Levi et al., 2009). In all three patient groups, hypothermia has been shown to reduce mortality and disability after lesions associated with these conditions.

Clinical data from hypothermia after focal ischaemia has indicated that hypothermia could also be useful for stroke. In fact, an observation has been made that patients with stroke who have higher core temperatures have a worse outcome (Reith et al., 1996, Castillo et al., 1998). The cause for this increase in temperature is unclear, but it has provoked the investigation into the idea that the inverse might be able to result in better outcomes after ischaemic injury. Additionally, failed neuroprotective strategies to date only inhibit a single element of a cascade that brings about cell death (Fisher and STAIR, 1999, Gladstone et al., 2002), but hypothermia affects the deterioration process more broadly, disrupting cell death mechanisms such as free-radical formation, the breaking down of the blood–brain barrier (BBB), and inflammation (Olsen et al., 2003). A meta-analysis has shown an improvement of at least a third in animals given hypothermic treatment within a clinically relevant time frame after induced stroke (van der Worp et al., 2007). Although moderate hypothermia has been assessed in a randomised trial and appeared to be well-tolerated (De Georgia et al., 2004), this consisted of only 40 patients and the data was insufficient for conclusions about safety and efficacy. Higher levels of cooling inflict a high risk of adverse events such as arterial hypotension and cardiac arrhythmias (Olsen et al., 2003). As a treatment for ischaemic stroke, hypothermia is a strong potential therapy; there are two large Phase 3 trials underway (USA: ICTuS3, EU: EuroHYP-1), and results from these trials will elucidate its effectiveness as a mainstream therapy (Geurts et al., 2014, Lyden et al., 2014, van der Worp et al., 2014).

1.2.1.6 Endovascular treatment

Mechanical thrombectomy has been an exciting new technique for the treatment of patients who have suffered ischaemic stroke and who are either ineligible for tPA treatment, or patients in whom tPA has failed to recanalise the blood vessels and initiate reperfusion, and has recently been approved by NICE for routine use by suitable experienced clinicians (NICE Guidance, 2016). There has been enthusiasm for the use of

endovascular recanalisation in clinical trials, although earlier intervention, more effective devices, and appropriate patient selection have been associated with improved outcomes (Romano and Sacco, 2015). In the Mechanical Embolus Removal in Cerebral Ischemia (MERCI) multi-centre trial, a device was used to remove the clot in patients that were ineligible for tPA therapy or those patients in which the tPA was ineffective (Smith, 2006), thus providing an alternative for patients that are either too late to receive tPA or those who are eligible for combination treatment within an 8-hour time window (Nogueira et al., 2009). Thrombectomy with the stent retriever plus intravenous tPA has been shown to reduce disability at 90 days and improve functional independence (60%) compared with a control group (35%) (Saver et al., 2015). It also has been shown to be of particular benefit in young patients (Zanaty et al., 2014, Vega et al., 2015), but information on whether this is useful enough in the adult or elderly population has yet to be definitively established. Stent retrievers have now been recommended by the Food and Drug Administration (FDA) for intra-arterial use in middle cerebral or common carotid artery occlusions, as tPA has not yet been approved for intra-arterial interventions (Powers et al., 2015). Endovascular interventions require the patient to be at a stroke centre with access to specialised equipment and treatment.

1.2.2 Shortcomings of current treatments

Of 1,026 therapies and procedures successful in animal models of ischaemic stroke, only one, tPA, has been validated for use in the human condition (O'Collins et al., 2006). A failure for these successes in animal studies to be translated in the clinic has led to a loss of confidence in stroke research, potentially leading to difficulties in research groups receiving funding. This may have been partly due to failures in clinical trial design (Fisher and Stroke Therapy Academic Industry Roundtable II, 2001). Although many clinical trials have been done, few have been considered effective (NINDS Stroke rt-PA Trial, 1995, Furlan et al., 1999, Sherman et al., 2000). The failures of clinical trials are likely to be because of insufficient power, inadequate definition of endpoints, or that the therapies do not work effectively in humans. More stringent trial inclusion and more relevant endpoints are required to eliminate agents at Phase 1 and 2 to prevent waste of resources (Fisher and Stroke Therapy Academic Industry Roundtable II, 2001).

Most therapies in trials currently are either neuroprotective, with the main aim to save the dying penumbra, or stem cell therapies designed to rebuild the area of cortex after stroke and bioengineering concepts to facilitate this (Massensini et al., 2015).

1.3 Animal models of stroke

1.3.1 Justifying the use of animal models

One important component of the initial stages in planning experiments for drug discovery is the use of animals: whether they are needed, whether they can be avoided and, if they must be used, how experiments can be planned ethically.

Excitotoxicity, peri-infarct depolarisation, inflammation, and apoptosis have been identified as the most important pathways following ischaemia (Dirnagl et al., 1999). Although some of the apoptotic mechanisms are retained in organisms such as the nematode *Caenorhabditis elegans* (Danial and Korsmeyer, 2004), a closer association is observed between rodents and humans, as the Circle of Willis is conserved from rodents and larger animals through to humans (Ziles, 1990, Howells et al., 2010). However, some scepticism still lies in the use of rodents for modelling a disease as complex as stroke, and some argue that rodent models are only of use for dissecting molecular and disease mechanisms (Cenci et al., 2002).

Rodents are currently the most widely used animals to model stroke and, indeed, most stroke models were developed in rats; this is the animal model most used for assessing the effect of therapeutic agents (de Leciñana et al., 2001), as rats have relatively low costs for transport, storage, and feeding; although it is possible to train measureable behaviours for assessing functional outcome. Mice, in comparison, are less sensitive to behavioural tasks, but have a wider range of available genetic strains, which can be used to good effect in the stage of drug development exploring mechanisms of drug action.

The National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) is an organisation that has been set up to monitor animal use and promote their ethical use; they have created guidelines for reporting animal research, the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines (Kilkenny et al., 2010b). If researchers continue to use animals to model stroke, adhering to the NC3Rs to minimise the use of animals where possible is imperative. Responsible research by diligently planning experiments and ensuring that results are useful will provide more reliable information about therapies and agents that can be subsequently used in clinical trials.

1.3.2 Preclinical recommendations and the translational roadblock

Research in animal models has made, and will continue to make, important contributions to the understanding of the pathophysiology of stroke and research into new treatments.

However, knowledge from the clinic is still not effectively being transferred into the animal models; young and otherwise healthy animals are being used in animal experiments, even though more than 75% of ischaemic events occur in people over the age of 65 and commonly suffer from one or more chronic conditions (Kelly-Hayes et al., 2003). This mismatch between animal research and the clinic has partly been blamed for the ineffective translation of neuroprotective drugs from benchside to the clinic.

One organisation committed to changing this mismatch between basic science and the clinic is the Stroke Therapy Academic Industry Roundtable (STAIR), whose purpose is to improve assessment of therapies in a pre-clinical setting with the final objective for clinicians to better select therapies that may be translated successfully in the clinic. After the failures of 1,026 therapies to get to the clinic (O'Collins et al., 2006), the main question is what can be done to better achieve goals to take therapies to the clinic?

Many neuroprotective and restorative agents tested to date do not have dose-response curves in the literature; although animal models mimic the human condition as closely as possible, the pharmacodynamics and pharmacokinetics of an agent are likely to vary between species and, therefore, simply scaling up the drug dose is not necessarily appropriate. The use of a target tissue concentration or other measurable tissue-level effect should be used so that it may be better translated in the clinic (Fisher and STAIR, 1999, Macleod et al., 2008).

Previous studies with tPA have shown that the window for treatment is very important following an ischaemic event (NINDS Stroke rt-PA Trial, 1995, Hacke et al., 2008), as late delivery of the treatment can trigger reperfusion injury or a haemorrhagic event and substantially increase the risk of mortality in patients. This could also be true of other stroke treatments and, therefore, the window of efficacy for any new drug should always be assessed. A small therapeutic window is thought to have led to the failure of N-methyl D-acetate (NMDA) antagonists such as MK801, which was well-tolerated and showed good efficacy pre-clinically, but failed in clinical trials (Hoyte et al., 2004a). The required duration of treatment is another component that needs addressing and preclinical studies including such factors are more likely to be included in future clinical trials (Fisher and STAIR, 1999). Finally, clinically relevant treatments must be tested appropriately; many pre-clinical studies use experimental designs that involve controlled time windows, which is not the case in human beings. Some experimental designs involve pre-treating animal models with the therapeutic agent prior to ischaemic insult or at the time of reperfusion with little attention to more clinically relevant time frames.

The importance for a therapy to be applied first in rats, as these animals have better functional tests available, and on success in small animals, to subsequently study the therapy in larger animals (Fisher and STAIR, 1999, Hoyte et al., 2004b) has also been made apparent. The STAIR recommendations suggest that mice are best used when transgenic animals need to be used. Even the strain used is important; some species of rat are more susceptible to larger lesion volumes following MCAO than others (Bardutsky et al., 2005). Another factor to consider is that as the current "gold standard" treatment is tPA, and treatments, particularly those that are neuroprotective, should be tested alongside tPA to ensure that they are compatible for use in combination. Studies should always be randomised, blinded, have sample size calculations, and, where possible, be repeated by another group.

Outcome measures from pre-clinical studies should include both infarct volume and functional assessment. However, it is important to note that rats in particular can show robust plasticity, so the use of a second, larger species for validation is likely to increase success in clinical trials.

1.3.3 Modelling Human stroke

Stroke is a complex and heterogeneous disorder comprising different underlying pathologies. Patients have a broad range of underlying conditions and the territory in which the infarct is located determines the presentation of the disease, which varies from individual to individual. Genetic profiles, environmental factors and life habits may contribute to ischaemia onset, which cannot be easily reproduced in an animal model. Although stroke is an acute event, it is the result of long-term damage to the cardiovasculature, caused by many years of arterial hypertension, cholesterol, diabetes and obesity, damaging the arteries. However, it is important to recognise that no single model will be able to effectively emulate all elements of the human condition. Motor and sensory effects can be assessed using behavioural tests in animal models, however, additional factors, such as aphasia, agnosia, and other memory effects are more difficult.

Assessing therapies in an animal model, in an inbred strain, a single stroke model, and within a timeframe for maximal efficacy, is difficult to translate into a heterogeneous stroke population of various origins and wide time-to-treatment windows (Fisher and STAIR, 1999, Fisher and Stroke Therapy Academic Industry Roundtable II, 2001, Hoyte et al., 2004b).

Rat models of MCA occlusion are useful approximations of focal cerebral ischaemia in humans (Ginsberg and Busto, 1989), but the clinical symptoms are not well translated;

in particular, anaesthesia and artificial ventilation, that are often used during small animal surgery to maximise recovery, might interfere with the formation and maturation of the ischaemic injury (Kurumaji and McColloch, 1989). Particular anaesthetic agents have been proposed as potential neuroprotective treatments for stroke (Hossmann, 1982). Anaesthesia may not only result in gross disturbances in cerebral function but can unpredictably change the effects of other pharmacological agents on brain function (Grome and McColloch, 1983). Such interactions between anaesthesia and the neuroprotective agent dizocilpine (MK-801) have been described previously (Kurumaji and McColloch, 1989). Problems associated with anaesthesia have long been recognised, and models that allow the animal to recover from anaesthesia during transient MCAO are available, with good prospect for use in preclinical models for acute treatment (Nagasawa and Kogure, 1989, Buchan et al., 1992). However, the potential confounds of using anaesthesia can be adequately controlled for by applying it equally to all animals.

Additionally, comorbidities such as age and spontaneous hypertension may be important and, historically, rarely considered. Importantly, this seems to be starting to change, with preclinical stroke studies now being established in aged (Soleman et al., 2010); hypertensive (Henning et al., 2010), diabetic (Rewell et al., 2010), obese (Vannucci et al., 2001), and nicotine dependent (Wang et al., 1997) animals. There even has been a stroke model that offers 3 co-morbidities; elderly, spontaneously hypertensive rats, with either acute or chronic diabetes (Rewell et al., 2010). The use of comorbid models results in larger infarcts compared to younger controls, in accordance with previous studies (Barone et al., 1992). Furthermore, mortality tends to increase with overall disease burden, but not sufficient to render modelling impractical.

Different models have been developed to simulate different elements of the human condition. Some of the main models used are discussed below (for an excellent review, see Macrae (2011)).

1.3.3.1 Intraluminal MCAO

This model was initially developed by Koizumi et al. (1986) and is the most widely used model due to the ease of surgical technique, the ability to reproduce the model easily in both mice and rats, and the choice to use either a transient or permanent form of the technique.

In short, the procedure consists of inserting a nylon thread into the internal carotid artery (ICA) and advancing the thread towards the brain to block middle cerebral artery

(MCA) blood flow. This is either achieved by introducing the suture into the common carotid artery (CCA) (Koizumi et al., 1986) or the external carotid artery (ECA) (Longa et al., 1989). The suture is typically advanced to the MCA, also occluding collateral circulation from the anterior communicating arteries. The key advantage to this model is the ability to study the effects of different durations of occlusion; the study of temporary models of ischaemia when the filament is removed after a period of occlusion (Nagasawa and Kogure, 1989), and permanent models can be studied when the filament is left inside the artery.

This model of stroke is a proximal model; the occlusion the MCA in this way also interrupts blood flow to the lenticulo-striatal arteries, which supply the basal ganglia. As these are the only arteries that supply this subcortical region, vessels become extremely ischaemic, and cortical areas have a more gradual reduction in blood flow. This model resembles human large artery occlusion; the resulting stroke can produce large, variable infarcts that include the lateral cortex, striatum, frontal, and parietal regions, and this can result in many deficits. Importantly, areas of the brain that are rarely affected in the human condition, such as the hypothalamus, can be affected in this model (Li et al., 1999). This can be a disadvantage, as it could prove difficult to assess any positive benefit from any therapies tested due to the large and sometimes variable extent of deficits. An additional consideration is that non-central side effects such as weight loss and water intake can correlate with diminished recovery after stroke (Trueman et al., 2011); these must be taken into consideration in the analysis of the results, as an observed recovery in behavioural tasks could be due to improvements in these factors rather than clinical outcomes from stroke improvement.

Permanent intraluminal MCAO can cause massive hemispheric infarcts and low survival (Fisher and STAIR, 1999), and there is some evidence that the filament itself damages the wall of the vascular endothelium to produce a 12% incidence of subarachnoid haemorrhage (Carmichael, 2005). Additionally, there have been reports that reproducibility can be poor due to the diameter of the blood vessels and the comparative suture size, insertion length of the suture, and even the coating of the filament in silicone or poly-D-lysine. Leaving the suture without a coating can lead to large variations between animals that are often not well reported (Belayev et al., 1999).

1.3.3.2 Thromboembolism and thrombin models

First described in 1955, the thromboembolic stroke models aim to produce reversible focal cerebral ischaemia while replicating the pathophysiological mechanisms during and

after the stroke insult (Hill et al., 1955, Zhang et al., 1997a, Zhang et al., 1997b). Disruption of blood flow in the brain from a thrombus is attributed to the migration of a peripheral clot or a build up of a clot *in situ,* and stroke models of this type involve either the injection of autologous clots (Overgaard et al., 1992, Busch et al., 1997) or the application of thrombin to the middle cerebral artery (Zhang et al., 1997b, Orset et al., 2007). Since 80% of human strokes are caused by thrombosis or embolism (Mohr et al., 1978), the use of the thromboembolic stroke model is useful for studying the early time course of stroke onset (Kito et al., 2001). This model permits the use of thrombolytics to study treatments that are compatible for use alongside tPA (Papadopoulos et al., 1987, Del Zoppo et al., 1990, Lapchak et al., 2000); this is due to the physiological and biochemical parameters during ischaemia and reperfusion being consistent with the human ischaemic event. The model requires no craniotomy when the thrombus is delivered peripherally to the common carotid arteries (but not when the thrombus is injected directly into the middle cerebral artery), is capable of producing both cortical and subcortical infarcts depending on the location of the clot and has been delivered under mild sedation in place of anaesthesia (Watanabe et al., 1977, Orset et al., 2007).

However, although this method has its advantages, the use of this model might not be suitable for the study of sustained deficits in rats. The variability in the lesion volume and location is high and, although this is a clinically relevant attribute, this makes it more difficult to assess outcome measures. Additionally, thromboembolic stroke models are susceptible to spontaneous lysis (Busch et al., 1997, Mayzel-Oreg et al., 2004); the subsequent reperfusion would require the use of more animals to, potentially, investigate an effect of treatment (Overgaard et al., 1992). The use of tPA often results in a secondary ischaemic damage or reperfusion injury, which is also observed in the clinic. The use of the thrombotic stroke model is preferred over the embolic, as infarcts are more reproducible because of more accurate placement of the clot, resulting in a lower mortality rate. Furthermore, the use of a smaller volume of thrombin permits the study of transient ischaemic attacks. This is an excellent model for the study of reperfusion injury and thrombolytics, but is unlikely to be useful in the study of plasticity.

1.3.3.3 Embolic micro/macrosphere stroke model

This model was first described in rodents as a model of embolic stroke with the intention of reducing variability compared to the multi-location embolic strokes described previously (Bralet et al., 1979). It requires the injection of either many small spheres into the cerebral blood stream (Yamaguchi et al., 2000, Mayzel-Oreg et al., 2004) or a

small number of large spheres (Watanabe et al., 1977, Gerriets et al., 2003) into the internal carotid artery, which are then lodged in the middle cerebral artery. Both of these described methods result in permanent focal ischaemic lesions, preserving the blood supply to the hypothalamus, damage to which has been shown to impair survival in the intraluminal MCAO model (Gerriets et al., 2003).

However, this model is associated with a 10–20% mortality (Bralet et al., 1979) and, although stroke is associated with similar mortality outcomes in the clinic, it is not possible to continue to use animal models with such high probability of mortality. Additionally, this model still can result in multifocal lesions in various brain regions in approximately 10% of cases (Bralet et al., 1979, Miyake et al., 1993). A consequence of these models is a widespread reduction in perfusion (Mayzel-Oreg et al., 2004); these factors, in combination, limit reproducibility and statistical power or require the use of more animals to produce the same result.

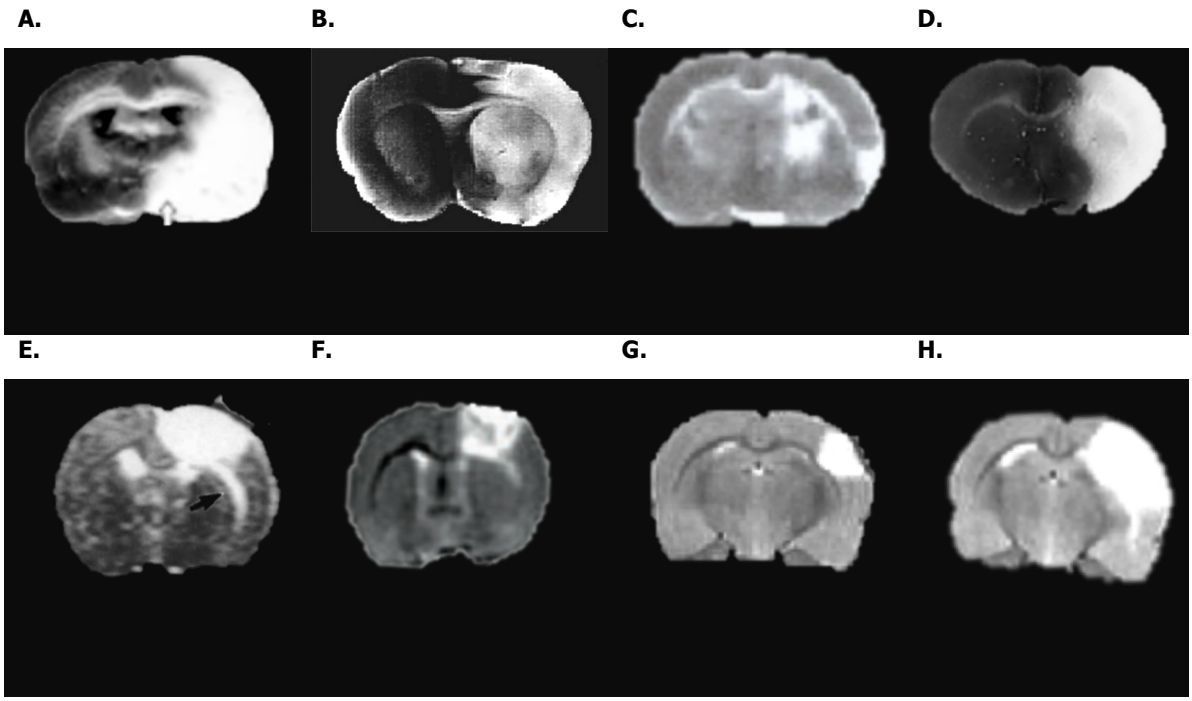


Figure 1-2 : Structural MRI 24 hours after stroke in various models of ischaemic stroke.

A. Permanent proximal model (Carmichael, 2005). B. Thromboembolic stroke (Busch et al., 1997). Microsphere model (Mayzel-Oreg et al., 2004). D. Macrosphere model (6 x 300 µm spheres) (Gerriets et al., 2003). E. Photothrombotic model (van Bruggen et al., 1992). F. Endothelin-1 model of stroke (Duricki et al., 2016b). G. Single vessel surgical occlusion model (Figure 2-3A). H. Three-vessel surgical occlusion model of stroke (Figure 2-3B).

1.3.3.4 Photothrombosis

The photothrombotic model is a commonly used model in rodents first described in 1977 as a platelet aggregation model but was improved to reduce the incidence of

subarachnoid haemorrhage (Rosenblum and El-Sabban, 1977, Watson et al., 1985). In mice, the model requires the photochemical dye Rose Bengal to be injected systemically and the application of an external light source (530–590 nm) through intact tissue for between 5 and 20 minutes. Oxygen is produced from the dye, causing peroxide damage to the vessel endothelium and subsequently vasoconstriction and platelet adhesion and aggregation of a thrombus and vascular occlusion (Watson et al., 1985, Dietrich et al., 1986). Animal preparation is straightforward and does not involve mechanical manipulation of cerebral blood vessels, which renders it an attractive option for stroke researchers (van Bruggen et al., 1992). Other advantages of this model include the fact that the lesion can be reproducibly positioned and the size, severity, and location can be altered by the intensity of the beam and duration of irradiation.

The main disadvantage of this model is that there is disruption of endothelial integrity; the blood brain barrier is damaged and local vasogenic oedema has been shown to occur almost immediately following induction of this model of stroke using T2-weighted imaging. This is in contrast to humans, in whom oedema is normally delayed by several hours. Consequently, the rapid occurrence of this oedema is more similar to traumatic brain injury than stroke (Schneider et al., 2002). Additionally, there is little ischaemic penumbra and therefore a reduced region of collateral blood flow is observed. The oxidative damage stems from the infarct core rather than being distributed into the peri-infarct area, which more closely resembles the human stroke condition and is seen in other stroke models (Kim et al., 2000, Katsman et al., 2003). It is unclear what exact downstream processes occur during this model; although the model has achieved much praise for being the most similar thrombus formation compared with the human condition, it has become evident that platelet aggregation is not the only cause of the ischaemic onset and *in vivo* blockade of platelets does not impact the infarct size (Kleinschnitz et al., 2008). Finally, there is conflicting evidence on the use of photothrombotic models; some studies have shown that photothrombotic models can produce large strokes with the use of wider diameter fibre optics (Wahl et al., 2014), while others show that this rat model of stroke may not be as informative when testing functional recovery following candidate therapies as the lesion volumes are smaller and the deficits more modest than mechanical stroke models (Choi et al., 2012), although this might be due to recruitment of the cortex contralateral to the lesion (Reinecke et al., 2003, Shanina et al., 2006).

1.3.3.5 Endothelin-1

Endothelin-1 is one of the most potent vasoconstrictors, and its administration produces ischaemic injury through its ability to produce a prolonged but reversible reduction of local blood flow (Yanagisawa et al., 1988, Masaki et al., 1992, Palacios et al., 1997). Endothelin-1 has the potential to model small focal ischaemic stroke of the MCA by applying topically (Robinson et al., 1990, Macrae et al., 1993, Fuxe et al., 1997, Windle et al., 2006) or injecting into territories surrounding the MCA (Sharkey and Butcher, 1995, Biernaskie and Corbett, 2001, Biernaskie et al., 2004), resulting in up to 96% blood flow reduction to the MCA, which is similar to the affect achieved with mechanical occlusion models (Macrae et al., 1993, Sharkey et al., 1993). Endothelin-1 has also been delivered into various regions of the parenchyma to induce localised subcortical focal cerebral ischaemia in rats (Hughes et al., 2003, Frost et al., 2006, Lecrux et al., 2008) and monkeys (Virley et al., 2004).

Endothelin-1 use in stroke models reduces blood flow rapidly in a dose-dependent manner (Yanagisawa et al., 1988, Fuxe et al., 1992, Macrae et al., 1993, Beyer et al., 1994), resulting in a robust and reproducible lesion, although ischaemia occurs immediately and reperfusion occurs over several hours (Biernaskie et al., 2001, Matsuo et al., 2001). This reduction and return of blood flow disrupts the ischaemic cascade and reduces the damage to the blood–brain barrier, thus rendering it similar to the human condition (Matsuo et al., 2001, Hughes et al., 2003). Endothelin-1 can be applied topically, injected, or a combination of these two administration routes, thus resulting in less variable lesions; it has been shown to be safe in comorbid models of age (Sharkey and Butcher, 1995, Soleman et al., 2010, Soleman et al., 2012, Duricki et al., 2016b) and spontaneous hypertension (Lecrux et al., 2008). Additionally, this model can be achieved in awake rats to remove the confound of anaesthesia (Sharkey et al., 1993). Another positive element of using the endothelin-1 stroke model is the documented behavioural outcomes following a lesion to the sensorimotor cortex (Biernaskie and Corbett, 2001, Adkins et al., 2004, Luke et al., 2004).

This model has the potential to be quite variable as the blood vessel architecture changes and the size of the lesion is dose dependent with the use of endothelin-1. Additionally, endothelin-1 can elicit vasospasm and can cause subarachnoid haemorrhage (Robinson et al., 1990, Masaki et al., 1992, Barone et al., 1994, Fuxe et al., 1997). Endothelin-1 is released extracellularly following a focal stroke event and may cause exacerbated ischaemic episodes following the lesion, leading to penumbral region deterioration (Barone et al., 1994, Hama et al., 1997, Windle et al., 2006). Endothelin-1

receptors are not only present on the endothelial cell, making up the walls of the blood vessels, but also in astrocytes (Hama et al., 1997, Nakagomi et al., 2000); glia (MacCumber et al., 1990); and neurons of the brain, spinal cord, and dorsal root ganglia (Giaid et al., 1989, Peters et al., 2003). This has the potential to confound results as endothelin-1 has been shown to induce astrocytes to their active state (Hama et al., 1997), also facilitating axonal sprouting in spinal cord injury (Uesugi et al., 1996). These processes are mediated by the two different subtypes of the endothelin-1 receptor, $ET1_A$ and $ET1_B$. $ET1_A$ receptor mediates the ischaemic events seen after the application of endothelin-1 after stroke, and antagonists mediate a vasodilation effect. Antagonists of the ET_{1A} receptor also reduce degeneration after injury through action on this receptor, possibly through being involved in glial scar formation (Fuxe et al., 1992, Uesugi et al., 1998, Nakagomi et al., 2000). The $ET1_B$ receptor permits endothelin-1 to act as a potent growth factor, involved in regeneration after CNS damage via astrocytic growth (Uesugi et al., 1996, Uesugi et al., 1998). This knowledge brings doubt to the clinical relevance of this stroke model, as the naturally-occurring human stroke is significantly more complex.

1.3.3.6 Surgical (electrocoagulation) MCA occlusion and Three Vessel Occlusion

The MCA is reported to be the vessel most commonly affected in human ischaemic stroke (Mohr et al., 2004), therefore, the use of a stroke model that is produced by occlusion of the rat MCA is both appropriate and relevant (Tamura et al., 1981). The model has been shown to produce anatomical similarities (Yamori et al., 1976) and lesion volumes of comparable ischaemic damage (Carmichael, 2005) to those seen in human ischaemic events (Molinari and Laurent, 1976, Garcia, 1984). The model requires surgical intervention to expose the MCA followed by electrocoagulation or mechanical clipping of the vessel (Tamura et al., 1981, Osbourne et al., 1987).

The surgical method of stroke created by Tamura et al. (1981) is one of the standard methods for studying permanent MCAO in rats and has also been adapted for use in mice (Yanamoto et al., 2003). The lesions produced are reproducible, and with a low mortality due to the release of intracranial pressure by doing a craniotomy. This craniotomy does increase the risk of infection, but with good aseptic technique, craniotomy models are associated with a lower mortality rate than closed skull models because of the reduction in intracranial pressure and the increase in space for the growth of oedematous tissue (Macrae, 2010, Macrae, 2011).

Additionally, the severity of stroke given to animals can be closely controlled, depending on the segment of MCA occluded; the model first described by Tamura was a permanent, proximal model of the MCA, but adaptations have been made to produce distal occlusions (Coyle, 1982, Shigeno et al., 1985), transient models (Buchan et al., 1992), and additional occlusions of one or more common carotid artery (CCA) to create a model with a sustained deficit after infarction (Bederson et al., 1986b, Chen et al., 1986, Rubino and Young, 1988). A distal occlusion of the MCA produces small infarcts isolated to the cortex, though longer and more proximal occlusions produce both cortical and subcortical ischaemia (Roof et al., 2001).

A comparison is made in the second chapter of this thesis of the permanent distal MCAO model by coagulation and a model with the additional occlusion of the CCAs ("three-vessel occlusion"). The CCAs supply blood to the MCAs and smaller, end arteries such as lenticulostriatal arteries, and thus reducing blood flow through the CCAs is associated with more reproducible infarcts and sustained deficits after MCA occlusion, although variation is seen from strain to strain (Rubino and Young, 1988, Buchan et al., 1992). One key feature of this model is that it has been shown to be a valuable model for the study of stroke recovery (Tamura et al., 1981, Kawamata et al., 1997, Fisher and STAIR, 1999), since the occluded vessels subserve the sensorimotor area. Indeed, motor symptoms in the human condition are determined by the site and location of the lesion. This model is associated with a low mortality when simultaneous occlusion of the carotid arteries is short; with good surgical technique, lesions can be produced reliably and reproducibly. The three vessel occlusion with permanent occlusion of the CCA ipsilateral to the coagulated MCA and 60-minute transient occlusion of the contralateral CCA to the lesion has been shown to be the most successful in the production of sustained deficits (Chen et al., 1986).

This model has been shown to be feasible in aged animals (Wayman et al 2016), spontaneously hypertensive rats (Brint et al., 1988, Buchan et al., 1992) and mice (Yanamoto et al., 2003). The isolation of the sensorimotor cortex is an attractive feature of this model and will be used for the duration of this thesis.

1.3.4 Caveats of using lesion studies

Rodents have little white matter compared with humans; therefore pre-clinical studies in these species should concentrate on mostly cortical factors, or lesions in the grey matter. Models that result in white matter lesions, although often used, are unlikely to have the same severity as observed in humans. Another factor to consider is that rodents are

lissencephalic rather than gyrencephalic like humans, which may have implications for the presentation of the ischaemic damage (Hoyte et al., 2004b).

Plastic changes occur after a lesion is induced under the right conditions. Functional outcomes that occur result from changes in the whole motor network, including plasticity in spared fibres; for instance, CNS injury has been shown to cause long-term changes in the rubrospinal tract. This can also be seen as a positive; learning how to manipulate this pathway could pave the way to future treatments (Lemon, 2008a).

Selective lesions may not be an optimal model for such a heterogeneous disease; however, it does give an insight into neuroplasticity in different pathways (Lemon, 2008a). It is more productive to concentrate on symptoms that are common to rats and humans rather than trying to pinpoint the differences in symptoms between the two species. Instead researchers should identify how the same human stroke symptom would manifest in a rat and design appropriate behavioural tests to assess these outcomes.

1.4 Motor and sensory tracts of the sensorimotor area

To measure outcome measures after stroke in the sensorimotor cortex, it is important to understand the role of the affected area and the descending and ascending tracts that can be manipulated to measure functional outcome.

1.4.1 Descending tracts

The largest of the descending tracts is the corticospinal tract (CST) (Watson and Harvey, 2009), the function of which includes descending control of afferent inputs (Cheema et al., 1984, Wall and Lidierth, 1997), control of spinal reflexes (Evarts and Tanji, 1976), excitation and inhibition of motoneurons (Jankowska et al., 1976, Alstermark and Lundberg, 1992, Maier et al., 1998), and long term plasticity of spinal cord circuits (Wolpaw, 1997). The multifunctional nature of the CST is partly because of the multiple sections of the brain that contribute axons, including motor areas for the execution of tasks, the supplementary motor area that is important for planning, the premotor cortex for sensory guidance, the sensory cortex for proprioceptive feedback (Miller, 1987), and the cingulate cortex for autonomic control (Lemon and Griffiths, 2005).

The CST is the principal motor system for controlling skilled movement. This pathway has been conserved through evolution in mammals and forms the only uninterrupted descending pathway between the cerebral cortex and the spinal cord in humans (Bernhard and Bohm, 1954, Iwaniuk and Whishaw, 2000). These direct neurons have been shown to be important in skilled digit movement in humans and other higher species (Kuypers, 1964, Lawrence et al., 1985), however, monosynaptic corticospinal neurons are sparse in rats and have not yet been definitively shown to be involved in the CST pathway (Babalian et al., 1993); they may be compensated for by an increased role for other descending tracts such as the corticoreticulospinal pathway (Georgopoulos and Grillner, 1989).

As the corticospinal motor system matures, adaptive motor behaviours develop fully and damage to this system can severely impair this skilled control (Martin, 2005). The CST originates in pyramidal neurons in layer V in motor and somatosensory areas, in addition to frontal and parietal areas, and then projects down the length of the spinal cord. The tract descends through the ipsilateral ventral brainstem to the medullary pyramids. Here, the majority of the axons (~90%) decussate at the spinomedullary junction: the decussated axons form the dorsal CST at the base of the contralateral dorsal funiculus. The 10% of axons that do not decussate descend ipsilaterally through the ventromedial

funiculus in the ventral CST (Brösamle and Schwab, 1997). Furthermore, a second minor percentage of axons form the dorsolateral CST, formed by decussation of axons, which descend in the dorsolateral white matter (Steward et al., 2004).

The fact that the CST is implicated in control of skilled motor function means that injury to projections of the CST results in deficits in fine control and movement in all mammalian species (Lemon, 2008b). Injury to the CST causes a breakdown in fine sensorimotor control, thus affecting not only motor function but also the capacity to correctly process sensory feedback from the forelimb (Lemon and Griffiths, 2005). Deficits in skilled forelimb reaching, ladder walking, and dexterity are key features following CST lesions in experimental models and analogous deficits are seen after human CST injury (Whishaw et al., 1993, Whishaw and Metz, 2002, Starkey et al., 2005, Ward et al., 2006). Therefore repair of a damaged CST is of key importance when trying to regain functional recovery after CST insult.

However, it is important to note that the CST is not required for the performance of skilled movements in amphibian species such as the frog (Iwaniuk and Whishaw, 2000) and thus the involvement of other descending pathways is likely to occur. Furthermore, lesion studies of the CST have been shown to impair, but not completely remove, the ability to complete skilled forelimb tasks (Whishaw et al., 1993). Other descending tracts, such as the rubrospinal, tectospinal, and reticulospinal tracts are likely to be responsible for conserved function after CST injury.

1.4.2 Ascending tracts

The CST also projects from the somatic sensory cortex to sensory processing systems in the dorsal horn and brain stem, resulting in regulation of proprioceptive and cutaneous information necessary for the generation of movement (Martin, 2005). The CST terminates in a wide number of laminae, which indicates involvement in a variety of functions, including nociceptive, autonomic, reflex, somatic motor, and somatosensory functions (Zemlan et al., 1978, Lemon, 2008a). Complex reflexes such as nociceptive reflexes (Liebeskind and Paul, 1977) depend on information relayed through the anterolateral columns; in contrast, dorsolateral columns convey signals from stretch, touch, and pressure receptors (Kennard, 1954, Kerr, 1975). The fibres that transmit this information form bundles in the spinoreticular, spinothalamic, and spinocerebellar tracts (Zemlan et al., 1978).

1.5 Functional recovery through plasticity of collateral sprouting from CST axons

Mechanisms promoting functional recovery after ischaemic stroke are likely to be dependent on structural and functional recovery of the remaining brain or spinal cord. Clinical studies have shown that recovery in the first 3 months is robust in many patients, but there is a large variation in patient recovery, and further recovery may occur after this time, which opens up opportunity for drug development at later timepoints (Nakayama et al., 1994).

1.5.1 Spontaneous recovery and functional reorganisation after stroke

In both patient and animal models, various degrees of spontaneous recovery in the weeks or months following ischaemic damage can occur in different phases. Early improvement after stroke is thought to be due to the resolution of oedema and survival of the ischaemic penumbra, with later recovery being associated with spontaneous compensatory plasticity and anatomical reorganisation from intact or spared fibres (Ward, 2005, Cramer, 2008, Blesch and Tuszynski, 2009, Lipsanen and Jolkkonen, 2011). Although most stroke patients show some degree of spontaneous recovery after the initial event (Ward and Frackowiak, 2004), the amount of recovery strongly depends on the size and location of the lesion (Chen et al., 2000).

1.5.1.1 Stroke promotes spontaneous plasticity

Animal studies have provided insights into the cellular and molecular events resulting in spontaneous recovery that is seen after stroke. One of the main mechanisms for spontaneous recovery is through collateral sprouting, where uninjured neurons from intact or spared circuits branch to connect with a denervated target. The resulting new connections take over the role from the lost fibres. Furthermore, it has been shown in many studies that injury to one hemisphere often causes an increased number of axons from the intact hemisphere to cross to the denervated side from between midbrain and brainstem nuclei in the brain and the crossing axons in the spinal cord (Kartje-Tillotson et al., 1986, Z'Graggen et al., 2000, Wiedner et al., 2001, Papadopoulos et al., 2002, Lapash Daniels et al., 2009, Soleman et al., 2012, Duricki et al., 2016b).

Stroke disrupts the pathways responsible for sensorimotor skills. This has been shown in rodent and primate models of ischaemic stroke, where recovery is associated with anatomical reorganisation within the brain and pathways to the spinal cord from the cortex and brainstem (Emerick et al., 2003, Emerick and Kartje, 2004, Lindau et al., 2013). The CST originates in the cortex and has been shown to undergo spontaneous collateral sprouting in the cord after stroke in mice (Lapash Daniels et al., 2009). Some spinal cord injury studies have shown that sprouting of injured axons onto spared, propriospinal neurons occurs after spinal cord injury and leads to increased connectivity with motoneurons (Bareyre et al., 2004). The recovery associated with increased sprouting after CST axotomy can be reversed following subsequent lesions to the ventral CST (Wiedner et al., 2001). Furthermore, following injury to the CST, sprouting of the rubrospinal tract compensates for much of the lost function from the initial injury in rats (Raineteau et al., 2001). Spontaneous plasticity of corticospinal projections can reconstitute up to 60% of pre-lesion axon density arising from sprouting of spinal cord midline crossing in monkeys (Rosenzweig et al., 2010).

Studying cortical connections in monkeys after ischaemic injury to the primary motor cortex can reveal novel connections through propagation of terminal fields and alterations in the trajectory of intracortical axons adjacent to the lesion (Dancause et al., 2005). Furthermore, cortical areas corresponding to somatosensory finger inputs have been ablated by microlesions in owl and squirrel monkeys, resulting in the loss of learnt behaviours. Detailed maps using intracortical stimulation have shown a post-lesion re-emergence of fingertip representation during the behavioural task and a new representation in a zone that had formerly been excited only by proprioceptive inputs (Xerri et al., 1998).

These studies show that following experimental stroke, reorganisation of the sensorimotor areas and collateral sprouting of descending pathways occur spontaneously (Benowitz and Carmichael, 2010). The findings have been mirrored in human studies (Ward et al., 2003, He et al., 2007). However, although there is some spontaneous repair after CNS injury, it is most often incomplete. Further recovery will require either overcoming the inhibitory environment of the injured neurons or establishing factors that will increase levels of regeneration, or preferably both.

1.5.2 The use of neuroimaging to measure spontaneous recovery

The longer the stroke treatment is delayed, the less likely the affected brain can be salvaged or indeed, that treatment will be helpful. The diagnosis of the type of stroke

can only be made with certainty with the use of imaging techniques (mainly CT), although approximately 50% of strokes cannot be seen on a CT scan (Warlow et al., 2003). The current recommendations require health professionals to scan immediately if there are indications for stroke, particularly if the patients are already on anticoagulant treatment or have a bleeding tendency in order to determine the correct treatment (National Collaborating Centre for Chronic Conditions (UK), 2008). In the next section, the use of how imaging can be used to better understand changes in the brain following stroke and cortical reorganisation has been explored.

1.5.2.1 Human strokes often result in some spontaneous recovery and functional cortical reorganisation of sensorimotor circuits

Typically, approximately 80% of ischaemic stroke patients present at hospital with reduced manual dexterity, which can ameliorate over time (Bonita and Beaglehole, 1988). Functional imaging studies of the motor system in hemiparetic patients have shown task-related brain activation requiring movement in patients compared with control subjects. However, these studies are normally done in patients with good recovery. In stroke patients, activation-induced regional blood flow increases during motor and sensory tasks in brain regions that are not usually active in control subjects (Chollet et al., 1991, Weiller et al., 1992, Nelles et al., 1999). Indeed, a negative correlation is usually observed between patient outcome and the number of brain regions activated during the completion of a task; patients with a good recovery have similar activation patterns to healthy controls, although more severely affected patients are observed to activate more brain regions (Ward et al., 2003). The recruitment of bilateral secondary motor areas in more severely impaired patients permits a restoration of some function, but results in suboptimal recovery in patients relying on these regions to generate motor output. This finding implies that the remaining motor network architecture and recruitment of the unaffected hemisphere is potentially an important mechanism for recovery in these patients; and this finding has been previously observed in the clinic (Weiller et al., 1992, Cramer et al., 1997, Calautti and Baron, 2003, Ward, 2004). The pattern of recovery that occurs from the recruitment of these areas in the first 10–14 days persists even in the chronic stages of recovery, highlighting the plasticity of secondary areas and importance of designing the correct rehabilitative strategy early on after stroke onset to enable the recruitment of these regions (Ward et al., 2004, Maulden et al., 2005).

In motor recovery, disruption of blood flow to the dorsal premotor cortex (PMd) by use of transcranial magnetic stimulation either ipsilateral or contralateral to the lesion increases motor reaction times in chronic stroke patients, suggesting that this structure is important for recovery in both the affected and unaffected hemispheres (Johansen-Berg et al., 2002b, Fridman et al., 2004). Furthermore, the hemisphere contralateral to the lesion is essential for recovery in those patients with larger impairments (Johansen-Berg et al., 2002b), indicating that the unaffected hemisphere is used more by those with poorer outcome, particularly when the primary motor region (M1) is affected on the lesioned cortex. Both contralateral M1 and PMd can be involved in functional recovery of the stroke-affected arm in hemiparetic patients (Lee et al., 2003b, Strens et al., 2003), and, importantly, substantial recovery can occur through these mechanisms with appropriate rehabilitation, even if the deficits are severe (Maulden et al., 2005). Overall, outcome is clearly limited by the degree of damage to the normal motor network and in particular to direct corticospinal projections, but recruitment and adaptation of surviving secondary motor areas in both hemispheres may help patients to achieve the best results given the anatomical constraints (Johansen-Berg et al., 2002a, Miyai et al., 2003).

A normal function of transcallosal inhibition between hemispheres controls the function of the motor and sensory cortices. Some studies have described a reduced inhibition within the motor cortex contralateral to the lesion after cortical stroke, but others have found abnormally high interhemispheric inhibition over the motor cortex ipsilateral to the lesion during upper arm movement (Shimizu et al., 2002, Murase et al., 2004). Combined, these findings suggest that the increased inhibitory drive could contribute to impairment and consequently, transcranial magnetic stimulation (TMS) could be used to modulate levels of activity at certain points in the brain to normalise any erroneous activity.

Longitudinal functional magnetic resonance imaging (fMRI) studies allow for a subject's recovery to be monitored throughout a given timeframe, which can further enhance understanding of functional changes within the brain over time, compared with cross-sectional or endpoint studies that provide a single snap shot of brain activity. From these studies we know that patterns of activation in chronic stroke patients evolve over time (Cramer et al., 1997). In one such study, the study of patients for over a year after stroke indicated a negative correlation between recovery and task-related recruitment of several brain regions, including the premotor cortex and the M1 region (Ward et al., 2003). There was an initial over-activation of these regions approximately 2 weeks after stroke, but this diminished with functional improvement in all stroke patients. This is in

line with other studies that suggest patients with poorer recovery begin with bilateral activations and fail to progress to a more normal activation pattern (Feydy et al., 2002). In support of this, some groups have shown a greater shift in activation from the hemisphere contralateral to the lesion to the lesioned sensorimotor cortex in those patients with most clinical improvement, which supports a more normal activation pattern in those with better recovery (Jang et al., 2003).

Chronic stroke patients that have no residual impairment tend to have relatively similar activation maps to control patients, but patients with stroke with more marked impairments show greater recruitment in ipsilesional primary motor cortex and bilateral secondary motor areas (Johansen-Berg et al., 2002a, Ward et al., 2003). This suggests that following ischaemic injury to the brain, the brain will use those surviving structures and networks that can generate some form of motor signal to spinal cord motoneurons in the most efficient way (Cramer, 2008). Studies show that chronic stroke patients show improvements following interventions. Stimulating peripheral nerves in chronic patients indicated that stroke subjects improved on measures of grasp compared to sham treated patients in which there was no improvement after 60 hours of electrical stimulation over a 3-week period. Furthermore, using fMRI and a finger-tracking task, increased activation was observed in the ipsilateral somatosensory cortex through probability maps compared with the baseline scan in patients with stroke (Kimberley et al., 2004).

The degree of corticospinal tract damage correlates well with the extent of motor recovery (Ward, 2005). Outcome might be restricted in some patients by the degree of damage to direct corticospinal projections, but recruitment and adaptation of surviving secondary motor areas in both hemispheres can help patients to achieve the best results. Many fibres in the corticospinal tract originate from the primary motor cortex, with contributions from other regions such as PMd, supplementary motor area, and insular cortex (Rouiller et al., 1993). This gives evidence that there is a need for integration of motor and sensory functions for successful motor performance.

fMRI has transformed the field of stroke and has substantially improved the understanding of what is occurring in the brain during recovery at a systems level. This should, when possible, be translated into animal models to better translate therapeutic research into human patients. This thesis reports the use of fMRI in Chapter 3 to measure activation in the brain after stroke longitudinally in animals after stroke. We will test the hypothesis that electrical stimulation of the forepaws will result in blood oxygen level-dependent activations that correlate with areas of the brain with increased activity, and could be related to functional recovery. Resting state network analysis will also be

used to assess connectivity between different brain regions at rest to identify changes in connectivity after stroke that may play a role in recovery.

1.5.2.2 Elucidating patterns of activation in animal neuroimaging studies to understand recovery mechanisms in human studies.

Similar to human studies, fMRI experiments in experimental stroke models have reported diminished blood-oxygen-level dependent (BOLD) activation in the lesioned sensorimotor cortex (Reese et al., 2000, Abo et al., 2001, Dijkhuizen et al., 2001, Dijkhuizen et al., 2003, Weber et al., 2008). In longitudinal studies, animals have been shown to re-gain the activation of the peri-lesional somatosensory cortex in response to forepaw stimulation, which corresponded to a restoration of sensory-evoked potentials (Weber et al., 2008). Additionally, enhanced activity as observed using analysis of fMRI is observed bilaterally in motor regions following stroke in rats, which normally subsides and returns to an activation state that is more normal in the sensorimotor cortices (Abo et al., 2001, Dijkhuizen et al., 2001, Dijkhuizen et al., 2003), although these studies did not show behavioural recovery of function. More precise cortical mapping of sensorimotor response after focal strokes to the forelimb sensorimotor cortex shows compensatory rewiring resulting in a shift of cortical sensorimotor responses to the peri-infarct area (Dijkhuizen et al., 2001, Dijkhuizen et al., 2003, Weber et al., 2008), and also redistribution of function of forelimb responses to other regions, including hindlimb regions (Winship and Murphy, 2008, Sigler et al., 2009). Intracortical stimulation of the sensorimotor cortex can also show corticospinal plasticity and shifts in cortical representation following spinal cord injury (SCI). For instance, in a study by Fouad et al. (2001), 4 weeks after a dorsal bilateral CST transection in the thoracic spinal cord stimulation of the hindlimb cortex was found to evoke novel forelimb electromyogram (EMG) responses which were associated with collateral sprouting of hindlimb CST fibres into cervical segments.

fMRI experiments can also be used to assess therapies upon restoration of function after stroke. An early neuroprotective strategy, as well as being able to prevent ischaemic damage, can show recovery of neural function and activation following electrical forelimb stimulation through recovery of peri-lesional penumbral regions after treatment (Sauter et al., 2002), though a late neuroprotective intervention might not reduce the size of the lesion but, at a delayed timepoint, compounds such as lithium provide enhanced fMRI activity in response to injury, promoting neurorestoration (Kim et al., 2008).

Non-human primate studies have characterised cortical map changes after focal ischaemic injury, which has greatly enhanced the knowledge of the effects of rehabilitation, axonal projections, and infarct size in stroke research (Nudo, 2007, Cramer, 2008). A study by Nudo and Milliken (1996) used intracortical microstimulation to map forelimb movement representations in the primary motor cortex, before and a few months after focal ischaemic lesion. They found that the infarcts caused a marked but transient deficit in the use of the contralateral forelimb to the lesion, with a corresponding increase in the use of the ipsilateral forelimb for retrieval tasks. Functional reorganisation has been shown by several studies, which show substantial reorganisation within the motor cortex after focal ischaemic stroke. An MCAO lesion placed in the region of the brain corresponding to the forepaw resulted in a larger forepaw representation area that expanded into regions formerly occupied by representations of the elbow and shoulder (Nudo et al., 1996a). Functional organisation in the undamaged motor cortex correlated with behavioural recovery of skilled hand function. These results suggest that after damage to the motor cortex, rehabilitative training shapes subsequent reorganisation in adjacent intact cortex, which could play an important role in motor recovery.

Contrary to human studies that allow for deeper understanding at a systems level, animal studies that use functional imaging techniques after focal brain damage can show changes at the molecular, cellular, and systems level, particularly when using techniques such as pharmacological MRI, histological tracing, and electrophysiological recordings. Many of the techniques used in animal studies have also been used to gather an insight into how the human brain responds to focal injury and how researchers can manipulate this to enhance functional outcome (Baron et al., 2004).

1.5.3 Enhancing recovery by overcoming inhibitory signals:

Following injury to the CNS there are several mechanisms that lead to spontaneous functional recovery. However, after a period, the functional improvement is no longer seen since an increase of inhibitory factors form a glial scar that severely disables regrowth or sprouting of existing fibres. There are a number of inhibitory factors within this post-injury environment, but for the purpose of this section the focus will be on the main obstacles that specifically impede axonal plasticity. Targeting these inhibitory factors could provide a mechanism to improve axonal growth and has been a concept behind some neuroplasticity strategies (for a review see Benowitz and Carmichael (2010)).

1.5.3.1 Nogo A

Nogo-A was the first growth inhibitory molecule to be discovered and purified from myelin extracts (Caroni and Schwab, 1988), and since then it has been independently cloned and characterised (Chen et al., 2000, GrandPre et al., 2000). Nogo-A is present in both the developing and established CNS, and is the most prominent of the myelin associated inhibitors (Huber and Schwab, 2000). This molecule can be found in many neurons (GrandPre et al., 2000) and has been localised at both branch points and synapses of cultured neurons (Hunt et al., 2002)—an ideal location for manipulating axonal growth after injury.

Interfering with Nogo-A through the blocking of its receptor NgR has now been studied in various *in vivo* models of CNS injury and has resulted in increased regeneration, compensatory sprouting, and functional recovery (Buchli and Schwab, 2005). Studies from injuries to the spinal cord have shown that downregulation of Nogo-A signalling induces axonal sprouting and increased functional recovery (Zorner and Schwab, 2010). Various other studies have shown that compensatory sprouting and axonal outgrowth can be significantly enhanced by application of anti-Nogo-A antibodies after CNS injury (Bregman et al., 1995). This treatment has also been shown to increase regenerative sprouting of CST and serotonergic (5HT) fibres in the caudal spinal cord (Schwab et al., 2005) and subsequently leads to functional recovery following injury. Following unilateral pyramidotomy, Nogo-A neutralisation caused an increase of sprouting of fibres across the midline to the de-afferented red nucleus and pons (Z'Graggen et al., 1998). Sprouting of uninjured CST fibres and rubrospinal fibres into the denervated cervical spinal cord in turn leads to improved forelimb functional recovery (Thallmair et al., 1998). Neutralising Nogo-A also proved effective in recovery of function by promoting repair of CST fibres after cervical hemisections in marmosets and macaques (Fouad et al., 2004). After cervical lesion in adult primates, Nogo-A-specific antibody treatment resulted in enhanced sprouting and functional recovery on a manual dexterity task (Freund et al., 2006, 2009), although more experiments are necessary to draw a conclusion on the plasticity of different spinal pathways. These findings in spinal cord injury models have paved the way for a role of Nogo-A in ischaemic stroke research.

Antibodies against Nogo-A have been suggested as a good method to counteract inhibition. The anti-Nogo-A antibody, IN1, has been shown to improve sprouting of intact axons after unilateral stroke injury, resulting in functional recovery in fine motor tasks in the paw contralateral to the lesioned hemisphere (Papadopoulos et al., 2002). IN1 also

promotes changes in dendritic formation of layer V pyramidal cells in the contralateral hemisphere to the stroke injury (Papadopoulos et al., 2006). However, neutralisation of Nogo-A does not reduce the stroke lesion size (Tsai et al., 2007), which implies that the antibody does not act as a neuroprotective molecule. Some studies have shown functional multilevel plasticity using IN1, which have shown benefit in elderly rats (Markus et al., 2005), hypertensive rats (Wiessner et al., 2003) and with treatment at delayed timepoints (Tsai et al., 2011); however, these benefits did not convey benefit in human clinical trials.

1.5.3.2 Chondroitin Sulphate Proteoglycans

Other molecules that mediate the inhibitory signals seen in the glial scar after stroke injury are chondroitin sulphate proteoglycans (CSPGs). Proteoglycans are a diverse family of molecules that contain a core protein and a number of covalently bound glycosaminoglycan (GAG) side chains. There are four known types: CSPGs, dermatan sulphate proteoglycans, heparan sulphate proteoglycans, and keratin sulphate proteoglycans (Hardingham and Fosang, 1992). CSPGs are important in the development and maintenance of the CNS and are the most abundant proteoglycan subtype (Properzi et al., 2005). Following spinal cord injury, CSPGs are one of the main growth inhibitory molecules within the glial scar, which severely decreases axonal regeneration by accumulating around dendritic synapses and forming peri-neuronal nets (Yamaguchi, 2000). CSPGs also form a barrier through the inhibitory influence of their GAG chains, which was first shown by a study by Snow et al. (1990) where DRG neurons avoided areas rich in CSPGs.

The enzyme chondroitinase ABC (ChABC) mediates the shortening of inhibitory side-chains that then promotes the axonal growth after injury (Bradbury et al., 2002, Soleman et al., 2012). CSPG levels have been shown to decrease in the peri-infarct area after stroke, which might contribute to the initial spontaneous recovery seen after injury (Carmichael, 2005). Many studies have now shown the beneficial effect of ChABC after injury to the nervous system (Moon et al., 2001, Bradbury et al., 2002, Barritt et al., 2005, Cafferty et al., 2008, Garcia-Alias et al., 2008). ChABC administration into partially denervated cervical spinal cord of elderly rats 3 days after focal ischaemic stroke promoted reorganisation of the CST within the spinal cord and induced functional recovery (Soleman et al., 2012). This indicates that degradation of CSPGs following ischaemic stroke may provide a future therapeutic strategy.

1.5.3.3 Inhibitory axon growth molecules

Other chemorepulsive axon growth inhibitors that emerge in the adult CNS after injury include Ephrin B3, Semaphorins and Netrin-1 (Huber et al., 2003, Benson et al., 2005, Liu et al., 2006). These are present in oligodendrocytes and contribute to the inhibitory environment within the myelin of the CNS following injury (Bundesen et al., 2003). Semaphorin4 is involved in microglial activation and oligodendrocyte differentiation after CNS injury (Toguchi et al., 2009), while ephrins and other semaphorins are important in establishing boundaries in the injured CNS (Pasterkamp et al., 1999). Therefore, blocking these molecules will inhibit a complex cell signaling system, resulting in beneficial effects on axonal sprouting following stroke injury (Carmichael, 2010).

1.5.4 Agents to enhance recovery through enhanced plasticity

Another way to mediate plasticity is to activate the growth potential of neurons rather than overcoming inhibitory signals. CNS neurons have growth potential during development but typically lose this ability perinatally (Goldberg et al., 2002). By promoting the intrinsic regenerative ability of the neuron and protecting damaged cells from apoptosis, neuronal cells should benefit from enhanced plasticity as a consequence of increased collateral sprouting.

1.5.4.1 Amphetamine

Administration of the stimulant amphetamine is known to induce neuronal release of noradrenaline, dopamine, and serotonin. Following stroke injury in spontaneous hypertensive rats, amphetamine has been shown to increase synaptogenesis and axonal growth through increasing levels of GAP43 and synaptophysin in the neocortex compared with vehicle treated animals (Stroemer et al., 1998). Preclinical studies in rats have shown that amphetamine treatment induces axonal plasticity and collateral sprouting after cortical injury (Stroemer et al., 1998, Adkins and Jones, 2005). However, several clinical trials measuring the effect of amphetamine application after stroke have resulted in highly effective outcomes after amphetamine (Crisostomo et al., 1988, Walker-Batson et al., 1995) but other studies have not shown this association (Sonde et al., 2001, Treig et al., 2003, Platz et al., 2005). There is no clear evidence to support or discourage routine amphetamine use after stroke injury.

1.5.4.2 Rehabilitation and training

Rehabilitation of stroke patients is one of the key components of treatment and management after stroke. Regular physical therapy of affected regions has been shown to improve the rate and extent of functional recovery. Importantly, the time of rehabilitation initiation is also an important aspect of therapy, as even severe deficits after stroke can benefit from recurrent physical therapy (Maulden et al., 2005).

Unilateral electrical stimulation of the CST promotes axonal reorganisation and synaptogenesis in mature rats even without injury. Injury of the CST stimulates sprouting of axons from fibres in the remaining ipsilateral and contralateral cortices, and this sprouting becomes even stronger when unilateral CST injury is combined with electrical stimulation of the intact CST. This results in an increase in the number and length of axon collaterals crossing from the intact side of the spinal cord into the denervated side compared with either treatment alone (Brus-Ramer et al., 2007). Transcranial magnetic stimulation (TMS) of undamaged pre-motor areas show improved use of an impaired limb in both patients and animals, although early clinical trials have failed to show a significant benefit (Yozbatiran et al., 2009). Activation under more physiological conditions strongly improves the use of a disabled forelimb after unilateral stroke or CST injury. This phenomenon is illustrated in the so-called forced use paradigm, which avoids compensatory use of the unimpaired forelimb (Jones et al., 2009). Following unilateral stroke, use of the impaired forelimb is required because of constraint of the unimpaired limb, resulting in improved performance with the affected forelimb (Taub et al., 1993, Wolf et al., 2006). After unilateral transection of the CST, forced use of an impaired limb doubles the number of collateral branches that extend from the intact CST into the denervated side of the spinal cord. This growth is associated with formation of new synapses and improved motor performance (Maier et al., 2008). Indeed, the process of retraining skilled hand use after ischaemic damage to the hand motor area alters cortical representations of the digits, wrist, and forearm, expanding into the intact cortex that had been formerly occupied by elbow and shoulder representation. In contrast, subjects without training experienced a loss of the digit and wrist-forearm area in surviving tissue (Nudo et al., 1996a). These data suggest that pharmacological or cellular therapies that promote recovery should be effective within an environment with altered behavioural activity patterns. This could then result in axonal sprouting and recovery leading to repair of cortical circuits (Maier et al., 2008).

Other learned behaviours can occur after stroke, such as compensation and learned disuse, or neglect, of the affected paw. Compensatory behaviour develops when the task

is completed through a different strategy than that used prior to the injury, which can be inefficient and lead to musculoskeletal damage (Dobkin and Carmichael, 2003). In contrast, learned disuse occurs when stroke lesions result in a reduced observation of the affected function—eg, after stroke lesions to the sensorimotor cortex, the affected limb would not be used in favour of the less-affected forepaw. Axonal sprouting occurs in response to increased activity (Carmichael and Chesselet, 2002, Butz et al., 2009), and these patterns of activity after stroke result in adaptive and maldaptive plasticity.

1.5.4.3 Neurotrophins

Neurotrophic factors are key modulators of neural development, survival and plasticity (Huang and Reichardt, 2001). There have been many studies showing the extensive role of neurotrophins on the CNS and evidence that they may have a role in neuronal plasticity following injury (Barde et al., 1982, Giehl and Tetzlaff, 1996, Bradbury et al., 1998a, Bradbury et al., 1999a, Mendell et al., 2001, Hollis et al., 2008). Transient expression of various neurotrophic factors might identify the boundaries of the critical period for recovery. In non-ischaemic regions, basic fibroblast growth factor (bFGF) (Speliotes et al., 1996) and nerve growth factor (NGF) mRNA are transiently altered after focal ischaemia and can be influenced further by behavioural experience (Humm et al., 1998, Dahlqvist et al., 1999).

In one study, grafts of NGF-expressing fibroblasts were delivered into a dorsal bilateral T7 lesion and resulted in a significantly higher amount of axons compared with control animals (Grill et al., 1997a). Similarly, delivery of brain-derived neurotrophic factor (BDNF) following SCI resulted in enhanced growth of reticulospinal and raphespinal axons (Ye and Houle, 1997). The application of fibroblasts expressing neurotrophin 4/5 following a thoracic hemisection resulted in reticulospinal and propriospinal axonal growth (Blesch, 2004). Neurotrophin 3 (NT3) has been shown to promote the sprouting of damaged CST axons within the spinal cord (Schnell et al., 1994) and is involved in regeneration of injured dorsal column sensory axons after injury (Bradbury et al., 1999a). Additionally, the application of neurotrophic factors can ameliorate the cell bodies' response to axotomy (Giehl and Tetzlaff, 1996). Therefore, delivery of neurotrophic factors to areas of CNS injury could be beneficial in promoting axonal sprouting and result in functional recovery (Grill et al., 1997b, Ramer et al., 2002, Zhang et al., 2008). The role of neurotrophins in the reorganisation of spinal cord circuitry following stroke has been shown previously (Duricki et al., 2016b). This thesis focuses on the role of NT3 in functional outcome after permanent focal ischaemic stroke injury.

1.6 Neurotrophins

1.6.1 History of Neurotrophins

Neurotrophic factors were first classed as a family of survival factors for sensory and sympathetic neurons. However, these factors have since been shown to be vital in aspects such as cell fate, axon growth, development, synapse formation, plasticity, pain, and the survival and function of both the peripheral and central nervous systems (PNS and CNS) (Lewin et al., 1992, Bibel and Barde, 2000).

There are four known members of the neurotrophin family. The first of these to be discovered was NGF. It was purified as a survival factor for cultured sympathetic and sensory neurons and is synthesised and secreted from sensory target organs (Levi-Montalcini and Angeletti, 1968). This discovery led to the birth of the neurotrophic theory that the targets of neuronal innervation secrete a factor that controls the size of target tissue innervation. The discovery of NGF was shortly followed by the discovery of a second member of the neurotrophic family: BDNF. BDNF was purified from pig brain as a survival factor that activated a neuronal population unresponsive to NGF (Barde et al., 1982). These discoveries led to the subsequent discoveries of NT3 and NT4/5.

1.6.2 Structure of neurotrophins

The neurotrophins share conserved function, structure, promoters, and sequence. Each protein sequence contains a prodomain that is cleaved off by furin and other prohormone convertases to produce the mature protein (Seidah et al., 1996). Once translated, these proteins form a tertiary structure through the use of a cysteine knot, which are common in several other growth factors. Interestingly, neurotrophins have the ability to form dimers to stabilise themselves; homodimers are common, but heterodimers of NT3 with BDNF are also possible due to similar levels of hydrogen bonding (Butte et al., 1998).

Proneurotrophins themselves do have biological roles and are secreted from cells. Proneurotrophins bind with a high affinity to the p75 neurotrophin receptor ($p75^{NTR}$) and sortilin, resulting in activation of downstream targets that promote apoptosis (Lee et al., 2001). Studies have found an upregulation of proneurotrophins following neuronal injury, which could enhance unwanted neuronal loss (Harrington et al., 2004).

1.6.3 Mechanisms of action

1.6.3.1 Tropomyosin-related kinase (Trk) Receptors

Neurotrophic signaling requires binding of the protein to its associated receptors. The receptors affiliated with the neurotrophin family are known as the tropomyosin-related kinase (Trk) family of receptor tyrosine kinases (TrkA, TrkB, TrkC) (Chao, 2003). These receptors have been shown to mediate processes including cell survival, axon and dendrite growth, glial interactions, and are an integral component of synapse formation and function.

Trk receptors are expressed on the terminals of many neurons—including sensory and corticospinal—on the cell body, axon, and terminals of neurons. These receptors consist of a transmembrane domain and a typical kinase domain followed by a short C terminal tail, although there are also splice variants that do not have the C terminal tail (Biffo et al., 1995). The extracellular segment contains two immunoglobulin (Ig) domains, which are essential for neurotrophin binding. Following the Ig domain is a cysteine rich cluster, three leucine rich repeats and another cysteine rich cluster (Friedman and Greene, 1999). Each receptor crosses the membrane once and is terminated with a cytoplasmic domain consisting of a tyrosine kinase domain surrounded by several tyrosines acting as phosphorylation-dependent docking sites for intracellular signaling complexes.

Dimerisation occurs between the Trk receptors and a $p75^{NTR}$ *via* ligand binding, although there could be other mechanisms (Longo and Massa, 2013). Activation of the receptors through transphosphorylation of the kinases present in the cytoplasmic domain results in the formation of a high-affinity receptor. The neurotrophin interacts with the Ig domain (Ultsch et al., 1999) and activation of this unit in turn activates a series of intracellular signalling cascades.

Each Trk contains a slightly different set of residues to associate with a specific ligand. The different neurotrophins bind to their respective receptors: with NGF activating TrkA; BDNF and NT4/5 activating TrkB and NT3 activating TrkC. NT3 has a unique feature compared with the other neurotrophins in that it can also bind all the other Trk receptors but at a lower affinity through differential splicing (Clary and Reichardt, 1994, Tsoulfas et al., 1996). The differential splicing adds short amino acid sequences into the extracellular Ig domains of the Trk receptor, resulting in an increased binding of non-preferred ligands at TrkA and TrkB (Meakin and Shooter, 1992, Shelton et al., 1995, Strohmaier et al., 1996).

Trk receptor activation promotes a variety of responses including survival and differentiation while activation of $p75^{NTR}$ can promote apoptosis (Teng et al., 2005).

There is also evidence suggesting that ligand binding to truncated isoforms of TrkC can effectively modulate intracellular signalling through the p75 receptor (Hapner et al., 1998).

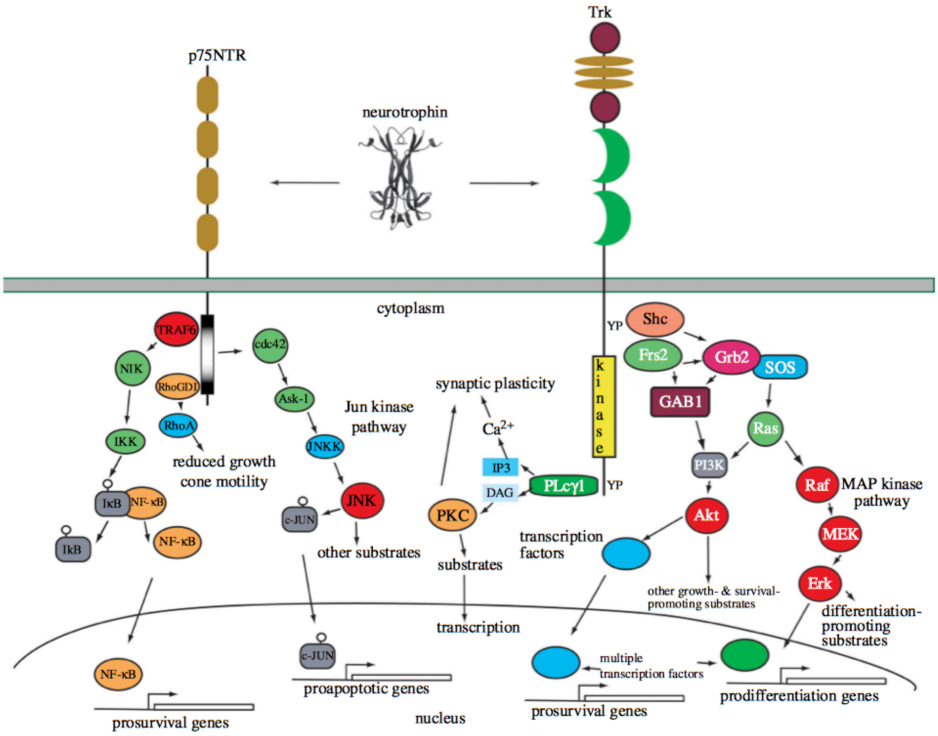


Figure 1-3 : Neurotrophin signalling.

From Reichardt (2006). This schematic depicts the key signalling cascades that arise from neurotrophin binding to the p75NTR and the Trk receptors. Activation of p75NTR promotes pro-survival mechanisms via NFkB. Conversely it causes reduced growth cone motility and apoptosis through RhoA and c-Jun pathways respectively. Trk receptor activation results in activation of the PLCγ1, PI3K and MAPK pathways to promote synaptic plasticity, transcription, neuronal survival and differentiation

Once a neurotrophin has bound to its Trk receptor, autophosphorylation of the tyrosine residues occurs (Jing et al., 1992). It is these residues that then interact with intracellular proteins to initiate a variety of processes. The main pathways activated include mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinases (PI3K), and phospholipase C gamma (PLCγ), which lead to increased synaptic plasticity, growth, survival, differentiation and an increase in transcription factors (Douville and Downward, 1997, Huang and Reichardt, 2001, Reichardt, 2006).

1.6.3.2 p75 Neurotransmitter Receptors ($p75^{NTR}$)

The $p75^{NTR}$ was first cloned in 1986 as a receptor for NGF and is also related to the tumour necrosis family (Johnson et al., 1986). This receptor consists of a single transmembrane domain, an extracellular domain featuring four cysteine rich repeats and a poorly-conserved cytoplasmic death domain (Liepinsh et al., 1997, He and Garcia, 2004). The cysteine regions have been shown to be important for binding NGF (Murray et al., 2004).

$p75^{NTR}$ is able to bind all of the proneurotrophins with similar efficacy (Rodriguez-Tebar et al., 1990, Frade and Barde, 1998). Mature neurotrophins can also bind to this receptor but at 1000 times less affinity (Lee et al., 2001). The presence of $p75^{NTR}$ has been shown to inhibit the activation of Trk receptors by non-preferred neurotrophins and also promotes retrograde transport of neurotrophins *in vivo* and may enhance axon growth through this mechanism (Curtis et al., 1995). The presence of $p75^{NTR}$ has been shown to both facilitate internalisation of Trk receptors (Geetha et al., 2005) and also suppresses degradation of TrkA and TrkB (Makkerh et al., 2005). There has been much work into the retrograde transport of neurotrophins (Hibbert et al., 2006) and on whether the mechanisms of transport differ between the positive effects of receptor activation or the activation of the detrimental apoptotic effects (Butowt and von Bartheld, 2001). Although it is well known that $p75^{NTR}$ modulates the binding of mature neurotrophins with their high affinity receptors (Bibel and Barde, 2000), activation of $p75^{NTR}$ alone by proneurotrophins has been shown to induce cell death (Casaccia-Bonnefil et al., 1998, Friedman, 2000), suggesting there are different mechanisms in place for these two seemingly opposite pathways. Additionally, inhibition of intracellular furin proteinase activity resulted in release of proNT3 and not the mature form (Yano et al., 2009). They showed that the proNT3 pathway induced sympathetic neuronal death that was dependent on $p75^{NTR}$ and sortilin, a protein found to enhance the transport and availability of Trk receptors at the neuron surface (Vaegter et al., 2011).

Upon activation of $p75^{NTR}$ three main downstream pathways are activated, these are the Jun kinase, NFkB and the RhoA signalling cascades (Reichardt, 2006). Activation of the NFkB pathway has been shown to promote neuronal survival (Wooten et al., 2001) whereas Rho family GTPase activity has been shown to inhibit neurite outgrowth and control cell motility and growth cone behaviour (Yamashita et al., 1999). Similarly, activation of the Jun kinase pathway is known to promote apoptosis (Bazenet et al., 1998, Kanamoto et al., 2000).

1.6.4 Retrograde transport

In 1980 it was discovered that neurotrophins and the receptor are internalised and retrogradely transported to the cell soma on substrate-receptor binding (Thoenen and Barde, 1980). The complex is internalised and then transported in endosomal compartments that set off a series of signalling cascades, allowing neurotrophins to affect local neuronal behaviour (Ye et al., 2003).

Retrograde transport studies have found that motoneuron cell bodies contain TrkC receptors (Merlio et al., 1992) and can retrogradely transport NT3 from peripheral muscle to potentially modulate proprioceptive and mechanoreceptive tasks (DiStefano et al., 1992, Zhou and Rush, 1995).

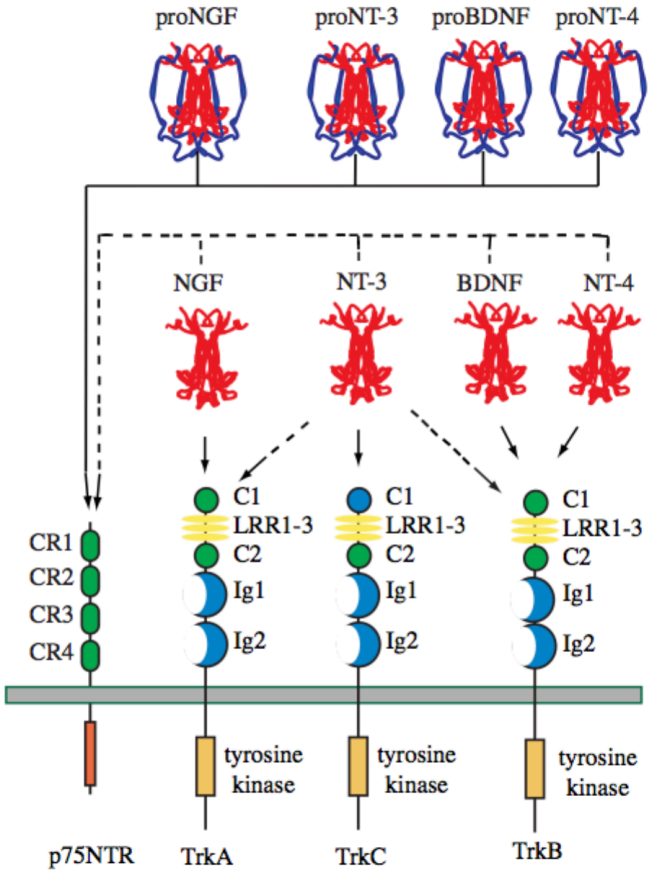


Figure 1-4 : Neurotrophin-receptor interactions.

From Reichardt (2006). This schematic shows the major interactions between the four NTs expressed in mammals and their receptors. All immature neurotrophins, termed proneurotrophins, are only able to bind to p75NTR. Following maturations the mature neurotrophins have the ability to bind to one or more Trk receptors. To achieve a binding site for the neurotrophins, the Trk receptors must first dimerise. In order to establish a high affinity receptor binding site, p75 interacts with the Trk receptors to form a high affinity unit. Once the neurotrophin has bound, it is internalised and proceeds to activate various signalling pathways.

1.7 Neurotrophin-3

1.7.1 NT3 in neuronal survival

The neurotrophin family was first discovered as a family of survival factors and the first studies focused on this role (Maisonpierre et al., 1990). Many studies have shown the survival effect that exogenously applied NT3 had on sensory neurons. Sensory neurons from NT3-/- cultures can be rescued by addition of exogenous NT3 (ElShamy and Ernfors, 1996), whereas studies using NT3 antibodies show the large loss of DRG neurons in culture (Gaese et al., 1994). NT3+/- mice have half the number of muscle spindles and proprioceptive neurons compared with wildtypes (Ernfors et al., 1994). Additionally, the application of NT3 antibodies at the end of gangliogenesis causes a 30% decrease in the number of surviving sensory neurons (Gaese et al., 1994).

There seems to be a large discrepancy between the neuronal numbers lost in NT3-/- mice and TrkC-/- mice. NT3-/- mice show a 60% loss in DRG neurons (Ernfors et al., 1994, Fariñas et al., 1994), whereas TrkC-/- only show a 17% loss (Klein et al., 1994), which includes all proprioceptive neurons (Minichiello et al., 1995). This difference could be due to the fact that NT3 supports many neurons in early development by acting through the other Trk receptors (Davies et al., 1995).

Animals with insufficient NT3 or reduced TrkC signalling never develop proprioceptive afferent projections to muscle (Kucera et al., 1995), suggesting that NT3 affects differentiation and survival (Ockel et al., 1996). NT3 is the only neurotrophin that exerts this control on the proprioceptive neurons. NT3-/- mice have a lethal phenotype in the early, post-natal period resulting from the loss of muscle spindle, which is not seen in NGF, BDNF, or NT4 mutant mice. NT3 mutation studies show deficits in populations of sensory neurons, in which NT3-/- mice at birth lack all TrkC positive sensory neurons (Ernfors et al., 1994). Furthermore, there are fewer Ia afferents and spindles in NT3+/- mice relative to wildtypes (Wright et al., 1997). This suggests that individual muscles can control the density of their proprioceptive innervation in development by regulating their production of NT3. Mice lacking TrkC show similar results to NT3 knockout studies in that there is a complete absence of Ia afferents, muscle spindles and gamma motoneurons (Kahn et al., 1999, Taylor et al., 2001).

1.7.2 NT3 for modulating synaptic strength

The monosynaptic spinal stretch reflex circuit is mediated by the synaptic connections between muscle spindle proprioceptive Ia afferents and motoneurons. Central Ia

afferents connect specifically with motor pools that innervate homonymous muscles and establish weaker synapses with synergistic motoneurons, but avoid motor pools innervating strict antagonists. Antagonistic motor neurons are not only excited by different sets of Ia afferents but also inhibited *via* Ia inhibitory interneurons.

The use of genetics has also enhanced our understanding of NT3's role in synaptic modulation. In one study, Egr3-null mutant mice were used in which muscle spindles were shown to degenerate progressively after birth, leading to proprioceptive impairments (Chen et al., 2002a). These mice had a significant functional deficit in the strength of sensory-motor connections, which could be fully restored following intramuscular injections of NT3, suggesting that NT3 regulates the synaptic connectivity between muscle sensory and motor neurons (Chen et al., 2002a). A second study showed that embryonic overexpression of NT3 using transgenic (mlc/NT3) mice induced inappropriate synaptic strengthening between the Ia motoneuronal connections (Wang et al., 2007).

1.7.3 TrkC and $p75^{NTR}$ expression in the adult

To gain a better understanding about how NT3 elicits its effects, it is important to understand where both NT3 and the receptors TrkC and $p75^{NTR}$ are located. Many early studies investigated the temporal and spatial locations of these two complexes. A specific NT3 antibody was discovered in the early 1990s (Zhou and Rush, 1993), but studies such as these use *in situ* hybridisation techniques whereby a radioactive probe for the TrkC mRNA is used to label the receptor transcripts, which can be cumbersome (Buck et al., 2000). The probe is usually targeted to the tyrosine kinase domain of the receptor to eliminate labeling of non-neuronal and truncated Trk isoforms (Merlio et al., 1992).

It is known that NT3 and its TrkC receptor show unique and overlapping mRNA expression patterns (Maisonpierre et al., 1990) and that the mRNA for both is widely distributed in both the PNS and CNS (Ernfors et al., 1990, Hohn et al., 1990). The highest concentrations of TrkC mRNA are located in the CNS (Martin-Zanca et al., 1990), however there are also high levels in the spleen and liver (Yamamoto et al., 1996). TrkC mRNA in the brain and spinal cord vary greatly during development, with the highest level being at one week after birth (Ernfors et al., 1992). Several studies have assayed for either NT3 or TrkC mRNA or NT3 protein (Maisonpierre et al., 1990, Copray and Brouwer, 1994) and have found their presence in the brain within the hippocampus, cerebral cortex, granular cell layer of the cerebellum (Merlio et al., 1992), in

approximately 10% of DRG cells (McMahon et al., 1994), in large diameter skeletal muscle afferents (McMahon et al., 1994, Griesbeck et al., 1995), in perisynaptic and myelinating Schwann cells (Hess et al., 2007), interneurons (Bechade et al., 2002), motoneurons (Merlio et al., 1992) and in several descending spinal cord pathways, including the CST, reticulospinal tract (RST), and bulbospinal (BST) (King et al., 1999).

In adult rats, TrkC mRNA is expressed in motoneurons (Merlio et al., 1992): however, there are substantial age-related decreases in the levels of TrkC and NT3 (Kaisho et al., 1994, Ulfhake et al., 2000). The decrease in TrkC mRNA in aged rats could be due to axonal dystrophy of proprioceptive sensory fibres, which would in turn lead to the decreased amounts of NT3 in muscle and spinal cord also observed with age (Fundin et al., 1997a). Interestingly, in ageing there is an increase in the $p75^{NTR}$ in motoneurons (Johnson et al., 1999). This lack of covariation between the TrkC and $p75^{NTR}$ indicates a changed role for $p75^{NTR}$ in aged motoneurons (Hess et al., 2007). Furthermore, the changes in TrkC expression that occur in ageing show the importance of using an elderly model of stroke when looking at NT3 as a potential therapy for an age related disease, as done in chapters 4 and 5 of this thesis.

1.7.4 Role of NT3 following injury

NT3 is known to be important in the maintenance of the adult nervous system and following injury to the nervous system. In the adult nervous system NT3 mRNA and TrkC are expressed on sensory neurons in dorsal root ganglia (DRGs) (McMahon et al., 1994), intrafusal fibres in spindles (Copray and Brouwer, 1994) and in tissues innervated by sensory fibres (Schecterson and Bothwell, 1992).

As homozygous NT3 knockout mice die within a few days of birth (Ernfors et al., 1994), it is more difficult to elucidate the role of NT3 in the mature nervous system (although heterozygous NT3 mice can live throughout adulthood) (Ma et al., 1999). With the use of Cre-loxP conditional NT3 knockout mice (Bates et al. 1999), it is becoming increasingly more feasible to investigate the local effects that low NT3 concentrations might have in specific tissues in adulthood. Much of the role of NT3 has been elucidated by assessing changes in NT3 concentrations, synaptic changes from electrophysiology studies, or by studying plastic changes on over-expression or down-regulation of NT3.

Since the discovery of NT3, there have been many studies done to establish the effects of this neurotrophin following injury to the CNS. NT3 has been shown to have a high permeability for the blood-nerve-barrier in adult rats (Poduslo and Curran, 1996), indicating it can come into contact with axons in nerves soon after reaching the systemic

circulation. A brief review of some strategies that have been used to deliver NT3 is included below, including local infusion (Bradbury et al., 1998a, Bradbury et al., 1999a) and viral vectors (Zhou et al., 2003, Zhou and Shine, 2003) in an attempt to promote axonal growth and repair.

The most studied delivery of NT3 has been by local infusion into the injured area to promote sprouting of spared neurons or form new functional connections. It is now well established that NT3 exerts powerful chemoattractive effects on a subset of neurons, which has been shown not only in injury, but also at embryonic stages of development (Paves and Saarma, 1997).

Studies have shown that NT3 can promote functional regeneration and sprouting of spared fibres when given directly at the site of injury. Treatment with NT3 has been shown to promote regeneration of injured dorsal column axons (Bradbury et al., 1999a), cause reinnervation of denervated dorsal column nuclei (Alto et al., 2009), result in regeneration and sprouting of the injured CST (Schnell et al., 1994), and establish functional recovery after CNS injury (Houweling et al., 1998). Cellular delivery of NT3 using secreting fibroblasts was found to promote increased corticospinal axon growth and partial recovery after dorsal hemisection lesions (Grill et al., 1997b). This method of delivery has also been used up to three months following a thoracic hemisection resulting in increased CST growth and partial recovery (Tuszynski et al., 2003). This provides evidence that NT3 can also elicit growth-promoting effects, which would be beneficial following a stroke injury and importantly even when given in a delayed timeframe.

The studies mentioned above administer NT3 locally to the area of damage. Although this method might be effective, it is not practical when searching for a clinically relevant therapy for stroke, as SCI therapies that administer NT3 in this way often had the opportunity for this delivery through spinal surgery after injury. This has led to studies using gene therapy in which NT3 can be delivered peripherally and transported to central targets, with the aim of causing long-term gene expression of NT3, allowing persistent delivery of this gene.

The use of adenoviral vectors *in vivo* with the human NT3 gene encoded has shown reinnervation of sensory axons after DRG injury. After injection of an adenovirus encoding NT3 directly into the ventral horn, long-term transgene expression in motoneurons was observed as well as substantial axonal re-innervation of sensory neurons (Zhang et al., 1998).

Furthermore, another study showed a significant increase of NT3 after direct delivery to the sciatic nerve after a cut to the CST at the level of the pyramids. Promisingly, the adenovirus-associated (AAV)-NT3 resulted in significant growth of CST axons across the midline (Zhou et al., 2003). Unfortunately, functional recovery was not measured because the nerve was transected, however, other studies have shown that increased CST sprouting results in functional recovery (Thallmair et al., 1998). Further to this, the same group showed that AAV-NT3 promoted this sprouting of CST axons but only in the acutely injured rat; this was not seen when therapy was chronically delayed or in an uninjured animal (Thallmair et al,. 1998). This led to the idea that processes associated with the injury itself, for example, Wallerian degeneration, could have a role to play in neuroplasticity; it has been shown that chronic delay in AAV-NT3 delivery can cause CST sprouting following a CST lesion but the spinal cord has to be 'immune activated' for sprouting to occur, suggesting a key role in the immune system in the regenerative role after injury (Chen et al., 2006).

1.7.5 Potential role for NT3 in stroke therapies

In a cortical microinjection endothelin-1 model of stroke, treatment of the disabled triceps with either NT3 protein or viral vector encoding pro-NT3 24 hours after stroke injury leads to contralesional corticospinal tract sprouting in the spinal cord (Duricki et al., 2016b). It is important to note that this study was a model for neurorestoration, and no neuroprotection was observed. This was associated with sensorimotor functional improvement. This is one of the first studies that show that a viral vector injected into the muscle resulted in NT3 synthesis and leads to sprouting of axons in the injured CNS. Additionally, other studies in our group have shown that recombinant NT3 can improve outcome after CNS injury when administered peripherally (Duricki et al., unpublished). This project will concentrate on extending this treatment into stroke models involving a larger lesion than those previously used in our lab.

Although the full mechanism of action of NT3 is still largely unknown, there is research being done to explore where NT3 goes in the body after peripheral administration, and this is something that our future experiments hope to address.

1.8 Aims of the thesis

The aim of this thesis was to see if NT3 could be used to enhance functional recovery following a large, permanent, ischaemic stroke with a delayed treatment.

The work in this thesis was inspired by David Shine's work indicating that synthesis of NT3 in the cord led to CST sprouting in the lumbar cord, even when delivered at severely delayed timepoints. We also knew from Jenny Fortun's work that AAV delivery of NT3 into muscles led to functional recovery after bilateral spinal cord injury (Fortun et al., 2007, Fortun et al., 2009). Furthermore, work from Denise Duricki showed that AAV delivery of NT3 into the affected muscles 24 hours after stroke could result in some functional recovery in focal ischaemic stroke in elderly rats, and that this might be a clinically relevant treatment for elderly people, without the limitations of a small therapeutic window (as compared with tPA). Therefore, the aim for the experiment reported in chapter 2 was to see if delayed, intramuscular treatment of AAV-NT3 improves sensory and motor recovery in young adult rats 4 weeks following ischaemic stroke injury. AAV-NT3 did not cause functional recovery, compared with stroke rats treated with a control viral vector. However, spontaneous recovery was observed in all the stroke groups.

A functional magnetic resonance imaging analysis was used in chapter 3 to try and identify where the origin of the recovery could be observed, using both forepaw stimulation and resting state functional imaging, to observe connectivity changes. Interestingly, these results contradict results currently published. The regions associated with recovery in these animals could be associated with spontaneous recovery seen in the previous chapter after large lesions to the somatosensory cortex.

In the experiment in chapter 4, an aged, male and female cohort of rats was used, since stroke affects both sexes, and after large cortical stroke, NT3 protein was infused subcutaneously for 4 weeks starting 24 hours after stroke. Recovery was assessed for 12 weeks using various sensorimotor tasks, resulting in a poor functional outcome. Some functional recovery was observed by use of H-reflex electrophysiology.

In the study reported in chapter 5, we used subcutaneous infusion of recombinant NT3 protein in elderly female rats after stroke, and observed a significant reduction of spasticity using H-reflex electrophysiology. An exploratory RNASeq analysis revealed interesting changes in mRNA regulation in cervical DRGs, opening up questions as to what mechanisms are involved in enhanced recovery seen following delayed treatment of NT3 after stroke injury. These are described in the general discussion in chapter 6.

Chapter 2 The effect of delayed AAV-NT3 delivery to affected forelimb muscles on sensory and motor function after permanent distal middle cerebral artery occlusion in young adult rats

2.1 Abstract

The experiments presented in this chapter aimed to identify a permanent model of stroke that would produce large, reproducible cortical infarcts in adult female rats. We first used the single-vessel MCAO model of stroke, in which craniotomy was done and the distal portion of the middle cerebral artery occluded by electrocoagulation. Functional deficits were measured using an array of behavioural tasks and lesions were confirmed through the use of structural MRI. Initial deficits were observed at 1 week after stroke in behavioural tasks, but by 3 weeks after stroke, all behavioural tasks showed substantial spontaneous recovery and the study was terminated. A second experiment was done to produce larger, more sustained deficits through simultaneous occlusion of the common carotid arteries (permanent occlusion of the common carotid artery ipsilateral to the lesion and transient, 60 minute occlusion of the common carotid artery on the contralateral side to the lesion) during MCAO occlusion. 4 weeks after stroke, AAV-NT3 or AAV-GFP was administered via forelimb intramuscular injection. Although work by others has indicated that functional recovery due to NT3 treatment was not likely in this chronic experiment, it was decided that the administration of the viral vectors worth trying as the resources were available. Outcome was assessed through the use of behavioural tasks for 8 weeks after treatment and ELISAs verified the presence of NT3 in the blood at the study end-point. The experiment was a success (in terms of inducing large cortical infarcts and a persistent deficit in sensorimotor function) and aligned with expectations even though there was no observed benefit of AAV-NT3. This experiment was the setting up of the surgical model for fine-tuning in later studies.

2.2 Introduction

Stroke takes a life every 5 seconds worldwide (World Health Organisation, 2014), and is the leading cause of adult locomotor disability (Kelly-Hayes et al., 2003). Tissue plasminogen activator (tPA) is only used in less than 10% of patients because of the narrow therapeutic window (<4.5 hours) and most patients are not admitted to hospital within this time (Saver et al., 2009). It is essential to find a treatment that can be of use to patients within a more feasible timeframe. The most prevalent outcome from having a stroke is locomotor disability (Langhorne et al., 2009); the use of lengthy rehabilitation is the only option for patients having had a stroke who suffer from this condition. A treatment that can improve the quality of life of stroke patients with chronic disability *via* a clinically relevant route and timeframe is therefore a target for new stroke therapies.

Following unilateral stroke, axons can be lost from one corticospinal tract (CST), resulting in loss of sensory and motor function, leading to reliance on the less-affected limb and possible neglect of the affected limb (Wiessner et al., 2003, Reep et al., 2004, Soleman et al., 2010). Spontaneous recovery that occurs after incomplete lesioning of the CST has been suggested to cause functional recovery (Wiedner et al., 2001). This has been shown previously by the sprouting of intact axons of the spinal cord after stroke (Carmichael, 2003, Nudo, 2006, Lapash Daniels et al., 2009). Experimental enhancement of spontaneous plasticity could promote further recovery after adult central nervous system injury. Indeed, many treatments include the use of axon growth promoters such as inosine (Chen et al., 2002b), or inhibition of inhibitory axon growth molecules such as chondroitinase ABC and anti-Nogo A antibodies (Seymour et al., 2005, Soleman et al., 2012), which have been shown to be beneficial in stroke models.

Neurotrophin 3 (NT3) is a growth molecule that has been shown to be important in the maturing neuron, as well as in cases of injury and repair, and many studies have shown the benefits of using NT3 for recovery after CNS injury (Bradbury et al., 1998b, Bradbury et al., 1999a, Zhou et al., 2003, Zhou and Shine, 2003). NT3 can modulate neuron differentiation, growth, branching and transmission; consequently, NT3 is a logical and appropriate potential stroke therapy. However, the short half-life of NT3 is a complicating factor in the delivery of NT3 for treatment, although it does cross the blood-brain barrier, and many studies have focused on delivery of NT3 intrathecally to provide direct application to the area of damage to maximise the effect of NT3 (Bradbury et al., 1998b). Although this has been effective, the infusion of NT3 would have to occur over sustained periods, which is often not convenient for use in the clinic, and results in a risk of infection. This has led to studies using gene therapy for delivery of NT3.

The use of adeno-associated viruses (AAV) has been successful to date, having been shown to be non-pathogenic, low toxicity vectors that are viable for delivery of gene therapy without integrating into the host genome (Kaspar et al., 2002, Carter, 2005, Hutson et al., 2012) and have been used safely in almost 100 clinical trials to date, including Parkinson's disease (Kaplitt et al., 2007), muscular dystrophy (Herson et al., 2012), and chronic heart failure (Jaski et al., 2009). The AAV1 serotype has also been shown to be safe in the long term (Zsebo et al., 2014) and expression of AAV vectors has been shown to last as long as 1 year (Mueller and Flotte, 2008b); this research has culminated in approval of an AAV1 for clinical use when delivered intramuscularly (Glybera (Miller, 2012, Salmon et al., 2014)) although there are concerns about the cost of this treatment (Ylä-Herttuala, 2015). AAV serotype 1 has been shown to be efficient at expressing transgenes and is also retrogradely transported, resulting in transgene expression in target tissues such as the spinal cord (Kaspar et al., 2002, Kaspar et al., 2003). This information, along with the knowledge that NT3 itself can be retrogradely transported (DiStefano et al., 1992), allows us to consider that if used in combination, AAV-NT3 may be able to target the spinal cord if given peripherally.

Previous preclinical studies that have used AAV-NT3 after CNS injury have reinforced this analysis. After unilateral CST lesions, AAV-NT3 induces the sprouting of uninjured CST axons from the intact side of the spinal cord towards the injured side when delivered peripherally to motor neurons, resulting in a functional recovery (Zhou et al., 2003, Chen et al., 2006, Chen et al., 2008, Fortun et al., 2009). These experiments indicate that not only is there increased sprouting in the spinal cord but also an increased synthesis of NT3 in the spinal cord after AAV-NT3 delivery (Petruska et al., 2010). In a model of small focal ischaemia, AAV-NT3 has also been shown to show a functional recovery in both young and aged animals after endothelin-1 lesions when AAV-NT3 was delivered at a delayed timepoint of 24 hours (Duricki et al., 2016b). These exciting data support the use of AAV-NT3 in a model of small cortical ischaemia, *via* a clinically relevant route and time frame. There is evidence that NT3 can produce an effect in CNS lesions at delayed times of 24 hours after the lesion (Chen et al., 2008). To discover how narrow a therapeutic window might be, efficacy of a treatment should be assessed at more than one time after the lesion onset, and this will be covered in this chapter. A stroke model involving a larger cortical infarct could be required to assess the effectiveness of NT3 further in stroke, to support previous data before going forward towards a clinical trial.

This aim of this chapter was to measure the effect of delayed (4 week) AAV-NT3 delivery to affected forelimb muscles on sensory and motor function after permanent distal middle cerebral artery occlusion in young adult rats.

2.3 Methods and Materials

2.3.1 Experimental Design

All procedures, behavioural testing and analyses were done using a randomised block design (Altman and Bland, 1999, Moher et al., 2010, van der Worp et al., 2010), and all procedures remained blinded until the end of the study. The experiments were designed in accordance with guidelines from the Stroke Therapy Academic Industry Roundtable (STAIR) (Fisher et al., 2005, Fisher et al., 2009) and others (Macleod et al., 2009). All procedures were done in accordance with the UK Home Office guidelines and Animals (Scientific Procedures) Act of 1986. Experimenters remained blinded to the surgery type (stroke or sham) by all animals having identical ventral midline incisions of the neck and incisions of the dominant temporal region of the head. At 4 weeks, Dr Lawrence Moon relabelled solutions containing viral vectors so that blinding was maintained throughout the study; the groups were only unblinded once all analysis was complete.

The permanent distal middle cerebral artery occlusion model was used in this study; a comparison was made between the use of a single vessel occlusion of the MCA and MCA occlusion with additional ligation of the carotid arteries, resulting in a three-vessel occlusion. Schematics for the models and the design of the two experiments are shown in Figure 2-1. The lesion was applied to the hemisphere corresponding to the dominant forepaw of the rat, as measured by the cylinder test (see section 2.4.4.1 on page 86), resulting in deficits in motor and sensory function in the first week of testing. At 24 hours after stroke onset, a structural MRI scan was obtained for each rat to determine the lesion volumes produced by use of this stroke model. Behavioural tests were done at baseline and weekly after stroke lesion for 3 weeks in animals with single vessel occlusion (see section 2.3.3.1.1), and 12 weeks in the three-vessel occlusion model (see section 2.3.3.1.2) after initial indication from the single vessel occlusion model indicated that this was ineffective for initiating sustained deficits in motor and sensory tasks. At the end of the study, animals were euthanised and dissected for tissues.

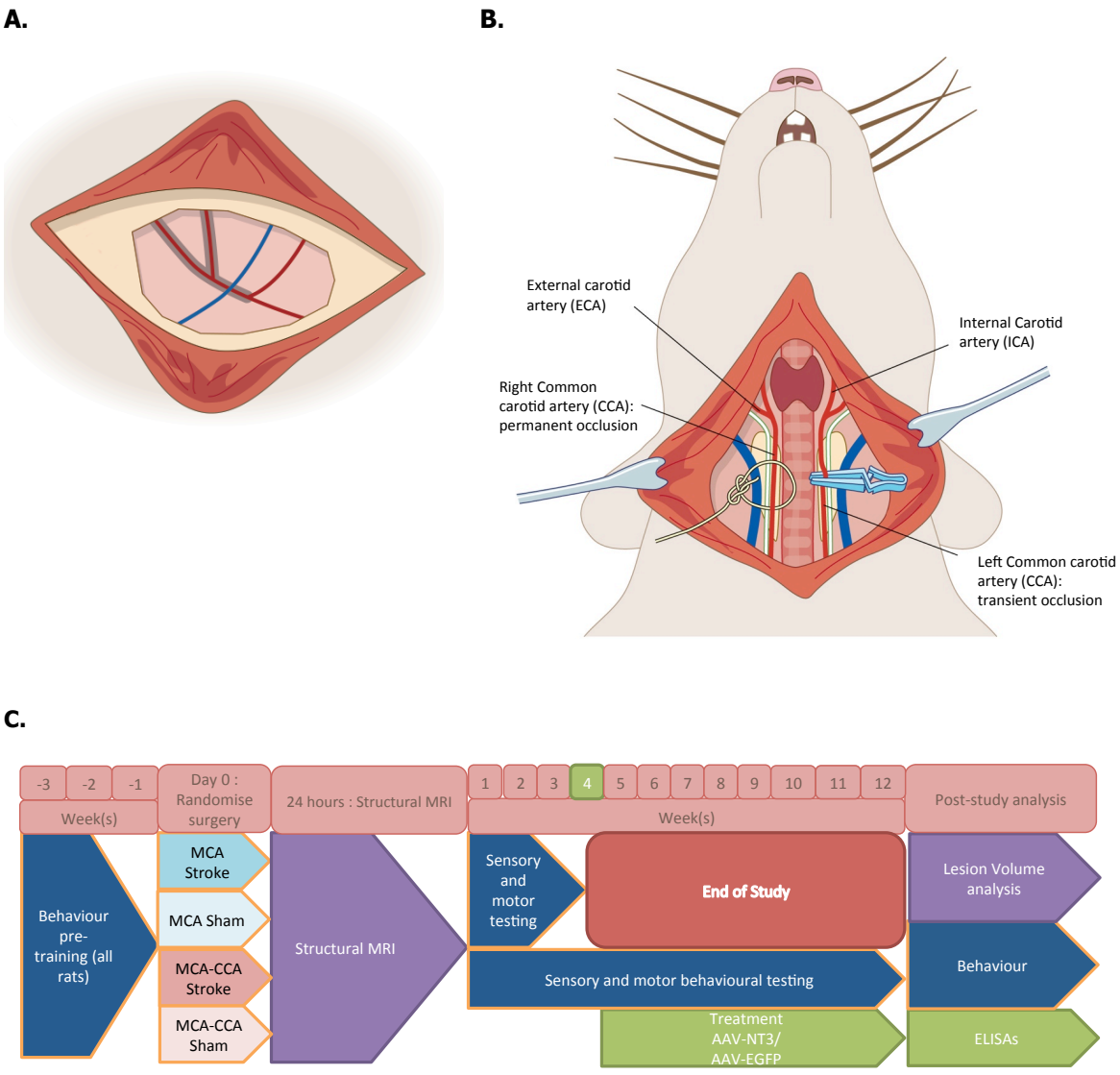


Figure 2-1: Overview of young adult MCAO study.

A. Schematic of the key vasculature for exposure and coagulation of the middle cerebral artery (MCA) in both models used in this chapter. The hemisphere corresponding to the dominant paw (in this case, the right hemisphere and the left paw are dominant) is occluded by electrocoagulation of the middle cerebral artery (red). The inferior cerebral vein (blue) is shown, and the shaded area indicates where coagulation of the artery occurs. Cutting the MCA below the inferior cerebral vein confirms the permanent occlusion.

B. Schematic of the exposure and coagulation of the common carotid arteries in the model with three-vessel occlusion (MCA-CCAO). The right common carotid artery (CCA; on the left-hand side of the image) is permanently occluded by tying a silk suture (5/0) around the blood vessel. The left CCA is occluded for one hour by using a microvascular clamp to reduce blood flow through the vessel. Surgeries were performed taking care not to make contact with the vagus nerve on each side (white).

C. Timeline of the study in this chapter. Before surgery to induce stroke, animals underwent three weeks of behavioural training and baseline scoring. Rats were then randomly allocated to either stroke or sham group for either of the two surgical models used in the study (MCA stroke n=15, sham=8; MCA-CCAO stroke n=36, sham n=10). At 24 hours after stroke, a structural MRI scan was taken for lesion volume calculations. Animals then underwent behavioural assessment for 3 weeks. Animals of the single vessel occlusion were terminated at this point. At 4 weeks, three vessel occlusion stroke rats (MCA-CCAO) were then randomised into groups receiving AAV-NT3 (n=12) or AAV-GFP (n=12). At the end of the study, lesion volumes and behavioural scoring were assessed in both cohorts, and ELISAs were produced on samples from the three-vessel occlusion cohort. The experimenter was blinded to all treatments until the end of the study.

2.3.2 Subjects

In this chapter, sixty-nine Lister hooded outbred female adult rats aged approximately 3 months (200–300 g) were obtained from Charles River, UK. Animals were housed in groups of 3 or 4 on a 12:12 light-dark cycle and had access to food and water *ad libitum*.

2.3.3 Surgical procedures

2.3.3.1 Stroke Surgery

Before surgery, animals were allocated randomly to either stroke or sham surgery groups. Procedures were done in a randomised block design to minimise bias. The lesions were allocated to the left or the right hemisphere depending on the pre-operative cylinder test results, which indicated on which side the dominant paw was; lesions were positioned on the contralateral hemisphere to the dominant paw. Surgeries were done with the help of D Duricki.

2.3.3.1.1 MCAO in the absence of carotid occlusion

23 rats were anaesthetised with ketamine (0.6 mg/kg) and Domitor (0.25 mg/kg) *via* intra-peritoneal injection. Body temperature was monitored using a rectal thermometer and maintained at 36°C with a heating blanket.

The stroke model used in this study was diathermy of the distal middle cerebral artery (Tamura et al., 1981). Stroke rats (n=15) were placed in a lateral position and an incision was made in the midpoint between the orbit and the external auditory canal on the side of the lesion, the skin retracted and the temporalis muscle separated to reveal the skull. A craniotomy was performed (5 mm x 5 mm) using a dental drill with a 1.6 mm coarse diamond-coated drill burr at approximately 8000 rpm, and the dura removed using a pair of #5 Dumont forceps, with one of the tips bent backwards approximately 180° to form an arc. The middle cerebral artery was occluded from the crossing of the inferior cerebral vein to the point of artery bifurcation using Jeweler diathermy bipolar forceps (0.25 mm angled tips, Eschmann; 8330349). The vessel was coagulated until occluded, when the artery appears black and no sign of blood flow was present. Heparinised saline was applied topically as needed with a syringe to keep the area cool and to clean the tissues. The craniectomy was then covered with bone wax, the soft tissues allowed to fall back in place, and the skin sutured (Vicryl, 4/0, absorbable sutures, Ethicon). Sham animals (n=8) received procedures up to but not

including craniotomy, as this procedure can produce behavioural deficits (Adams et al., 1994). The anaesthesia was reversed by administering Antisedan subcutaneously (0.25 mg/kg), and animals were kept in a heated recovery box until the animals regained consciousness. Saline and pain relief (Carprofen, 0.25 mg/kg, *s.cut*) were given perioperatively and for recovery every 24 hours for 48 hours and thereafter as necessary. One animal died during surgery, and one was humanely killed within 24 hours because of evidence of herniation during structural MRI scanning (Table 2-1).

	Stroke animals (n=15)	Sham animals (n=8)
Baseline assessment	15	8
Died during surgery	1	0
24-hour MRI	14	8
Humanely killed after MRI scan	1	0
Total animals included in behavioural analysis	**13**	**8**

Table 2-1: Numbers of animals in single-vessel occlusion MCAO analysis

2.3.3.1.2 MCAO with bilateral common carotid artery occlusion

As the previous study used a model of stroke which did not produce persistent deficits (see Results 2.4.4.1–2.4.4.4 below), we next assessed permanent occlusion of the MCA combined with permanent common carotid artery occlusion on the ipsilateral side and transient occlusion of the common carotid artery on the contralateral side as this method is reported to have the best outcome with the lowest mortality rate (Chen et al., 1986). Stroke rats (n=40) were anaesthetised with ketamine (0.6 mg/kg) and Domitor (0.25 mg/kg) *via* intra-peritoneal injection. First, the animals were laid in the supine position and a ventral midline cervical incision was made to allow isolation of both common carotid arteries using a silk suture (5/0, non-absorbable sutures). Animals were then placed in a lateral position and the middle cerebral artery occlusion surgery was done as in Section 2.3.3.1.1. Once the MCA was occluded, the rats were put back in the supine position, the common carotid on the same side as the occluded middle cerebral artery was tied off permanently with the silk suture, and the carotid artery on the conralesional side was occluded for 60 minutes using a stainless steel artery clip with approximately 125 g of pressure. Sham animals (n=10) received the ventral midline cervical incision and exposure of the skull, but no further procedures as the craniotomy alone could produce behavioural deficits (Adams et al., 1994). Anaesthesia was reversed by administering Antisedan subcutaneously (0.25 mg/kg), and animals were kept in a

heated recovery box until they regained consciousness. 12 animals (26%) died within 24 hours, with 2 animals dying after this time point (

	AAV-NT3 stroke animals (n=20)	AAV-GFP stroke animals (n=20)	Sham animals (n=10)
Baseline assessment	20	20	10
Died during surgery	6	6	0
24-hour MRI	14	14	10
Did not recover 24 hour MRI scan	0	1	0
Animals completing behavioural tasks	11	11	10
Humanely killed at week 3	1*	0	0
Total animals included in behavioural analysis	13	13	10

Table 2-2). One animal did not recover from isoflurane anaesthesia at 24 hours. Animals in the MCA-CCAO cohort received treatment of AAV-NT3 (n=13) or AAV-GFP (n=13) 4 weeks after stroke onset. Half of sham animals (n=5) received saline, and those remaining (n=5) did not receive injections. Saline and pain relief (Carprieve, 0.25 mg/kg, *s.cut*) were given perioperatively and every 24 hours after surgery for 2 days and

** One animal was humanely killed part way through the study. This animal was included in the behavioural analysis up until this time point, since our statistical method can handle missing values (Duricki et al., 2016b)*

thereafter as necessary.

	AAV-NT3 stroke animals (n=20)	AAV-GFP stroke animals (n=20)	Sham animals (n=10)
Baseline assessment	20	20	10
Died during surgery	6	6	0
24-hour MRI	14	14	10
Did not recover 24 hour MRI scan	0	1	0
Animals completing behavioural tasks	11	11	10
Humanely killed at week 3	1*	0	0
Total animals included in behavioural analysis	13	13	10

Table 2-2: Numbers of animals in three-vessel occlusion MCAO analysis

2.3.4 Confirmation of infarct using structural MRI

Structural MRI was done 24 hours after stroke and at the same time point as all functional MRI scans (section 3.3.4). MR imaging was done on an Agilent 7 Tesla (T) horizontal bore scanner (Agilent, Palo Alto, CA, USA). Animals were anaesthetised with isoflurane (5% induction, 1–1.5% maintenance). Once anaesthetised, rats were secured

in a head frame inside the quadrature birdcage MR coil (43 mm ID) and placed in the scanner. Each animal's physiology was monitored during the scan using a respiration monitor (BIOPAC, USA) and a pulse oximetry sensor (Nonin, USA) that interfaced with a computer running BIOPAC software. Additionally, a fan heater directed at the animal responded to any alterations in body temperature identified by rectal probe, and was maintained at 37±1 °C. The T2-weighted MR images were acquired using a fast-spin echo sequence: effective echo time (TE) 60 ms, repetition time (TR) 4000 ms, field of view (FOV) 40 x 40 mm, acquisition matrix 128 x 128, acquiring 20 x 1 mm thick slices in approximately 8 minutes. Data were analysed using a semi-automatic contour method in Jim software (Xinapse systems Ltd). The bright hypertense signal surrounding the lesion was measured as the lesion volume. D Duricki aided with scan acquisition.

2.3.5 Treatment of MCA-CCAO animals

2.3.5.1 AAV construction

pAAVsp (Salk Institute) has a CMV promoter, a synthetic intron flanked by splice donor/splice acceptor sites, and a multiple cloning site (MCS) terminated by a betaglobin polyA sequence. The transcript is flanked by AAV2 inverted terminal repeats. AAVsp-NT3 was cloned using the human neurotrophin-3 CDS (CDS 8538.1, 774 bp) corresponding to transcript variant 2 (NM_002527.4), which encodes the isoform 2 precursor protein (257 amino acids) including the secretory signal sequence. The hNT3 CDS was restriction digested from a modified pBluescript plasmid (SKsp) using Sfi1 and Pme1 sites and then cloned into AAVsp with these same sites. AAV-GFP constructs were produced in the same way, but with the EGFP gene inserted between the Sfi1 and Pme1 restriction sites. Prof Fred Gage (Salk Institute, CA) provided these plasmids: the NT3 transgene was originally cloned by Dr Kevin Jones. AAV (serotype 1) were generated using pHelper plasmid and the capsid plasmid encoding Rep/Cap1. The plasmids were sent to University of Pennsylvania Vector Core for production of high titre AAVs.

2.3.5.2 Intramuscular delivery of AAV-NT3 or AAV-EGFP

4 weeks after surgery, rats were anaesthetised with isoflurane (5% in O_2 for induction) and then transferred to a station where anaesthesia was maintained at approximately 1.5% in O_2 delivered *via* a facemask. The skin on the forelimb contralateral to the infarct was shaved and swabbed with 1% chlorhexidine in 70% ethanol before making a small incision between the elbow and axilla. Stroke rats were randomly allocated to receive AAV serotype 1 encoding either enhanced green fluorescent protein (EGFP) or human NT3. Injections were given into triceps and biceps as depicted in (Figure 2-2E) using a

10 µl Hamilton syringe bearing an ultrafine, bevelled non-coring 32-gauge needle. In total 90 µl was injected; in triceps, 25 µl was injected deeply into both the long head (5 x 5 µl) and the lateral head (5 x 5 µl) and 7.5 µl was injected superficially into the long head (3 x 2.5 µl) and the lateral head (3 x 2.5 µl). For biceps, 15 µl was injected deeply (3 x 5 µl) and 10 µl superficially (2 x 5 µl). Rats received 4.7 x 10^{11} viral genomes. Half of sham rats received skin incision only and the other half received saline injection into the triceps and biceps in the same volumes as the stroke animals received AAV. Skin was sutured and analgesic administered as above.

2.3.6 Behavioural Techniques

Rats were handled daily for 4 weeks before the commencement of the study, and fully trained by the operators on the horizontal ladder and staircase tasks before the study began. Further details on pre-training are given in the relevant sections, and preoperative baseline scores for all tasks were collected 1 week before surgery. Sham rats of the two different sham groups in the AAV study were pooled for analysis.

2.3.6.1 Vertical Cylinder test

Forelimb use for lateral posture support during exploratory behaviour was assessed using the cylinder test (Kozlowski et al., 2000). Naive animals usually have a preference for a forelimb, but use both paws for postural support. The rearing exploratory behaviour of rats was filmed when placed in a transparent cylinder (Plexiglas, measuring 20 cm diameter, 30 cm height) for 2 minutes per week. Mirrors were placed behind the cylinder to enable observation from all angles. Behaviours were scored after slow-motion video playback as use of the left, right, or both paws simultaneously during rearing or lateral movements; during a rear, the first forepaw to make contact with the cylinder wall was scored as an independent paw placement, and subsequent placements of the other forepaw while maintaining the initial paw placement was marked as a "both" movement. Forelimb asymmetry was calculated with the following formula adapted from Hsu and Jones 2005:

$$forelimb\ asymmetry = \frac{contrateral\ forelimb\ use + \frac{1}{2} bilateral\ forelimb\ use}{total\ forelimb\ use}\ x\ 100$$

2.3.6.2 Horizontal ladder

The horizontal ladder test was used to test forelimb and hindlimb function. The skilled paw placement required from the animals on this task allows an analysis of skilled locomotor co-ordination to be measured (Metz and Whishaw, 2002). The apparatus consisted of Plexiglas side walls, 1.2 m in length, 50 cm height and an adjustable width to prevent animals turning during the task. Metal rungs were placed at a height of 20 cm, spaced unequally (between 1 and 4 cm apart) and repositioned weekly to prevent pattern learning.

Rats were trained to cross the ladder from a neutral cage to reach their home cage in one direction. Baseline behavioural training occurred in stages over the course of 3 weeks: in the first week, animals were permitted to habituate to the apparatus by spending 15 minutes per day freely exploring the ladder with other animals from their cage with the adjustable width set wide to prevent anxiety. During the second week of baseline training, animals were encouraged to begin crossing the ladder from one end of the ladder to the other, by placing the home cage at the end of the ladder, although animals were still in groups from their home cage. The width of the ladder was reduced by 2 cm a day over the course of the week to prevent multiple animals from being able to cross, resulting in animals having to cross one at a time by the end of the week. In the final week of baseline training, animals were put on the ladder one by one with their home cage at the end of the ladder. Midway through the week, the rungs were changed to familiarise the animals to the changing foot positions, and rungs were changed daily thereafter during training. One baseline recording was made for each animal before surgery, and every week for 12 weeks afterwards.

Animals were filmed three times per session. Videos were played back in slow-motion and the number of left and right forepaw and hindpaw errors was quantified and represented as percent slips of total steps taken. Limb placement errors were classed as slight paw slips, deep paw slips and complete misses; the number of errors per step per traverse of the ladder was calculated and averaged for the three trials.

2.3.6.3 Staircase Test

The staircase test is designed to measure changes in both skilled and gross skilled movements following motor system damage (Montoya et al., 1991). The staircase (Campden Instruments Ltd., UK) consists of seven steps containing wells on each side of a central platform. Baseline training occurred in stages over 4 weeks: in the first week

animals were introduced to the treats (in this study we used Tesco own-brand chocolate pops) that we would use over the course of the study; rats were given chocolate treats in their home cage with the detachable stairs of the apparatus. In the second week of training, animals were introduced to the rest of the apparatus; animals were given 15 minutes per day, with the rest of the animals from their cage, to freely investigate the insides and outside of the staircase boxes, which had chocolate treats scattered throughout to encourage exploration inside the boxes. On the last day of the week, each rat was placed inside the staircase with the lid closed. In the third and fourth weeks, animals did daily tests on the staircase under test conditions (see below), until the animal had retrieved 75% of pellets on its preferred side. The final test was used as the baseline score.

Three sugar pellets were placed in each well during a behavioural session (21 pellets per side). Rats were placed in the staircase for 10 minutes and the number of pellets retrieved and the number of pellets displaced on each side was recorded and are displayed as a percentage of the total number of pellets available for that paw. To be included in the task, rats had to achieve a minimum of 75% retrieval of pellets at baseline (Montoya et al., 1991). Rats were food restricted to 10 g of food per rat the day before testing.

2.3.6.4 Bilateral Tactile Stimulation Test

The bilateral stimulation test was used to assess the extent of somatosensory asymmetry and changes in sensorimotor tactile extinction after stroke (Upchurch and Schallert, 1982, Kozlowski et al., 2000).

An adhesive label (13 mm diameter) was attached to the plantar surface of each forepaw, and the time was recorded for the animal to first sense the label and then the time taken for the subsequent removal of the sticker. To determine whether rats showed bias for their affected or unaffected forelimbs, the side of label removal was recorded until a ≥75% preference was observed.

The extent of the asymmetry was determined using seven pairs of different sized stimuli (Schallert et al., 2000). The size of the label on the affected forepaw was gradually increased and the unaffected forepaw stimulus was simultaneously decreased on each trial until the rat removed the label from the affected forepaw first, reversing the natural bias towards the non-impaired side. This behavioural test was done by D Duricki.

2.3.7 Enzyme-linked Immunosorbent Assays (ELISA)

12 weeks after stroke surgery, and 8 weeks after treatment with one of AAV-NT3, AAV-GFP or saline, rats were terminally anaesthetised with Pentobarbital Sodium (Euthatal) and two 1 mL samples of blood taken from the heart with a regular syringe. One sample was left to coagulate at room temperature for 15 minutes before spinning down for 15 minutes at 13,000 rpm and the supernatant taken for serum sample. The final 1 mL sample was kept as a whole blood sample. The rat was then perfused transcardially using 50 mL Phosphate buffered saline (PBS: NaCl, 137 mM; KCl, 2.7 mM; Na_2HPO_4, 4.3 mM; KH_2PO_4, 1.4 mM) before dissecting the biceps and the long head of the triceps. Samples were frozen at –20°C for future analysis. The procedure was then carried out on the serum sample diluted 1:10 according to the ELISA kit manufacturer's instructions (DY008, Human NT-3 DuoSet ELISA Kit, R&D systems, UK).

Protein standard curve solutions ranging from 2000 ng/mL to 0 ng/mL were made by serial 1:2 dilutions from a protein standard (R&D systems). The protein standard curve was then used to calculate the NT3 protein concentration in the samples.

2.3.8 Statistical analysis

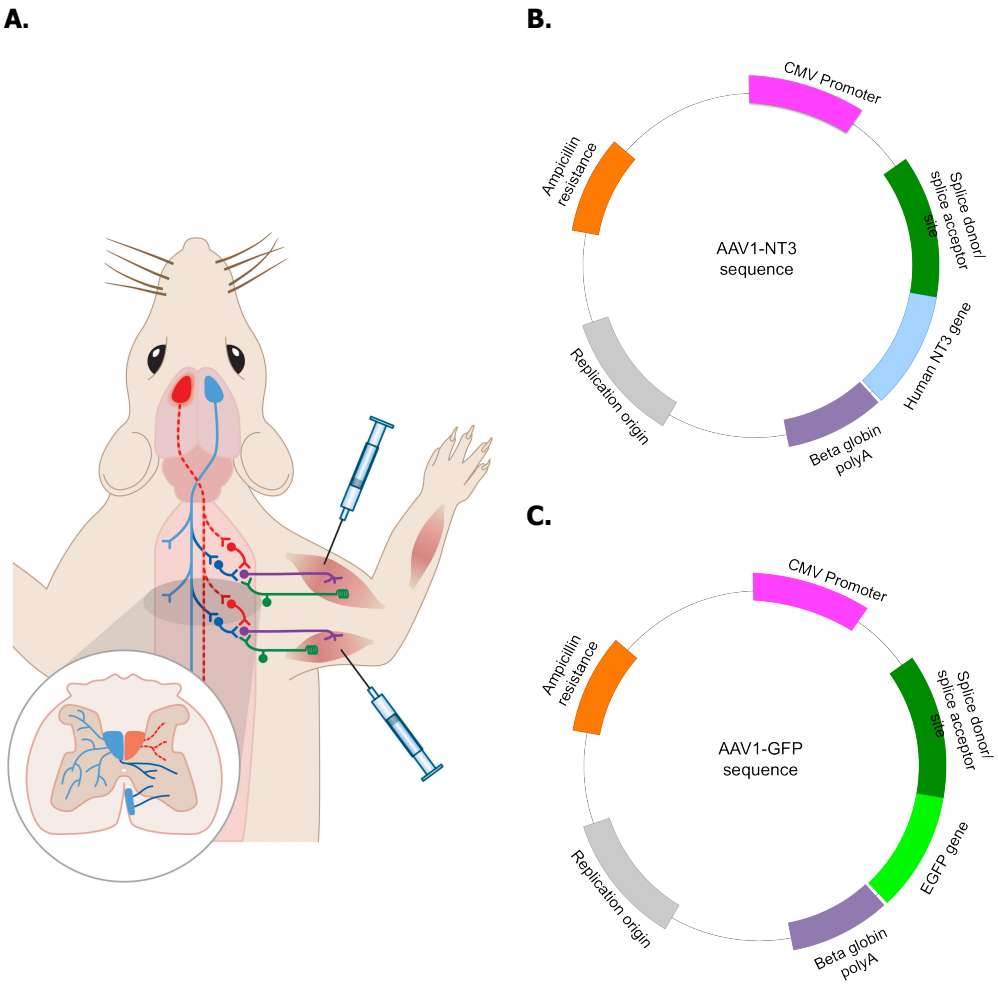
We did sample size calculations using G*Power. Means of sham and stroke animals from the first, pilot study, were used to calculate a sample size of a minimum of 12 animals per treatment group at an alpha of 0.05 and a power of 0.80. We rounded this to 20 animals to account for any losses or ineffective stroke lesions.

Statistical analyses were done using SPSS (version 18.0). Graphs show means ± SEMs (except where otherwise stated) and 'n' denotes number of rats. Asterisks (*) indicate $p \leq 0.05$. Threshold for significance was 0.05. ELISA and lesion volume data were assessed using Kruskal-Wallis and then Mann-Whitney tests. For correlation, linear regression was done and the square of Spearman's rank correlation co-efficient (R^2) is reported. Behavioural data were analysed using linear models and Restricted Maximum Likelihood estimation to accommodate data from rats with occasional missing values (Gueorguieva and Krystal, 2004, Krueger and Tian, 2004, Duricki et al., 2016b), because some rats died before the end of the study. Akaike's Information Criterion (AIC) showed the model with best fit for the behavioural data. Baseline scores were used as covariates. Degrees of freedom are reported to nearest integer. t-tests were two-tailed.

2.4 Results

2.4.1 Treatment of MCA-CCAO subjects using AAV-NT3

The benefit of NT3 to promote growth after models of CNS injury has already been shown previously (Houweling et al., 1998, Zhou et al., 2003), even in a transgene encoded in a viral vector (Zhou and Shine, 2003, Petruska et al., 2010). This method of NT3 delivery has already been shown to be useful in a small, focal model of stroke (Duricki et al., 2016b). In this study (Figure 2-2), the aim was to use the knowledge gained to date in this area to implement improvement in a larger model of stroke. Delivering NT3 24 hours after CNS injury has been shown to be effective, and in some cases, using an even later time point may enable the discovery of wider therapeutic windows. Studies looking at motoneuron pool locations in adult rats have shown that grasp function is associated with the cervical cord from C3–C7 (McKenna et al., 2000, Tosolini and Morris, 2012). By injecting the proximal forelimb muscles, the likelihood of retrograde transport of vector or NT3 protein along the short distance to the spinal cord or DRG is maximised.



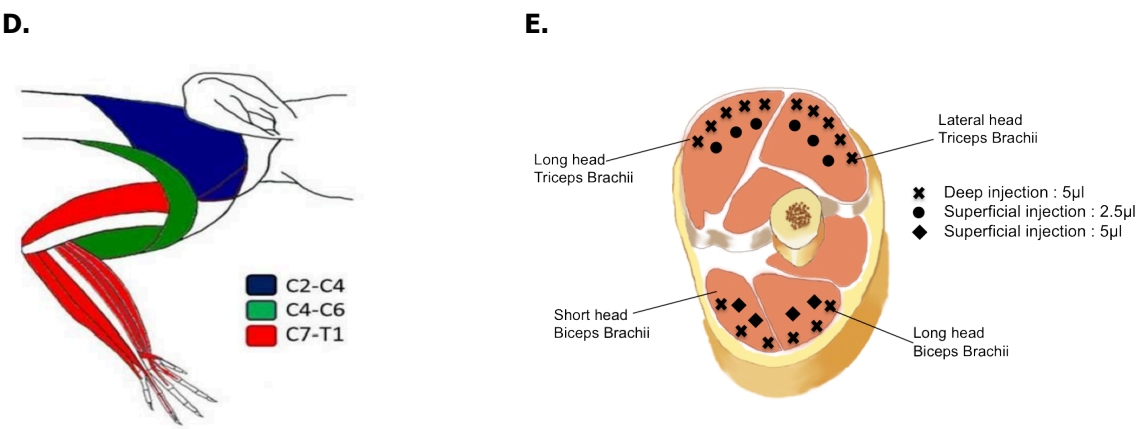


Figure 2-2 : Schematic of treatment with injection of AAV to peripheral muscles.

Schematic of the study (A). Stroke is surgically induced (red) and AAV-NT3 (B) or AAV-GFP (C), encoding NT3 or EGFP respectively, is delivered to the affected forelimb muscles 4 weeks after stroke. After injections of neurotrophins into the biceps and triceps muscles of a rat, which could cause crossing of intact axons in the spinal cord (D), retrograde transport into the motoneuron pools of cervical regions C3-C7 occurs (McKenna 2000). Schematic of approximate injection points (E). Rats received injections were into triceps and biceps using a 10 µl Hamilton syringe. Stroke rats received a total of 90 µl of 3.0 x 10^{10} vg encoding either NT3 or EGFP. A combination of deep and superficial injections were given into the long and lateral heads of the muscle. ■ represent deep injections and ♦ and • superficial injections. To try and maximise retrograde transport of the viral genomes, multiple injections of the virus aimed for the upper third of the muscle as this is the position of where the vascular and nerve bundles enter (Bertelli et al., 1995)

2.4.2 Animals receiving three-vessel occlusion have larger and less variable infarcts than middle cerebral artery occlusion alone

During surgery, 53.3% of animals in the MCA occlusion group had infarcts in the right hemisphere, and 55% of animals in the three-vessel occlusion model had an infarct in the right hemisphere. For assessment of lesion volumes, rats in all groups underwent isoflurane anaesthesia and structural T2-weighted MRI scans at 24 hours after introduction of the stroke lesion. In these scans, bright, hyperintense areas represent regions containing fluid such as CSF or oedema and the volume of these areas is correlated with the area of infarction (Gerriets et al., 2004). In Figure 2-3 the 24-hour infarction volumes from both studies can be compared. Lesion volumes of the MCAO group were 47.3±51.4 mm^3 (median±IQR), whilst the MCA-CCAO model resulted in a lesion volume of 112.3±27.4 mm^3. This is a highly significant difference ($p<0.0001$, Mann-Whitney *post hoc* test). Regions of the brain affected by the single-vessel MCA occlusion (Figure 2-3A') include only a small part of the forelimb, dorsolateral and upper lip portions of the sensory cortex (Paxinos et al., 1980). In contrast, the three-vessel occlusion model (Figure 2-3B') resulted in ischaemic damage to the same areas as the single vessel occlusion, with a larger proportion of the forelimb region, and also the secondary sensory cortex and a portion of the primary motor area.

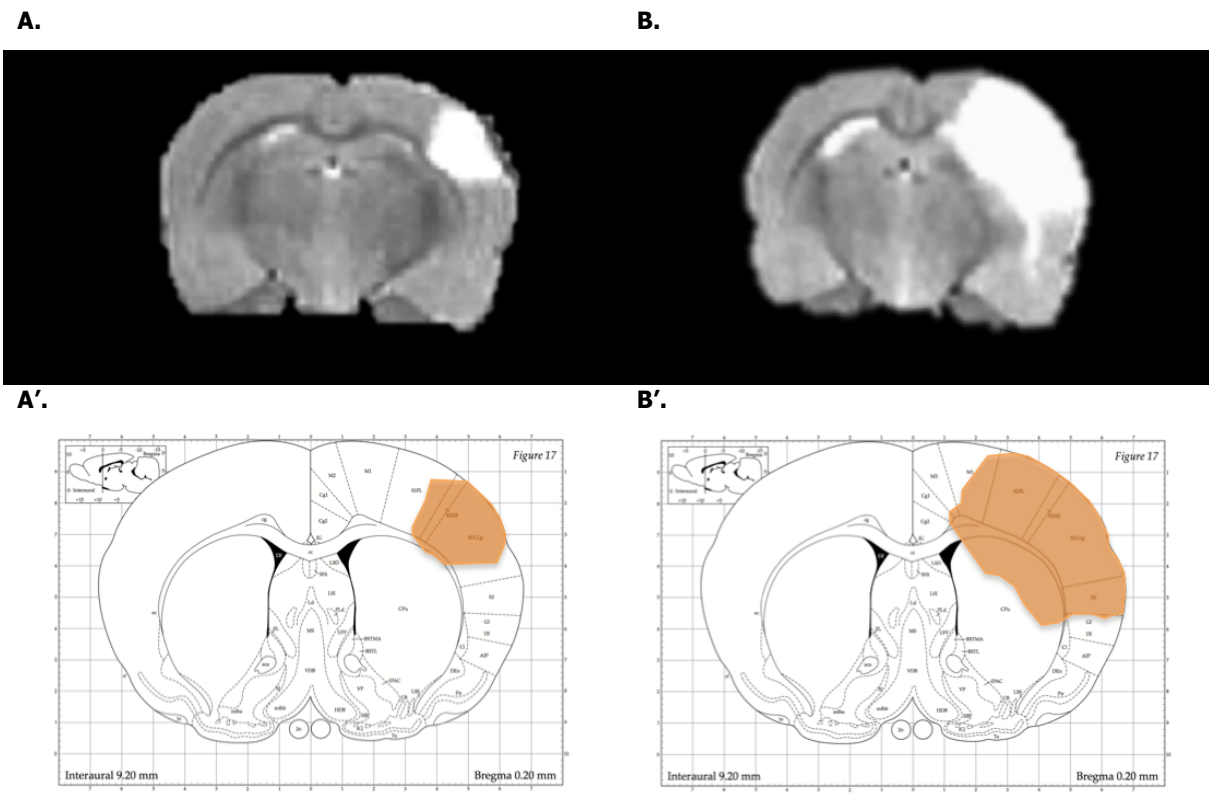


Figure 2-3 : Comparison of lesion volume between single- and three- vessel occlusion models.

T2-weighted imaging at 24 hours of (A) single vessel and (B) three-vessel occlusion models of stroke. White areas in the image indicate areas of oedema and lesion caused by the infarction. These areas affected by stroke correspond to A' and B' respectively. C) A box plot comparing the difference in median lesion volumes between the two studies. The box plot shows the median lesion volume and interquartile ranges, where the error bars indicate the minimum and maximum lesion volumes in the two studies (single vessel occlusion, n=14; three-vessel occlusion, n=28). Significance was identified by the Mann Whitney test (p<0.001).

2.4.3 Sham animals treated with no injection or saline in place of AAV treatment showed no difference in behavioural tests.

Many studies have been criticised for having irrelevant or ineffective sham data because of ineffective treatment element in the control group (Kilkenny et al., 2010a). In the experiment with NT3 treatment, animals were split into groups at 4 weeks; sham animals were either injected with saline or received only an incision of the skin for injection. The results in Figure 2-4 show that for the cylinder (top left, p=0.270), horizontal ladder (top right, p=0.304), staircase test (bottom left, p=0.998), and bilateral tactile extinction test (bottom right, p=0.486), there is no significant difference between sham groups. When compared with previous data with AAV-GFP delivery in sham groups (Duricki et al., 2016b), in which there was again no difference from previous, untreated shams, it is evident that these controls all indicate that there is no difference between untreated and saline treated animals in the sham group. Muscle injections at 4 weeks were not deleterious to behavioural function; therefore for the remainder of this study, sham animals from both groups were pooled in the behavioural analysis.

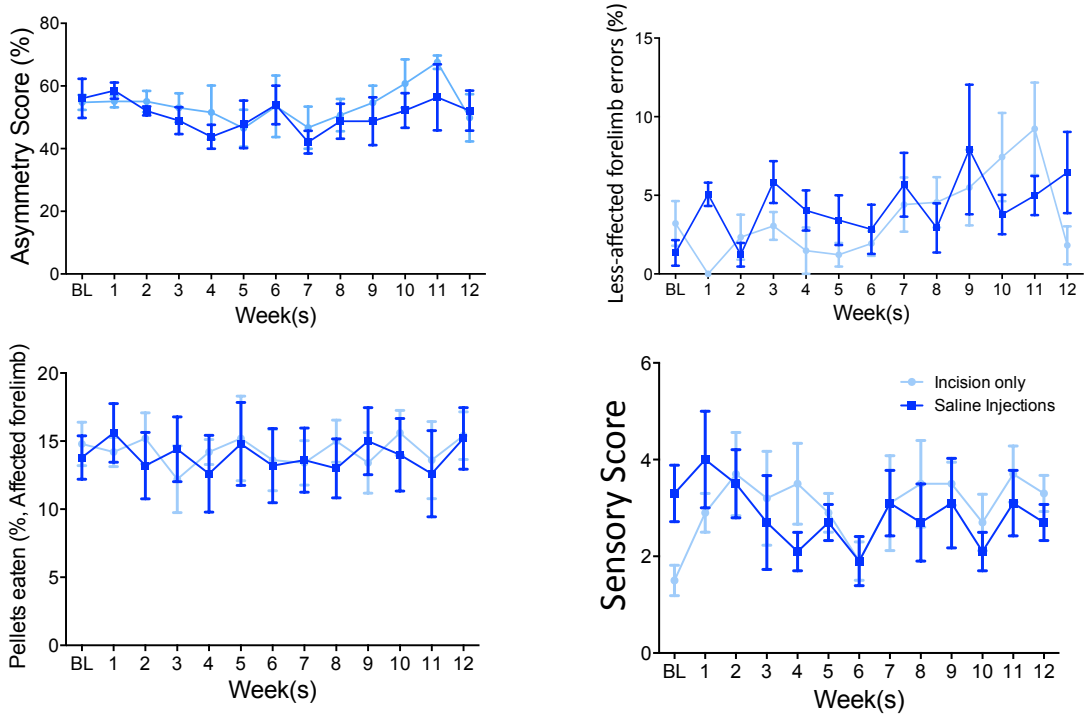


Figure 2-4 : Sham data indicate no difference between untreated and treated sham.

Sham animals in the three-vessel occlusion, treatment arm of the study, received either incision for delivery of intramuscular saline (n=5) or only incision (n=5) at 4 weeks after the commencement of the study. Mean and SEMs from the cylinder (top left), horizontal ladder (top right), staircase test (bottom left) and bilateral tactile extinction test (bottom right). RM ANCOVA revealed no significant difference between groups (p>0.05).

2.4.4 Functional recovery in young adult rats

Damage to the sensorimotor cortex after MCAO results in deficits in motor control and processing of sensory information. To determine whether the AAV-NT3 treatment can elicit an effect on stroke recovery, the cylinder, horizontal ladder, staircase, and sensory behavioural tests were done to test postural support, motor control, fine motor movements, and sensory neglect respectively. Animals were trained for 3 weeks before baseline on the horizontal ladder and staircase tests and both models underwent behavioural assessment for 4 weeks before proceeding with AAV treatment in the three-vessel occlusion model. Treatment was given after behavioural testing in week 4.

2.4.4.1 There is no change in use of forelimbs for postural support after distal MCAO stroke

The cylinder test was used to quantify forelimb asymmetry during exploratory rearing and therefore establish whether there is any recovery following stroke and treatment (Kozlowski et al., 2000). Over the duration of the single vessel occlusion study, there was no effect of MCAO surgery on use of the affected paw compared to sham animals (Figure 2-5A). The mean asymmetry score for sham animals at week 1 was 56 ± 4.8% compared with 57 ± 3.7% for stroke animals showing an approximately equal use of forelimbs during weight support. After stroke injury, stroke animals had no deficit compared with sham ($F_{1,18}$=0.031, p=0.861), showing no increase in asymmetry involving preferential use of their less affected forelimb for postural support. Additionally, there was no effect of time ($F_{2,38}$=0.490, p=0.616) and no interaction between time and group ($F_{2,38}$=1.03, p=0.366). In the three-vessel occlusion model (Figure 2-5B), the mean asymmetry 1 week after stroke was 54 ± 5.7%, 46 ± 19%, and 41 ± 17% for sham group, AAV-GFP and AAV-NT3 stroke groups respectively. No overall group difference was established ($F_{2,30}$=0.773, p=0.471), no overall effect of time was observed ($F_{11,336}$=0.525, p=0.886), and there was no interaction between group and time ($F_{22,336}$=1.38, p=0.120).

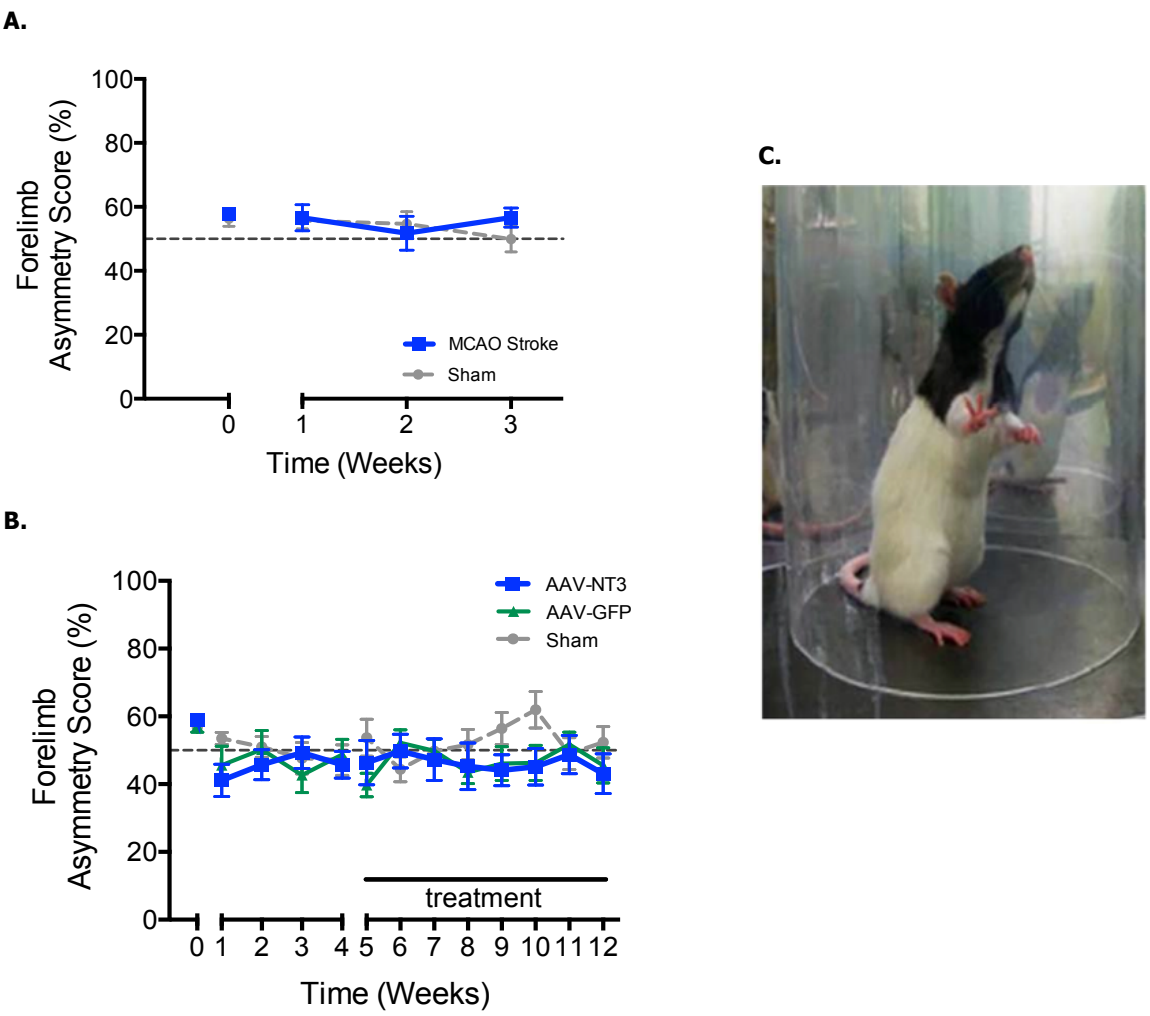


Figure 2-5 : The cylinder test is not a useful model for the study of single- or three-vessel occlusion model of stroke.

Following injury, both single- (stroke n=13, sham n=8) (A) and three-vessel occlusion (stroke groups n=12 per group, sham group n=10) model with 60-minute occlusion of the common carotid artery (B) rats showed no difference in asymmetry indicating no change in preferential use of their forelimb during weight support following stroke compared to sham groups. Therefore treatment with AAV-NT3 or AAV-GFP groups showed no difference in recovery with treatment. Results are displayed as mean ± SEM. Linear models indicated no significant effects of group or time ($p > 0.05$). (C) shows the use of the vertical cylinder to measure postural support and exploratory behaviour (Adapted from Duricki et al., 2016).

2.4.4.2 The horizontal ladder shows a significant impairment of the contralateral forepaw, but no difference between treated groups.

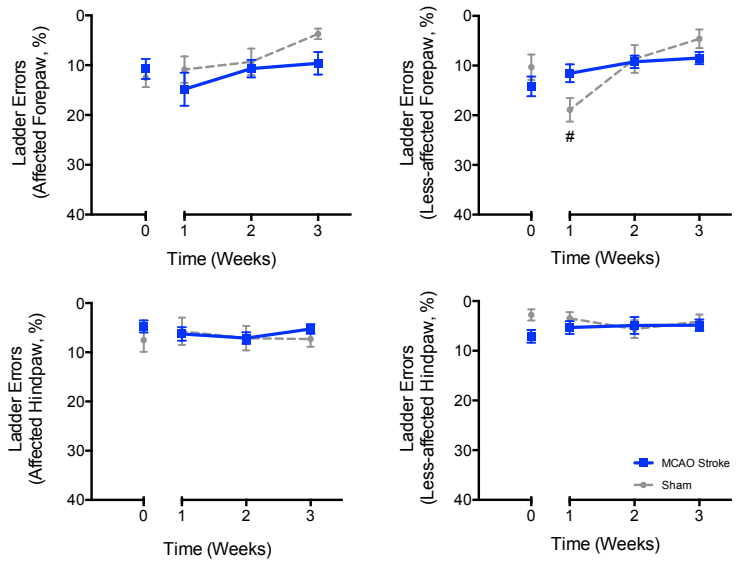
The horizontal ladder was used to assess motor recovery after stroke injury by testing precise sensory-guided paw placements and motor control (Metz and Whishaw, 2002). The horizontal ladder consisted of rungs spaced at 1–4 cm intervals and changed weekly to prevent the rats from learning the rung pattern. Data is displayed as percentage errors over the total number of steps taken in three sessions.

The results in Figure 2-6A show that there was no significant impairment in motor control on the horizontal ladder task following the single vessel MCAO lesion

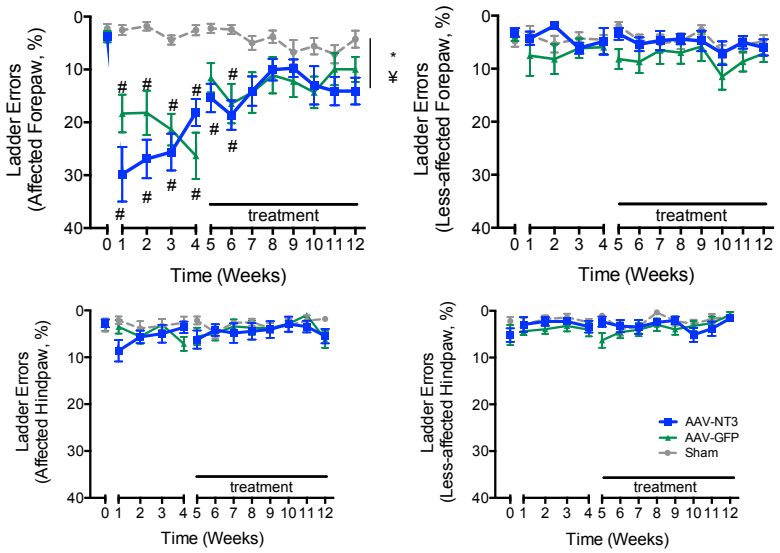
(7.1±0.78%) compared with sham (2.8±0.86%), paying particular attention to the forepaws (affected paw, top left graph, effect of group $F_{1,19}$=2.135, p=0.161). There was a trend towards an effect of time ($F_{2,36}$=2.94, p=0.066) and no interaction between group and time ($F_{2,36}$=0.507, p=0.607). The less-affected paw (top right graph) showed no effect of group ($F_{1,23}$=0.508, p=0.483), although there was a significant effect of time ($F_{2,40}$=11.8, p<0.001) and an interaction between time and group ($F_{2,40}$=4.91, p=0.012). It was the sham group that had an impaired performance in week 1 (*post hoc* week 1 sham *vs* stroke, p=0.006); this is likely to be a false positive result, although statistically significant. The rats in both groups consistently completed the task with fewer errors in both hindpaws with no effect of group ($F_{1,20}$=0.000, p=0.991), no effect of time ($F_{2,38}$=0.501, p=0.610) and no interaction between groups ($F_{2,38}$=0.451, p=0.640). This shows that unilateral stroke did not affect performance with any limb on this task with this stroke model.

The three-vessel occlusion model stroke groups both showed a large deficit in the performance of the dominant forepaw in the task after injury compared to sham (Figure 2-6B, top left, group $F_{2,30}$=10.4, p<0.001, *post hoc* test for sham *vs* AAV-NT3 and sham *vs* AAV-GFP, p<0.001), which improved gradually in both groups over the 12 weeks of the study (effect of time, $F_{11,336}$=6.07, p<0.001; group x time, $F_{22,336}$=4.41, p<0.001). The sham group consistently performed in this task with few or no errors, and both stroke groups show some recovery relative to the sham group over time. The less affected forepaw (Figure 2-6B, top right) showed no effect of group ($F_{2,30}$=2.46, p=0.103), no effect of time ($F_{11,336}$=1.51, p=0.126), and no interaction between group and time ($F_{22,336}$=0.604, p=0.921). The number of errors using the affected hindpaw (Figure 2-6B; bottom left) also showed no effect of group ($F_{2,30}$=2.16, p=0.133), no effect of time ($F_{11,337}$=1.15, p=0.323) and no interaction between group and time ($F_{22,337}$=1.16, p=0.285). Finally, the less-affected hindpaw (bottom right) showed no difference between any of the groups ($F_{2,30}$=1.88, p=0.170), no effect of time ($F_{11,336}$=1.70, p=0.072) and no interaction of group with time ($F_{22,336}$=0.767, p=0.766). This shows that unilateral stroke affected performance with the contralateral forelimb on this task, but the AAV-NT3 treatment did not alter recovery compared with the AAV-GFP group, indicating that the treatment given has not had an effect. All statistical analyses were performed on the mean percentage of errors made on each of the forepaws and hindpaws. Neither stroke model used in these experiments gave a sufficiently large deficit for testing the benefits of a potential therapy.

A.


Ladder Errors (Affected Forepaw, %)
Ladder Errors (Less-affected Forepaw, %)
Ladder Errors (Affected Hindpaw, %)
Ladder Errors (Less-affected Hindpaw, %)
Time (Weeks)
#
MCAO Stroke
Sham

B.


Ladder Errors (Affected Forepaw, %)
Ladder Errors (Less-affected Forepaw, %)
Ladder Errors (Affected Hindpaw, %)
Ladder Errors (Less-affected Hindpaw, %)
Time (Weeks)
treatment
#
*
¥
AAV-NT3
AAV-GFP
Sham

C.

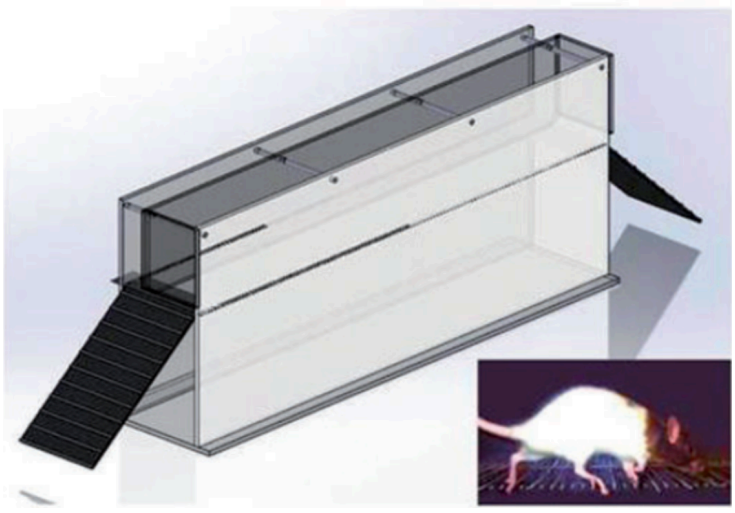


Figure 2-6 : There is spontaneous recovery of both stroke groups on the horizontal ladder task after three-vessel occlusion, but no improvement associated with AAV-NT3 treatment.

Following single vessel occlusion (A stroke animals n=13, sham n=8), rats did not produce significant impairments in the affected forepaw (top left), less-affected forepaws (top right), or either of the hindpaws (affected, bottom left; less-affected, bottom right) as measured by linear models and post hoc tests (p>0.05). B) Occluding the MCA with occlusion of both carotid arteries (stroke groups n=12 per treatment group, sham n=10), rats exhibited significant impairments in motor control of their affected forelimb (B, top left). The two stroke groups made a similar number of foot faults one week after injury, with both the AAV-NT3 and AAV-GFP groups beginning a recovery process following the three week time-point (group difference p<0.05, ¥ interaction of group with time p<0.05, # denotes an effect between stroke groups and sham p<0.05). The NT3 treatment in this study does not result in motor recovery of the affected forelimb following stroke injury. The percentage of foot faults on the less-affected forelimb indicates no change from sham in either of the stroke groups (B, top right). The hindpaws also have no effect of stroke throughout the study (B; affected hindpaw, bottom left; less-affected hindpaw, bottom right). Results displayed as mean±SEM, Linear models and two-tailed t-tests. C) a schematic of the horizontal ladder apparatus, and inset, a rat completing a trial for assessment (Adapted from Duricki et al., 2016b).*

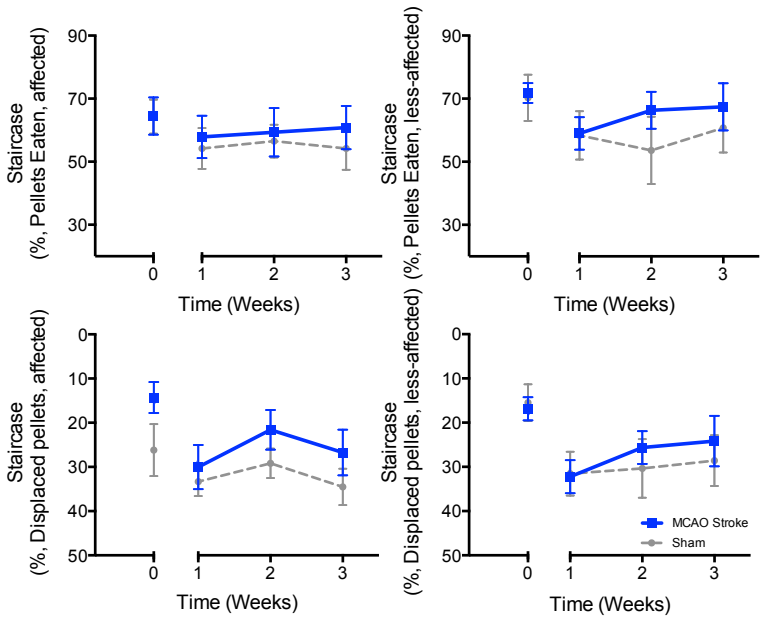
2.4.4.3 Fine motor skills are not affected by single vessel occlusion, and AAV-NT3 does not improve recovery after three-vessel occlusion MCAO.

The number of pellets that were eaten or displaced from the staircase by the affected or less-affected forepaws was counted to assess fine motor control involving precision reaching and dexterity and displayed as a percentage of total pellets available (Montoya et al., 1991). The performance of the stroke group in the single vessel occlusion model (Figure 2-7A) on this task demonstrated that unilateral stroke did not result in retrieval of fewer pellets, or a decrease in accuracy in retrieving pellets, measured by the number of pellets eaten and a first order autoregressive covariance linear model analysis. The retrieval of pellets with the affected paw (Figure 2-7A, top left) showed no effect of group ($F_{1,20}$=0.298, p=0.591) no effect of time ($F_{2,38}$=0.114, p=0.893) and no interaction between time and group ($F_{2,38}$=0.114, p=0.893). A similar observation was noted with the retrieval of pellets with the less-affected paw (Figure 2-7A top right), with no effect of group ($F_{1,18}$=0.528, p=0.477), no effect of time ($F_{2,38}$=1.00, p=0.377) and no interaction between time and group seen ($F_{2,38}$=1.15, p=0.328). Displaced pellets were counted to measure accuracy of retrieval. Stroke had no effect on the accuracy in the staircase test using the affected forepaw (Figure 2-7A, bottom left) indicated by no effect of group ($F_{1,18}$=0.371, p=0.550), no effect of time ($F_{2,38}$=2.65, p=0.084), and no interaction between group and time ($F_{2,38}$=0.372, p=0.692) using a linear models analysis. The less-affected paw (Figure 2-7A, bottom right) also showed no effect of group ($F_{1,18}$=0.363, p=0.554), no effect of time ($F_{2,38}$=1.73, p=0.190) and no interaction between group and time ($F_{2,38}$=0.495, p=0.613) following stroke lesion.

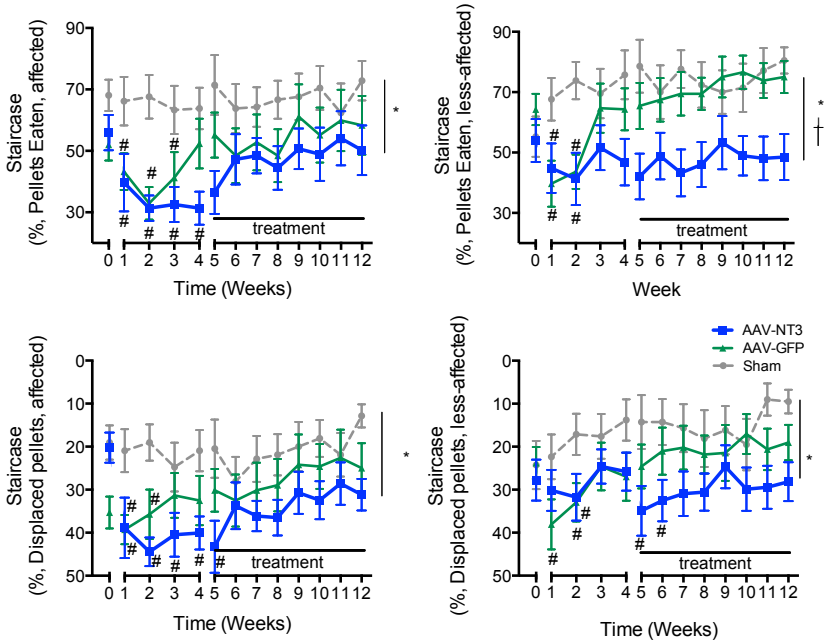
In the three-vessel occlusion model, the impairments observed in pellet retrieval were more severe. Using the affected paw, retrieval of pellets was not affected by group

(Figure 2-7B, top left, group $F_{2,32}$=3.00, p=0.064), and there was no interaction (group x time, $F_{22,32}$=1.87, p=0.052), although there was an effect of time, observed by the recovery of both stroke groups toward baseline over the experiment ($F_{11,31}$=3.95, p=0.001). There was a significant difference in performance of stroke animals between AAV-GFP animals and AAV-NT3 animals in the retrieval of pellets (Figure 2-7B, top right) with the less-affected forepaw ($F_{2,24}$=8.78, p=0.001, *post hoc* AAV-NT3 *vs* AAV-GFP, p=0.015). However, it was the AAV-GFP group that appeared to improve pellet retrieval towards baseline over time ($F_{11,31}$=2.48, p=0.023) but there was no interaction between group and time ($F_{22,31}$=1.65, p=0.098). A decrease in accuracy was seen in the affected paws of both stroke groups, although no difference between stroke groups was observed (Figure 2-7B, bottom left, effect of group $F_{2,30}$=4.77, p=0.016, *post hoc* AAV-NT3 vs. AAV-GFP p=0.207), measured by the proportion of displaced pellets at the end of the task. There was an effect of time ($F_{11,337}$=2.33, p=0.009), but no interaction between group and time ($F_{22,337}$=0.687, p=0.852). A similar degree of accuracy is seen with the less-affected forepaw (Figure 2-7B, bottom right, effect of group $F_{2,30}$=4.31, p=0.023), and there is a trend towards differences between treatment groups (p=0.073); which could possibly be an indication of compensatory mechanisms. An effect of time was observed as recovery took place over time ($F_{11,30}$=2.96, p=0.009), but there was no interaction between group and time ($F_{22,31}$=1.45, p=0.171).

A.


Staircase (%, Pellets Eaten, affected)
Staircase (%, Pellets Eaten, less-affected)
Staircase (%, Displaced pellets, affected)
Staircase (%, Displaced pellets, less-affected)
Time (Weeks)
MCAO Stroke
Sham

B.


Staircase (%, Pellets Eaten, affected)
Staircase (%, Pellets Eaten, less-affected)
Staircase (%, Displaced pellets, affected)
Staircase (%, Displaced pellets, less-affected)
Time (Weeks)
Week
treatment
AAV-NT3
AAV-GFP
Sham

C.

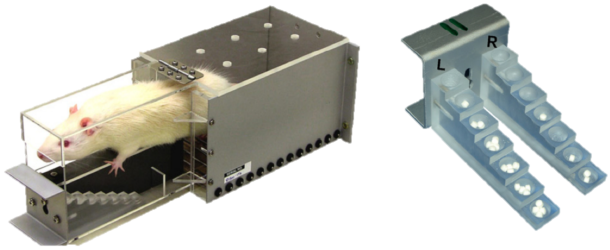

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Figure 2-7 : Skilled motor tasks are impaired following three-vessel occlusion but no improvements are seen following treatment with AAV-NT3.

A) The single vessel occlusion model did not produce significant impairments in retrieval of sucrose pellets with the affected forepaw (top left), less-affected forepaw (top right), or accuracy of retrieval (affected, bottom left; less-affected, bottom right) as measured by linear models and post hoc tests (stroke n=13, sham n=8, p>0.05).

*B) Occluding the MCA with occlusion of both carotid arteries, rats exhibited significant impairments in motor control of their affected forelimb (B, top left), less affected forelimb (top right) and the accuracy with which rats retrieved pellets with the affected (bottom left) and less-affected (bottom right) forepaws respectively (n=12 in each stroke group, sham n=10). * indicates group effect p<0.05, # denotes significant effect of stroke group compared to sham p<0.05, ¥ denotes significant interaction of group with time and † represents difference between AAV-NT3 and AAV-GFP groups. (C) The Montoya staircase test (Klein et al 2012), and the staircase, right, with sugar pellets placed in the wells of the staircase.*

2.4.4.4 Sensory neglect was a transient effect after single vessel occlusion, but AAV-NT3 does not affect recovery

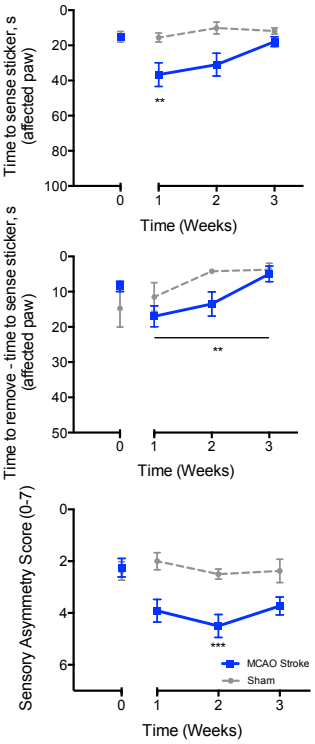
Somatosensory asymmetry was assessed using the bilateral stimulation test (Upchurch and Schallert, 1982). This was measured by recording the time it took the rat to contact an adhesive label followed by the time it took to remove the label from the affected forepaw (Figure 2-8A, top). After single-vessel MCA occlusion, analysis of the time it took for the rat to first contact the sticker revealed an overall group effect ($F_{1,19}$=9.98, p=0.005) and an effect of time ($F_{2,19}$=3.86, p=0.039), although no interaction between group and time ($F_{2,19}$=2.84, p=0.084), indicating a transient deficit in the stroke group. However, time taken to subsequently remove the sticky label showed a trend towards an effect of group (Figure 2-8A, middle, effect of group $F_{1,18}$=3.83, p=0.066), and no interaction between group and time ($F_{2,37}$=1.12, p=0.338). An effect of time was observed ($F_{2,37}$=6.24, p=0.005), shown by the performance of both sham and stroke animals improving over the course of the study, suggesting that this is a learned behaviour and that pre-training could be done in future experiments to make the deficit larger for detecting effects of stroke. A more sensitive measure of somatosensory function was used that measures asymmetrical sensory impairments after stroke (Schallert and Whishaw, 1984). This test involves 7 sets of labels (Figure 2-8C) in which the animal is started on level 3 (boxed), with the bigger label attached to its affected forepaw and the smaller label to its less-affected forelimb. A higher score implies that a rat neglected a larger stimulus on its affected paw and preferentially removed the smaller stimulus from its less-affected paw. Stroke rats showed a significant effect of group ($F_{1,20}$=23.2 p < 0.001). This shows that rats were biased to the stimulus on the less-affected forepaw and responded in removing this stimulus first (Figure 2-8A, bottom). This was a sustained effect, with no effect of time ($F_{2,36}$=1.19, p=0.316), and no interaction between group and time ($F_{2,36}$=0.374, p=0.691).

In the three-vessel occlusion model, the time to sense the sticky label on the affected forepaw was impaired in the first weeks following stroke (Figure 2-8B, top left, effect of group $F_{2,31}$=27.1, p<0.001), but no difference between treatment group was observed (*post hoc* p=0.646), although there was also an effect of time ($F_{11,31}$=11.6, p<0.001), and an interaction between group and time ($F_{22,31}$=8.05, p<0.001) as the lesioned animals recovered towards sham performance levels. A similar trend was seen in the less-affected forepaw (Figure 2-8B, top right), where there was an effect of group, but there was no difference between stroke groups ($F_{2,31}$=5.75, p=0.008, *post hoc* AAV-NT3 *vs* AAV-GFP, p=0.760). There was also an effect of time ($F_{11,31}$=2.60, p=0.018) but no interaction observed between group and time ($F_{22,31}$=1.12, p=0.381). These results show that after a larger stroke, sensation is reduced transiently with both paws but that this deficit almost completely resolves spontaneously over time.

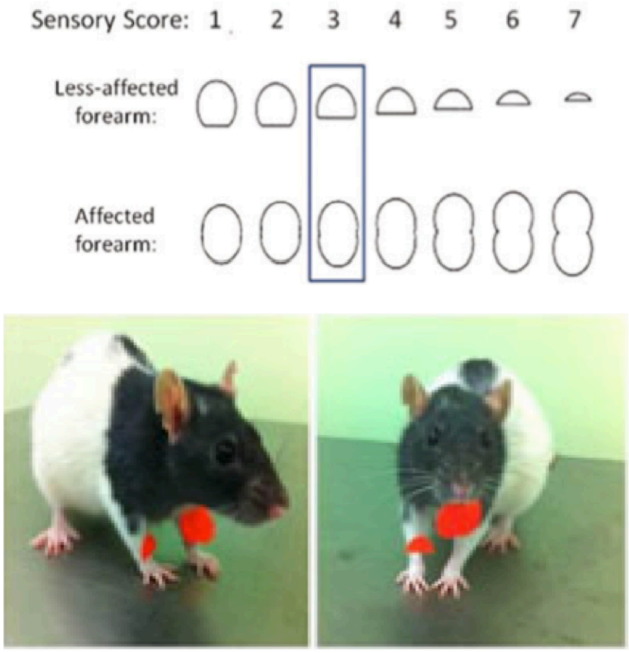
The time taken to subsequently remove the sticker indicates a difference in the ability of the stroke rats to process sensory information and produce a motor response to the stimulus. In the affected paw, there is a significant effect of group in the subsequent removal time (Figure 2-8C, middle left, effect of group $F_{2,29}$=9.16, p=0.001) with no difference between stroke groups (p=0.339). There was also an effect of time ($F_{11,335}$=10.1, p<0.001) but no interaction between group and time ($F_{22,335}$=1.46, p=0.086), indicating a recovery of both stroke groups towards sham ability on the task. Conversely, the subsequent time for removal after sensing the sticker in the less-affected paw showed no effect of group (Figure 2-8D, middle right, p>0.05), indicating no difference between the stroke and sham groups with the dexterity of the movement required to remove the sticker from the paw after sensing the sticker. Finally, the asymmetry score resulted in a significant effect of group (Figure 2-8B, bottom, $F_{2,30}$=32.8, p<0.001) but no difference was observed between treatment groups (p=0.262), and there was no interaction between group and time ($F_{22,337}$=1.34, p=0.140), although there was an effect of time ($F_{11,337}$=3.00, p=0.001); this correlates with the recovery seen at baseline.

In summary, these behavioural tests had been selected to identify features of these two methods of MCAO. The single-vessel occlusion model showed a deficiency in sensation that was consistent with the location in the sensory cortex. It was shown that the three-vessel occlusion model was able to produce more severe acute deficits compared with the single-vessel MCA occlusion model, but these impairments were not sustained. As a result of spontaneous recovery seen in both stroke models, there was an insufficient deficit from which to measure a benefit of treatment in any of these behavioural tasks.

A.


Time to sense sticker, s (affected paw)
Time (Weeks)
Time to remove - time to sense sticker, s (affected paw)
Time (Weeks)
Sensory Asymmetry Score (0-7)
MCAO Stroke
Sham
Time (Weeks)

C.


Sensory Score: 1 2 3 4 5 6 7
Less-affected forearm:
Affected forearm:

B.

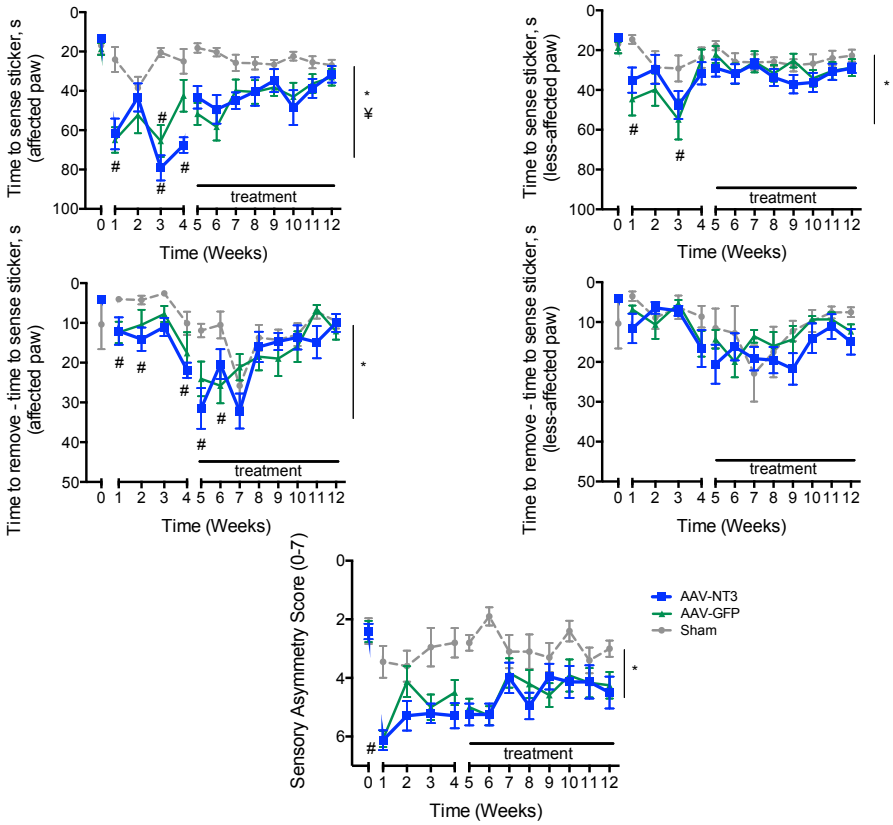

Time to sense sticker, s (affected paw)
treatment
Time (Weeks)
Time to sense sticker, s (less-affected paw)
Time to remove - time to sense sticker, s (affected paw)
Time to remove - time to sense sticker, s (less-affected paw)
Sensory Asymmetry Score (0-7)
AAV-NT3
AAV-GFP
Sham

Figure 2-8 : The bilateral tactile stimulation test does not indicate any change in sensory processing after treatment with AAV-NT3

In the single vessel occlusion model (A), the time it took rats to first contact the sticker label on the forelimb indicated a difference between stroke groups (n=12 per treatment group) compared with sham (n=8, top), the time it took to fully remove the label (middle), and the degree of asymmetry (bottom), but there was no effect of treatment (AAV-NT3 vs *AAV-GFP, p > 0.05). B) After three-vessel occlusion an initial impairment was observed in time to sense of the affected (top left) and less-affected (top right) forepaw, although the subsequent time to remove the sticker was less significant, there was still an effect of group in the affected forepaw (middle left), though not the less-affected forepaw (middle right). Regarding the ratio of asymmetry (bottom graph), the sensory deficit was similar for the AAV-NT3* vs *AAV-GFP. Graphs show mean±SEM. * denotes group difference, p<0.05; ¥ denotes interaction of group with time, p<0.05; # denotes difference between stroke groups and shams, p<0.05; Linear models. C) The sticky tape test. Top, the stickers required to do the asymmetry task and below, the asymmetry task being performed.*

2.4.5 Treatment of AAV-NT3 indicates sustained high levels of NT3 in the bloodstream 8 weeks after stroke.

One major aim of this study was to elucidate whether intramuscular delivery of recombinant AAV vectors could be used to improve outcome in an adult rat model of chronic stroke. To test whether the delivery of the AAV-NT3 was successful despite the no functional recovery of the AAV-NT3 treated rats, an ELISA was done on blood serum. The mature proteins for human and rat NT3 are identical and therefore the ELISA assay is unable to distinguish between them. Previous studies have shown that NT3 is retrogradely transported *via* the muscle to the spinal cord (Petruska et al., 2007, Petruska et al., 2010), but in this study, blood was taken 8 weeks after AAV-NT3 treatment to identify whether expression of NT3 from the vector was ongoing.

Analysis of the blood from the treated animals in this study revealed NT3 concentrations in the blood serum of 56.6±4.34 pg/mg (mean±SEM) total protein in AAV-NT3 treated animals compared with 8.193±1.71 and 8.459±1.819 pg/mg total protein in AAV-GFP stroke animals and sham, respectively (AAV-NT3 *vs* AAV-GFP p =3.47e-4, AAV-NT3 *vs* sham p=1.11e-4, *post hoc* p=2.29e-5). Although the behavioural results observed were not significant, a correlation analysis of the behavioural results indicated that the presence of NT3 did not correlate with week 12 scores in motor (p=0.847), sensory (p=0.4245), or neglect tasks (p=0.3778), but showed a correlation with increased pellet retrieval in the staircase test with higher levels of NT3 (p=0.0428), although this is a weak association given the performance of the AAV-GFP treated animals in this task. In this instance, the r^2 value (0.420) indicates that the amount of NT3 present in the serum samples accounts for 42% of the variance in the performance in this task.

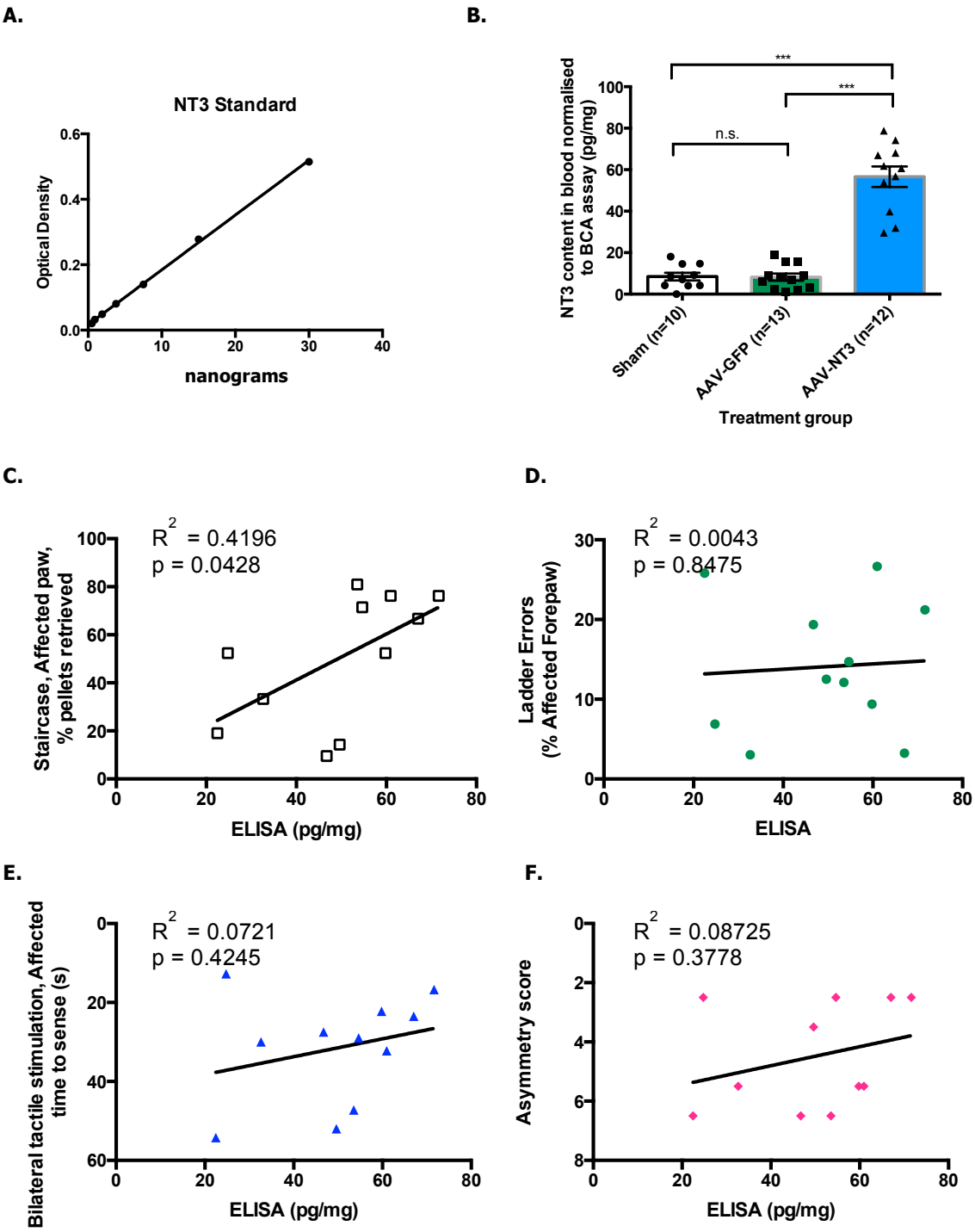


Figure 2-9 : Evidence of increased NT3 concentration in the blood after AAV-NT3 treatment is correlated to improved skilled motor reaching tasks.

The NT3 Standard curve, run in duplicate, from which ELISA data was calculated (A). Eight weeks after injection of AAV-NT3 or AAV-GFP rats (n=12 AAV-GFP, n=13 AAV-NT3, n=10 sham) were terminally anaesthetised and blood removed transcardially before being spun to acquire serum. Protein levels analysed by ELISA concluded that there was a significant difference in the levels of NT3 between the groups (B; $p<0.001$ post hoc Kruskal-Wallis $p<0.001$). Graph shows mean±SEM. (C–F) The amount of NT3 present in the ELISA of AAV-NT3 treatment animals did not correlate with motor performance (D), sensory (E), and neglect (F), but did correlate with skilled motor movements (C, n=12 AAV-NT3 group). The square of Spearman's rank correlation co-efficient (R^2) is reported.

2.5 Discussion

2.5.1 Study overview

The aim of this chapter was to establish a permanent, distal model of ischaemic stroke from which a treatment effect of AAV-NT3 could be measured through the use of functional improvements in behavioural tasks. Initially, a comparison was made in this chapter between a single-vessel MCA occlusion, and the three-vessel occlusion model, which involved the permanent occlusion of the MCA and ipsilateral CCA and transient occlusion of the contralateral CCA to the lesion. From 4 weeks into the study, the three-vessel occlusion animals were selected for treatment with AAV-NT3 or AAV-GFP. This delayed timeframe was chosen in part to simulate chronic delays, in addition to the fact that there had been indications that this timepoint was effective in spinal cord models of injury (Chen et al., 2008). Furthermore, in an endothelin model of stroke, AAV-NT3 delivered at 24 hours by intramuscular injection into affected biceps brachii and triceps brachii had already been shown to be effective in both young and aged rats (Duricki et al., 2016b). However, in this chapter, AAV-NT3 was not shown to be effective in improving functional outcome, and the purpose of this discussion is to explore the reasons why this might have occurred, when many groups have seen some effect of NT3 after CNS damage (Grill et al., 1997b, Zhou et al., 2003, Chen et al., 2006, Petruska et al., 2007, Fortun et al., 2009, Petruska et al., 2010, Duricki et al., 2016b).

2.5.2 Three-vessel occlusion results in larger lesion volumes compared to MCA alone.

Following human stroke, hemiparesis of the contralateral limb is the main deficit that occurs (Langhorne et al., 2009). The first aim of this chapter was to find a reproducible model of stroke targeting the forelimb sensorimotor cortex resulting in sustained deficits such as impairments in locomotion, skilled motor function and sensory processing. After previous successes in this lab using the endothelin model of stroke, which causes small, focal infarcts, the goal of this study was to investigate the role of AAV-NT3 after *large,* sustained stroke in otherwise young healthy rats.

After surgical occlusion of the MCA that corresponded to the dominant forepaw (measured by the cylinder test), lesions in the areas of the cortex corresponding to primary sensory areas were observed (Figure 2-3A'). However, these were not sufficient to cause functional deficits. This is particularly true of the behavioural tasks; in the cylinder (Figure 2-5A), the horizontal ladder (Figure 2-6A), and the Montoya staircase

test (Figure 2-7A), no deficit was seen even one week after injury. Sensory impairments were evident in this model (Figure 2-8A), but these impairments were not sustained and returned to sham levels over 3 weeks. These lesions are actually smaller than the volumes achieved in the endothelin model of stroke (47.3±51.4 mm^3 (mean±SEM) in this study compared to 78.3±10.1 mm^3 in the endothelin model in young rats (Duricki et al. *unpublished*)). The areas of the brain affected by the lesion in the single-vessel occlusion model, notably, a small portion of the sensory forelimb region and sensory areas corresponding to the upper face and lip, were not sufficient to produce a deficit in the behavioural tasks. This is most likely due to the proportion of spared CST using this method. This model has therefore been unsuccessful in achieving the goals set out in this chapter and will not be discussed further.

In contrast, the three-vessel occlusion—namely, the occlusion of MCA with ipsilateral permanent CCA occlusion (112.3±27.4 mm^3), and transient occlusion of the contralateral CCA—was shown to meet some of these criteria (Figure 2-3B'). The volume of these lesions were larger and less variable than the MCAO (47.3±51.4 mm^3), and larger, slightly more variable than the endothelin model mentioned previously, where variability was measured by the coefficient of variation (endothelin CV=0.999 *vs* three-vessel MCAO CV=1.195). The areas of the brain affected included a large portion of the forelimb region, and also the secondary sensory cortex and a portion of the primary motor area. These regions have been linked to the performance in the behavioural tasks discussed in this chapter (Plautz et al., 2000). In all the behavioural tasks used, the deficits achieved after the first week are significant compared to the sham groups, resulting from these large lesion volumes.

One thing to consider that may have played a role in the observed lesion volume is the use of ketamine as an anaesthetic. Ketamine is a known neuroprotective agent (Kohrs and Durieux, 1998), and previous studies have shown its potential for use as both a neuroprotective and, in the S+ isoform, a neuroregenerative agent in cultured neurons (Himmelseher et al., 1996, Himmelseher et al., 2000). *In vitro* and animal studies of up to 7 days in length have stated a neuroprotective effect of ketamine when used before, during or after various types of brain injury or insult. Indeed, it has been shown to be an effective neuroprotective agent in an MCA model of stroke in rats (Lin et al., 1996). In this study, it is important to note that both stroke groups in the treatment arm will have received, on average, the same length of anaesthesia time. This will convey a similar level of neuroprotection with or without NT3 treatment, but it is important to consider the use of anaesthetics to achieve the best possible outcome for the experiment. In this context, the potential of agents that either are not neuroprotective, or show

neuroprotection for the shortest period of time should be considered. Isoflurane, a commonly-used inhalation anaesthetic, has also been shown to confer a neuroprotective benefit in rat models of global ischaemia (Gray et al., 2005); this neuroprotection would be acute, but the long-term benefit can last for up to 3 months (Elsersy et al., 2004) compared to 6 months with ketamine (Koerner and Bambrink, 2006).

The three-vessel occlusion model has been shown to create large lesions of similar variability to the endothelin model. The use of ketamine may have produced neuroprotective benefits in these rats over the 12-week study, which, despite severe initial deficits after stroke surgery, could have contributed to the observed recovery through neurorestoration mechanisms. The use of isofluorane as an alternative anaesthetic will be explored in Chapter 4.

2.5.3 Delayed AAV-NT3 does not convey a functional benefit to young adult rats after three-vessel occlusion.

In order to assess functional recovery, it is possible to use behavioural tasks to assess forelimb asymmetry after a unilateral lesion. After three-vessel occlusion, there will typically be impairments of forepaw function of the limb contralateral to the lesion, which can be measured using motor and sensory tasks. There are parallels between rodent and human skilled reaching (Cenci et al., 2002, Sacrey et al., 2009) and occlusion of the MCA serves as an impairment to measure treatment success from. The staircase test can be used as a quantitative measure of skilled reaching of the rat (Klein et al., 2012); the horizontal ladder as a measure of gross motor skill (Metz and Whishaw, 2009), and the bilateral tactile test as a measure of sensory awareness and neglect (Kozlowski et al., 2000). The cylinder test in this study measures exploratory behaviour and is a measure of paw dominance.

In this study, we saw no benefit in delayed AAV-NT3 treatment in any of the behavioural tasks despite impairments being observed in motor tests, possibly because the deficit was too small in both groups by the end of the study from which to measure a benefit. In postural support during the cylinder test (Figure 2-5B), there was no observed significant impairment compared with sham group, and no difference was observed between treatment groups. The results acquired from this test may indicate that it is not sensitive enough to pick up motor deficits for this lesion type. In the horizontal ladder test of gross motor function (Figure 2-6B), the AAV-NT3 treated cohort returned to sham levels, indicating increased use of the affected forepaw after treatment. However, the AAV-GFP group also showed an improvement in the use of the disabled forelimb. An

interesting observation was that impairments were also seen in the ipsilateral, which is the unlesioned, less-dominant paw. The ipsilateral limb is usually affected in stroke patients as well. Movement elements of aim, supination, and advance are abnormal compared with healthy controls (Foroud and Whishaw, 2010).

The same was seen in the fine motor test of the Montoya staircase, which requires skilled movements for pellet retrieval (Figure 2-7B). In fact, there were differences between groups at week 3 (prior to treatment), which could relate to variability in lesion placement and size. AAV-NT3 did not modify the trajectory of recovery in these animals, and indicates that there was no functional effect of NT3 in this study.

The three-vessel occlusion model of stroke results in motor, but also sensory impairments. This is analogous to the human condition whereby sensory deficits are evident in the vast majority of stroke victims (Carey et al., 1993). Therefore, when trying to overcome stroke impairments it is vital that both motor and sensory function are assessed. The bilateral tactile stimulation test was used in this study to measure sensory responsiveness and neglect. However, no difference in the time the rat took to first contact or subsequently remove the sticky label was observed between stroke treated animals. There was an observed deficit using the asymmetry measure, simulating sensory neglect, although both paws seem to have suffered sensory (but not motor) impairments, but there was no effect of treatment with AAV-NT3. It is also important to observe that treatment of stroke animals with NT3 did not provide any evidence of detrimental outcome.

This is contrary to studies that have shown functional locomotor improvements following CNS injury and acute NT3 treatment (Grill et al., 1997b, Zhou et al., 2003, Zhou and Shine, 2003, Chen et al., 2006, Boyce et al., 2007, Fortun et al., 2009, Duricki et al., 2016b). Importantly, it has been shown previously that radiolabelled NT3 rapidly crosses the intact blood–brain barrier and accumulates stably in the cortex, striatum, and other regions of the nervous system when infused into the brachial vein (Poduslo and Curran, 1996). As the brachial vein arises from the triceps muscles, it is likely that infusion of the AAV-NT3 into these muscles leads to accumulation of NT3 in the brain and spinal cord after stroke given that the blood-brain barrier (BBB) is compromised. It is possible however, that the dose required to cross the BBB is higher than the amounts used in this study, and a high bolus dose might be required for detection in the brain (Poduslo and Curran, 1996). Recombinant human NT3 has a short half-life and is relatively stable in the bloodstream and is delivered mostly quickly from blood into the cervical spinal cord compared with the brain and other regions of spinal cord (Pan et al., 1998).

Searching for an explanation for why there was no effect of delayed NT3 in the study in this chapter that used AAV treatment of NT3, two papers (Chen et al., 2006, Chen et al., 2008) describe how delayed treatment of NT3 is possible even up to 4 months after CNS injury, but requires re-activation of the immune system using low-dose systemic lipopolysaccharide in order to initiate regeneration of spared CST axons, which is unfeasible in the clinical context of stroke treatment. Not only this, but there has been suggestion that Wallerian degeneration is an essential component of instigating collateral sprouting of the uninjured CST axons from the less-affected hemisphere with NT3; delayed timepoints, such as those seen in this study, should enable the recruitment of cells or molecules in the tracts undergoing Wallerian degeneration within the therapeutic window (Chen et al., 2006). This leads to the conclusion that any treatment with NT3 could take place within the window this degeneration is occurring, up to 90 days after CNS injury (Beirowski et al., 2005, Vargas and Barres, 2007).

However, functional impairments in the behavioural tasks used in this study were reversed, and, over the 12 weeks of the study, achievements of the stroke animals from both treatment groups reached those of sham animals. The most logical explanation for this is that spontaneous recovery of the animals in this study resulted in these behavioural gains. Distal MCAO lesions in the rat can result in lasting behavioural impairments (Marston et al., 1995). However, performance in these tasks can be improved by pre-training (Grabowski et al., 1993), and has been linked to an increase in compensatory behaviour (Hurd et al., 2013). Perhaps with repeated, weekly, motor training, post-stroke performance can reach near-normal levels. This is in line with observations that, after cortical stroke, rats develop a transient impairment in behavioural tasks that can recover to nearly pre-lesion performance (Carmichael, 2005). Additionally, this stroke model has been previously shown to produce spontaneous recovery in smaller lesions (Gonzalez and Kolb, 2003). By placing the occlusions in future studies more proximally in the MCA, this could result in larger lesions to include more of the rostral M1 area and, therefore, produce a more sustained deficit in rats.

Another mechanism of recovery could be the recruitment of regions surrounding the lesion, or peri-lesional activation, to bring about the functional improvement seen in these tests. There is evidence for this in both rats (Starkey et al., 2012) and humans (Cramer, 2008). Furthermore, the motor area of the cortex, M1, appears to be anatomically spared after stroke lesions, according to the structural MRI scans (Figure 2-3B'), yet behavioural function is impaired after stroke, which is line with other studies that also show that the M1 region has a smaller forelimb representation after stroke (Nudo and Milliken, 1996, Frost et al., 2003, Gharbawie et al., 2005) and these

representations change over time with increased use of the affected forelimb (Nudo et al., 1996b). This could be explained by the representation of an additional forelimb motor area in the rat that is more rostral than the M1 region (Neafsey et al., 1986). The mechanisms for the spontaneous recovery seen in this model will be explored in more detail in Chapter 3 with the use of functional MRI.

2.5.4 AAV-NT3 delivery at 4 weeks results in high blood levels of NT3.

In this study, the use of AAV-NT3 at 4 weeks after stroke resulted in high detectable levels of AAV-NT3 8 weeks later, and although this did not confer a benefit in behavioural outcomes, it is a topic that could be considered further in terms of relevant clinical delivery of NT3 for future studies.

Although AAV-NT3 has been shown to be safe and effective in several animal models (Blits et al., 2003, Fortun et al., 2009, Lu et al., 2011, Boyce et al., 2012, Hunanyan et al., 2013, Duricki et al., 2016b), it has not yet been used in clinical trials, because of the safety concerns of using viruses for gene delivery in the clinic and worries about immunogenicity despite having the safety go-ahead (Hasbrouck and High, 2008, Mueller and Flotte, 2008a). However, the high levels of NT3 present in the blood in this study and others following AAV-NT3 treatment (Sahenk et al., 2014), and the fact that two human clinical trials have shown that systemic, high doses of recombinant NT3 are well-tolerated make the delivery of recombinant NT3 an attractive treatment option (Parkman et al., 2003, Sahenk, 2007).

An increase in NT3 levels could be seen with the use of another promoter, namely the MCK, which is muscle-specific (Wang et al., 2008), and has been shown to result in higher levels of NT3 in the circulation after intramuscular injection of AAV-MCK-NT3 (Sahenk et al., 2014). Using the CMV promoter in the AAV-NT3 construct in this study (Figure 2-2B), NT3 serum concentrations are detectable after 8 weeks, in line with a previous group that were able to measure NT3 in the blood 3 weeks after treatment up to total of 10 months. Their suggestion was that AAV-NT3 gene transfer into the muscle results in NT3 secretion into circulation reaching therapeutic blood levels, and these levels of NT3 are sufficient to confer functional, histopathological, and electrophysiological improvements in peripheral nerves after inducing a model of neuropathy (Sahenk et al., 2014). Additionally, other studies using ELISA have shown that local increased concentrations of NT3 in the peripheral muscles results in high levels of NT3 in the DRG (Duricki et al., 2016b), suggesting that changes occurring to promote recovery, if any, might happen through retrograde transport from muscle to DRG and

modification of proprioceptive reflexes rather than delivery into the CNS. Therefore, a systemic treatment could be sufficient to elicit an effect in an acute model of CNS injury. Subcutaneous delivery of recombinant NT3 protein initiated 24 hours after stroke will be explored further in Chapters 4 and 5.

2.5.5 Conclusions

In conclusion, this study has shown that permanent distal MCA occlusion with bilateral carotid occlusion results in large, reproducible strokes and will be the preferred stroke model for this thesis. AAV-NT3 did not enhance functional recovery in young adult rats when initiated 4 weeks after stroke. This therapy involves a human transgene, which can be administered in a clinically straightforward route to peripheral muscles. High doses of NT3 have been shown to be safe in phase 1 and 2 clinical trials (Chaudhry et al., 2000, Coulie et al., 2000, Parkman et al., 2003, Sahenk, 2007).

Spontaneous recovery has been seen in young animal models of stroke previously (Gharbawie et al., 2005, Duricki et al., For submission). Mechanisms of this recovery will be explored in Chapter 3 using functional MRI. Perhaps the use of an aged model, although challenging, will provide a more stable deficit from which treatment benefits can be measured. This is something that will be addressed further in Chapters 4 and 5.

Chapter 3 Exploring recovery of sensorimotor areas after three-vessel occlusion stroke in young animals using functional MRI.

3.1 Abstract

Animals from the three-vessel occlusion model portion of the previous chapter had also undergone longitudinal functional magnetic resonance imaging (MRI) analysis to try and identify where the origin of the recovery could be observed, using both forepaw stimulation and resting state functional imaging, to observe connectivity changes. Animals were scanned at baseline, and at 1, 4, and 12 weeks after stroke. Resting state MRI showed increased probability of correlated activation between the seeded region of the forelimb region of the primary somatosensory cortex and the secondary somatosensory cortex, bilateral striatum, and the 4th to 6th lobules of the cerebellum. These regions have been shown to be important in processing of cutaneous and proprioceptive inputs – and recruitment of these regions in the weeks following stroke could indicate some processes that could be involved in the spontaneous recovery in the bilateral tactile extinction behavioural task observed in chapter 2. Additionally, differences observed during forepaw stimulation MRI at 4 and 12 weeks compared with the pattern of activation observed at 1 week after stroke indicated regions of the brain responding to the electrical stimulation of the affected paw; these included the regain of activation following stimulation in the areas of the primary somatosensory cortex surrounding the lesion, in addition to the secondary somatosensory cortex, which might relate to the changes seen in the bilateral tactile extinction task from the previous chapter. No interhemispheric activity was seen in this study, in contrast to previous studies. This suggests that maybe the size of the lesion and the amount of sparing of neurons determines the type of recovery that takes place after stroke, with larger lesion sizes resulting in less interhemispheric connectivity because of fewer spared neurons.

3.2 Introduction

Imaging methods have been applied to assess stroke pathophysiology both for clinical diagnosis and scientific research. Techniques such as transcranial magnetic stimulation (TMS), positron emission tomography (PET), electroencephalography (EEG), and magnetoencephalography (MEG) allow quick and non-invasive study of the brain and can be used to understand the mechanisms by which the brain attempts to preserve function following ischaemic damage; indeed, spatial and temporal patterns of reorganisation have been documented in both humans and animal models of stroke using fMRI (Rossini and Pauri, 2000, Dijkhuizen et al., 2001, Green, 2003). Among these, proton magnetic resonance imaging (MRI) is particularly versatile, and is the technique chosen for this study. Specifically, we used a combination of structural MRI, functional MRI (with somatosensory stimulation) and resting state functional MRI to probe the changes in brain structure, function, and connectivity in the stroke-lesioned rats.

Structural MRI is ideally suited for the detection and investigation of ischaemic brain infarcts because of the ability to detect oedematous changes. Although the pathological changes that take place during an ischaemic insult are complex, the water accumulation that occurs after a stroke is detectable using spin-echo imaging protocols. The T2 (spin-spin) relaxation time is prolonged in oedematous lesions, resulting in increased signal intensity in T2-weighted sequences; these sequences are the most sensitive to water content changes (Bederson et al., 1986a, Brant-Zawadzki et al., 1986).

In functional MRI (fMRI), differences in magnetic susceptibility of oxygenated and deoxygenated blood is used to indicate changes in brain activity. In particular, the paramagnetic deoxyhaemoglobin creates inhomogeneity in the applied magnetic field and slight lowering of the MR signal (McRobbie et al., 2007). During periods of increased neural activity and therefore metabolism, more oxygen is required, resulting in an overwhelming increase of fresh blood flow to the area. This reduces the concentration of deoxyhaemoglobin and subsequently increases signal intensity. The resulting blood oxygenation-dependent (BOLD) effects are short-lived (seconds) and detection of this haemodynamic response requires rapid sequences such as echo planar imaging (EPI) and the repetition of tasks or stimulations in order to measure activity (by averaging to enhance signal to noise)—for example during sensory paw stimulation—while scanning. Forepaw stimulation is the one of the most reported BOLD fMRI experimental protocols in rodents, and consists of a non-noxious electrical sensory stimulation to the animal's paw, giving rise to a robust BOLD response in the contralateral sensorimotor cortex in a

healthy animal. These changes in the BOLD signal are only in the region of a few percent in animals, but the use of higher strength magnets (≥7 Tesla) can increase the sensitivity of the scan (Harel et al., 2006). This has been used with some success already in animal models of stroke (Dijkhuizen et al., 2001, Dijkhuizen et al., 2003), and the results in this chapter will add to the existing literature in the detection of disruption to neural activity after stroke.

Resting-state MRI (rsMRI) is a particular type of fMRI that correlates BOLD responses spatially across the whole brain without the need for any specific stimulation, allowing the more extensive assessment of changes in organisation and connectivity of whole functional neural networks (Salvador et al., 2005, Fox and Raichle, 2007, Auer, 2008, He et al., 2009, Wang et al., 2009). This technique records baseline brain activity while the subject is at rest, and spontaneous BOLD fMRI signals are collected. rsMRI can identify temporal correlations of low frequency (<0.1 Hz) between regions with known functional similarities based on the BOLD signal (Biswal et al., 1995, Damoiseaux et al., 2006, Raichle, 2011). The simplicity in its design and image acquisition has made resting state analysis popular in fMRI studies.

Structural MRI is particularly useful for the detection and measurement of the stroke lesion, but in combination with functional MRI we can gain a greater insight into the pathology of the insult. For example, fMRI-detected activation responses could be absent in the affected area despite normal appearance on structural (eg, T_2-weighted MR) images, indicating that structural integrity around a lesion does not automatically result in preservation of function, and shows the importance of using functional imaging techniques such as fMRI to detect tissue dysfunction in these regions. Moreover, the ability of MRI to acquire structural and functional images longitudinally in the same rodent over a number of weeks is more advantageous than a single endpoint scan in terms of monitoring changes in the brain over time. Compared with human beings, the use of fMRI in animal models of stroke, in which the stroke is more homogeneous as it is experimentally induced, can provide more information and can be used in combination with other techniques such as immunohistochemistry to aid in the interpretation of the fMRI responses (Van der Linden et al., 2007).

fMRI studies while the patient is at rest provide crucial information on the neural mechanisms of motor recovery in patients, while also avoiding the use of forced or compensatory behaviour. This resting-state fMRI is therefore also applicable to patients with stroke who are not capable of proper performance of motor tasks. In stroke patients, one study used rsMRI to detect the well known symptom of neglect in the

affected arm and to follow up the changes that occur during progression from acute to chronic stage after stroke injury (He et al., 2007). This study required the patient to press a key on a keyboard with their affected hand after a visual stimulus. Reduced functional connectivity was shown (indicated by increased reaction time) at acute timepoints after stroke to the frontoparietal cortex, but these patients mostly recovered at the chronic stage. This neglect, or impairment in reaction to a visual stimulus, has been postulated to be due to the disruption of interhemispheric activity. Resting state MRI has also been useful in examining changes in brain connectivity with age (Stevens et al., 2009, Supekar et al., 2009, Ferreira and Busatto, 2013). The main advantage to using resting state fMRI is the ability to assess the changes in functional connectivity, especially in a longitudinal experimental design, such as the one used in this study, where the connectivity changes can be observed throughout the recovery period. These changes can then be correlated to loss and, potentially, return of function as the animals perform better at behavioural tasks over time following stroke injury.

Rehabilitation of the damaged brain can induce reconnection of damaged neural circuits after injury. In particular, a small loss of connectivity will tend to lead to spontaneous recovery, although a major loss of connectivity will lead to permanent loss of function; for these patients, a compensatory approach to recovery is required. A third scenario are patients that have potentially rescuable lesioned circuits, but recovery depends on providing precisely targeted inputs, maintaining adequate levels of stimulation, and avoiding activation of competitor circuits that could suppress activity in target circuits (Robertson and Murre, 1999). Neuroimaging techniques such as fMRI facilitate the visualisation of brain regions responsible for this recovery process. The recruitment of bilateral secondary motor areas in the more severely impaired patients permits a restoration of some function, but results in suboptimal recovery in those patients relying on these regions to generate motor output. This implies that the remaining motor network architecture and recruitment of the unaffected hemisphere is potentially an important mechanism for recovery in these patients and this has been previously observed in the clinic (Weiller et al., 1992, Cramer et al., 1997, Calautti and Baron, 2003, Ward, 2004). The pattern of recovery that occurs from the recruitment of these areas in the first 10–14 days is indicative of the pattern of long term recovery and is dependent on the severity of the initial lesion, highlighting the plasticity of secondary areas and importance of designing the correct rehabilitative strategy early on after stroke onset to enable the recruitment of these regions (Ward et al., 2004, Maulden et al., 2005). Additionally, it has been shown that functional reorganisation during learning motor tasks takes in the region of weeks in the primary motor cortex, but more rapid

changes are observed in the cerebellum, striatum, and other motor-related cortical areas (Ungerleider et al., 2002).

In the previous chapter (Chapter 2) we showed that although treatment with NT3 did not produce any behavioural benefits, a return to baseline was observed in both stroke groups in all of the tasks performed. We wanted to identify the mechanisms by which this recovery was occurring, and the use of longitudinal BOLD fMRI and resting state MRI in this study over a number of weeks enabled us to better characterise the affected networks and the extent of changes taking place. This spontaneous recovery might elicit the observed functional recovery seen.

In animal neuroimaging, anaesthesia is usually administered to reduce stress, stabilise physiology, and reduce movements, which can all interfere with the quality of the produced scans. These MRI studies typically use inhalation anaesthesia such as isoflurane, which is safe, easy to administer, and has a rapid recovery time. However, isoflurane is a vasodilator and has direct effects on cerebral vascular smooth muscle (Jensen et al., 1992, Zhao et al., 2007), also reducing neural activity in the cerebral cortex (Jansson et al., 2004, Duong, 2007). Some laboratories have successfully managed to detect fMRI BOLD signal changes under isoflurane but this requires complicated set ups with highly sensitive radiofrequency coils and an invasive mechanical ventilation and paralysis paradigm (de Celis Alonso et al., 2012). A less widely-used anaesthetic is alpha chloralose, which has been shown to preserve neurovascular coupling (Ueki et al., 1992). However, alpha chloralose has slow pharmacokinetics, which can produce metabolic acidosis and a reduction in body temperature. The fact that this anaesthetic is also used as a rodent pesticide has meant that alpha chloralose has been used as a non-recoverable anaesthetic in the past. A study in which stroke effects were measured by laser Doppler found alpha chloralose could be used productively as a recoverable anaesthestic (Luckl et al., 2008). Furthermore, another study showed a new low dose regime of alpha chloralose-Pestanal (with a β-isomer of $< 8\%$) can be used for longitudinal recovery scanning (de Celis Alonso et al., 2011). Using a low dose alpha chloralose anaesthesia regime we were able to conduct repeated longitudinal BOLD fMRI, allowing us to monitor changes following forepaw stimulation in the same rats pre-stroke and then at 1, 4, and 12 weeks following stroke.

3.3 Methods

3.3.1 Experimental Design

The model of stroke used, the size of lesion, and the performance in behavioural tasks of animals used in this study was assessed in the previous chapter (section 2.5.2). In brief, animals had MCAO stroke surgery with bilateral carotid occlusion as described previously (section 2.3.3.1.2). Animals underwent baseline rsMRI and fMRI with forepaw stimulation 2 weeks before stroke for a baseline assessment, and these scans were repeated 1, 4, and 12 weeks after the lesion. 34 animals were used overall; animals from both stroke treatment groups were pooled as the individual treatment groups showed no differences, and by pooling the data, the study has a greater power to detect changes between groups since there is an improved signal-to-noise ratio in fMRI maps. However, because inadequate quality of some images, there are fewer subjects in some of the group maps below (Table 3-1). Scans were excluded when quality control measures were not met, including; large artifacts in the scans, animal movement too large to be corrected in the realignment stage of image processing, unexpected signal drops in time- series, and low signal to noise ratio. Scans that failed the quality control criteria were excluded from the group analysis at that timepoint. Resting state MRI had

	AAV-NT3 stroke animals* (n=20)	AAV-GFP stroke animals* (n=20)	Sham animals (n=10)
Baseline scanned	20 (10)	20 (11)	10 (6)
Animals included in fMRI analysis[§#]	18 (8)	18 (7)	6 (4)
Animals in resting state analysis	5 (3)	5 (2)	9 (4)
1 week fMRI	13 (8)	12 (7)	10 (6)
Animals included in fMRI analysis[§]	12 (7)	6 (3)	10 (6)
Animals in resting state analysis	7 (5)	7 (4)	10 (6)
4 week fMRI	12 (7)	12 (7)	10 (6)
Animals included in fMRI analysis[§]	8 (7)	11 (5)	6 (5)
Animals in resting state analysis	7 (6)	10 (6)	6 (5)
12 week fMRI	12 (7)	12 (7)	10 (6)
Animals included in fMRI analysis[§]	6 (5)	7 (5)	6 (2)
Animals in resting state analysis	4 (1)	4 (3)	9 (5)

Table 3-1 : Numbers of animals included in all subgroups of fMRI analysis

*Numbers in brackets indicate numbers of animals in the dominant paradigm (Figure 3-1C). *Stroke animals were pooled, to increase power in the data analysis. [§]Animals were removed from fMRI analysis if there were movement artifacts, unsuccessful anaesthesia, and inadequate quality of images, through dropout of signal, and poor signal-to-noise ratio. [#]Baseline animals included animals from all three treatment groups.*

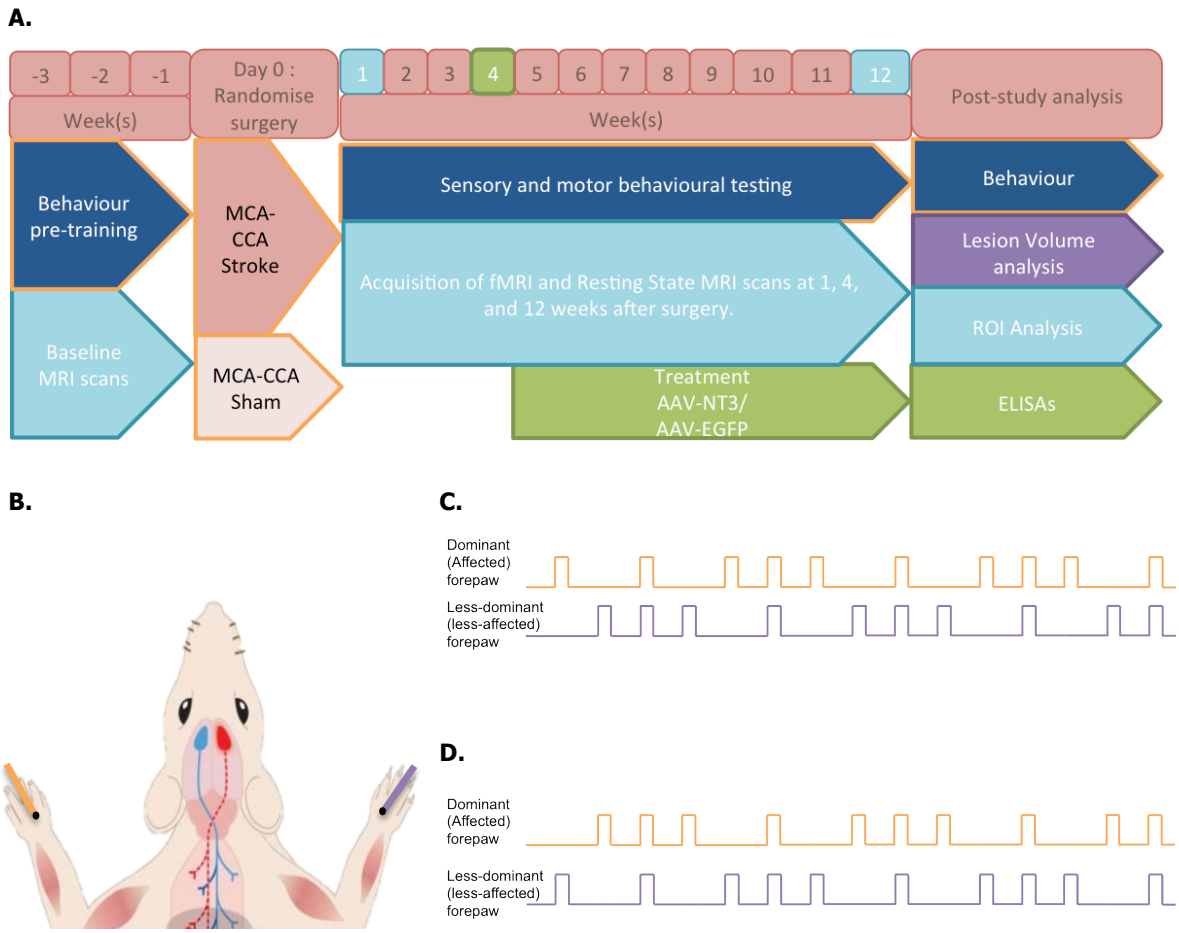


Figure 3-1 : Overview of MRI study

A) Timeline of the study in this chapter. Before surgery to induce stroke, animals underwent three weeks of behavioural training and baseline scoring. Rats had a baseline structural and functional MRI scan 2 weeks before being randomly allocated to either stroke or sham group (stroke n=40, sham n=10). Stroke animals were randomised into groups receiving AAV-NT3 (n=12) or AAV-GFP (n=12). Resting state MRI and fMRI during forepaw stimulation were acquired at 1, 4, and 12 weeks after stroke. B) Schematic of forepaw stimulation, with stimulation of the dominant (orange) and less-dominant (purple) paw. Animals were randomly assigned to one of two pseudo-random stimulation paradigms with either the dominant (C) or less-dominant (D) paw being stimulated first.

some additional exclusions due to the additional processing of images, which reduced the entry criteria. Some rats' dominant paw was on the left (53.3%) and for others on the right; we always lesioned the brain hemisphere contralateral to the dominant paw (section 2.3.3.1). All animals had MRI scans, and were randomly allocated to receive one of two paradigms for electrical forepaw stimulation. Animals remained in treatment groups for the analysis of MRI scans, and were unblinded on study completion.

To avoid confounding factors, the animals were divided into two forepaw stimulation groups equally: one in which the dominant paw (stroke-affected Figure 3-1C) was stimulated first and the remaining had the less dominant paw (less stroke affected, Figure 3-1D) stimulated first. On completing analysis, it was discovered that stroke

animals showed no difference between treatment groups (as with the behavioural results in chapter 2), and were pooled to improve the conviction of the results. The experiments were designed in accordance with guidelines from the Stroke Therapy Academic Industry Roundtable (STAIR) (Fisher et al., 2005, Fisher et al., 2009) and others (Macleod et al., 2009). All procedures were performed in accordance with the UK Home Office guidelines and Animals (Scientific Procedures) Act of 1986.

3.3.2 Subjects

This study used 47 Lister hooded female adult rats aged about 3 months (200–300 g) that were used in the three-vessel occlusion model group of the previous chapter. Animals were housed in groups of 3 or 4 on a 12:12 light-dark cycle and had access to food and water *ad libitum*.

3.3.3 Surgical procedures

3.3.3.1 Stroke Surgery

Animals from the second experiment of the previous chapter (three-vessel occlusion study) were used for fMRI analysis. Before surgery, animals were allocated randomly to either stroke or sham surgery groups, and the pre-operative cylinder test results indicated on which side the dominant paw was located; lesions were positioned on the contralateral hemisphere to the dominant paw, as described previously (Chapter 2, experiment 2; section 2.3.3.1.2).

3.3.3.1.1 MCAO with bilateral carotid occlusion

Stroke rats (n=37) and sham rats (n=10) were anaesthetised with ketamine (0.6 mg/kg) and Domitor (0.25 mg/kg) *via* intra-peritoneal injection. Body temperature was monitored using a rectal thermometer and maintained at 36°C with a heating blanket. Surgical procedures and aftercare were performed as in section 2.3.3.1.2.

3.3.4 Functional Magnetic Resonance Imaging.

3.3.4.1 Animal preparation

After behavioural testing at baseline, and 1, 4, and 12 weeks after stroke (described in section 2.3.6), rats (n=47) were initially anaesthetised with 4–5% isoflurane in 0.8 L/min medical air and 2 L/min oxygen. The tail vein was cannulated and the animal was

transferred to the MRI scanner, where a bolus of 40 mg/kg alpha chloralose Pestanal (Sigma) was given intravenously and the isoflurane switched off. An infusion line for continuous administration of alpha chloralose was attached to the tail cannula with an infusion rate of 10 mg/kg/hour for the duration of the scan. Medical air (0.8 L/min) and oxygen (2 L/min) were delivered continuously throughout the scanning period (de Celis Alonso et al., 2011). Alpha chloralose anaesthesia was prepared by mixing equal amounts of Borax Decahydrate (Sigma) and alpha-chloralose Pestanal (Sigma, <8% beta isoform) in physiological saline to a final concentration of 40 mg/mL for each component and dissolved in a glass beaker at 50–55 °C. The solution was filtered using a 22 µm filter.

Rats were immobilised with ear bars and a tooth holder inside a quadrature birdcage MR coil (43 mm ID) and placed in the scanner (7 Tesla [7T], Agilent). Each animal's physiology was monitored during the scan using a respiration monitor (BIOPAC, USA) and a pulse oximetry sensor (Nonin, USA) that interfaced with a PC running BIOPAC software. Additionally, a fan heater directed at the animal responded to any alterations in body temperature identified by rectal probe, and was thermostatically maintained at 37±1 °C.

During after-scan recovery, animals received oxygen-rich air (FiO_2 30%) in a heated recovery chamber (31 °C) until fully conscious (this takes approximately 3 hours because of the slow pharmacokinetics of alpha chloralose Pestanal), at which point animals were returned to their home cages with adequate soft food (Luckl et al., 2008). Animals were closely monitored following the end of the scan, given a subcutaneous injection of saline and the depth of anesthesia was monitored periodically by pinching the tail and extremities. If fluid was present in the trachea, this was syringed out.

3.3.4.2 Structural MRI

Structural MR imaging was conducted as described in Chapter 2 on a 7T horizontal bore scanner (Agilent, Palo Alto, CA, USA). The T2 weighted MR images were acquired using a fast-spin echo sequence: effective echo time (TE) 60 ms, repetition time (TR) 4000 ms, field of view (FOV) 40 x 40 mm, acquisition matrix 128 x 128, acquiring 20 x 1 mm thick slices in approximately 8 minutes. Data were analysed using a semi-automatic contour method in Jim software (Xinapse systems Ltd.) as described previously (Cash et al., 2001). The bright hypertense signal surrounding the lesion was measured as the lesion volume.

3.3.4.3 Resting State MRI (rsMRI)

3.3.4.3.1 rsMRI acquisition

Subsequently, rsMRI was conducted during 10 min with a T_2*-weighted gradient echo planar imaging (EPI) sequence (50° flip angle; TR= 360 ms, TEs= 5, 10, 15 ms; voxel size 0.5 x 0.5 x 1.0 mm^3, resolution 64 x 64 x 20) over 300 volumes (in 5 minutes).

3.3.4.3.2 rsMRI analysis

Images were converted from native to NifTI format and brains masked (excluding non-brain voxels) from structural scan data by use of locally written MATLAB scripts. The resulting images were analysed with SPM-8 (UCL, London; **http://www.fil.ion.ucl.ac.uk/spm/software**) or by locally written MATLAB programmes. The scans from all animals that had a dominant right paw were flipped to ensure all dominant hemispheres were on the same side for analysis. Intra-session movement correction (6-parameter, rigid body spatial transformation) of the time series was completed using the first image of the series (co-registered to the structural scan) as reference. Images were then spatially normalised to a high-resolution template provided by Prof Kawashima at Tohoku University (Valdés-Hernández et al., 2011). A Gaussian smoothing filter (2x in plane resolution) was applied to the images in order to reduce noise and normalise the distribution of the data (Mikl et al., 2008).

A seed-based correlation analysis used the primary somatosensory cortex as the seeded region in each hemisphere (Figure 3-2 and Figure 3-3). The obtained pairwise Pearson coefficient *r* at each voxel was Fisher-transformed to account for variance stabilisation (Fisher, 1958, Mathworks, 2006). Mean group maps (week baseline n=10; week 1 n=14; week 4 n=17; week 12 n=8; see section 3.4.1 for more information about group sizes) were obtained by averaging all individual z-correlation maps followed by a back transformation of z-scores to calculate regular correlation coefficients. Group correlation maps are shown as $r^2>0.5$. The contrast function in SPM was then created to observe differences between timepoints and between groups by mathematically subtracting the value (between –1 and 1) in each voxel.

All calculations to generate correlation maps were done in SPM8 and MATLAB using custom-written scripts.

3.3.4.4 Forepaw stimulation fMRI

3.3.4.4.1 fMRI acquisition during paw stimulation

Following rsMRI, fMRI scans were acquired using an identical EPI sequence and parameters as described previously (section 3.3.4.3.1). 900 volumes (in 15 minutes) were thus acquired with a pseudo-random on-off stimulation of the left, right or both paws simultaneously (3Hz, 400μs, 2mA pulse) using a platinum subdermal needle electrode and a transcutaneous electrical nerve stimulation (TENS) pad on each paw (Lowe et al., 2007). The order of the paw stimulation was also randomised (see Figure 3-1C and D for the two pseudorandom sequences). One animal died after the baseline scan due to breathing difficulties in recovery.

3.3.4.4.2 fMRI Analysis of data acquired during paw stimulation

Images were converted from native to NifTI format and brains masked (excluding non-brain voxels) from structural scan data by use of locally written MATLAB scripts, and analysed as mentioned previously (section 3.3.4.3.2). Statistical analysis was by general linear model in SPM8. First-level analyses were carried out using a regressor consisting of the pseudo-random stimulation paradigm convolved with the Haemo-Dynamic Response Function of a healthy rat (Berwick et al., 2002), and the estimated movement parameters of each individual rat. Contrast images from the 1^{st}-level analysis were then carried on to a 2^{nd}-level group analysis to find the difference between groups. These group maps were thresholded to show voxels with $p<0.001$ (uncorrected for multiple comparisons).

3.3.4.4.3 ROI analysis of fMRI data

ROI analyses of fMRI data can be used as an exploratory tool, as a statistical control, and/or to take apart functional information from fMRI data (Poldrack, 2007). ROI time course extractions were done with the use of the SPM toolbox MarsBaR. To quantify group-averaged percent signal changes for each fMRI modality, ROIs for forelimb sensory areas (S1FL) were defined for both hemispheres through a coregistered MR image and a digitised Paxinos and Watson's rat brain atlas (Paxinos and Watson, 2006). From these assigned voxels, the stimulus epoch and rest epochs were averaged separately across rats according to which randomisation cluster the rats belonged to (either "dominant-first" [Figure 3-1C], or "less-dominant-first" [Figure 3-1D]). For the

analysis of fMRI activation in somatosensory cortices, the acquired fMRI responses were split into affected and the less-affected hemispheres and analysed separately.

The tissue mean percent signal change in somatosensory cortex (ΔSI) was calculated for BOLD weighted responses, in which ΔSI for BOLD was defined as the averaged signal intensity for each second for the duration of the 900 volumes of fMRI scan.

3.4 Results

3.4.1 Resting state data show supporting network connectivity during spontaneous recovery after stroke.

Resting state data were acquired in 300 volumes (timeseries) in anaesthetised rats. Visual inspection of the resulting statistical correlation maps revealed correlations that were characterised by the seeding of the lesioned or unlesioned primary somatosensory cortices according to the rat brain atlas (Paxinos and Watson, 2006). The sensory cortices of the left and right hemispheres according to the atlas were used as ROIs to analyse intracortical signals and interhemispheric functional connectivity during rsMRI. In Figure 3-2 and Figure 3-3, the lesioned hemisphere is shown on the right. The region of interest of the S1 area corresponding to the dominant forepaw was seeded at baseline (Figure 3-2A), and the results from the resting state analysis showed levels of baseline connectivity within and between hemispheres. One week after stroke, less significantly correlated voxels were seen in the lesioned hemisphere (Figure 3-2B).

This reduction in correlation in rsMRI BOLD signal between the S1 and other brain regions is consistent with a proposed loss of interhemispheric connectivity due to loss of neurons with callosal axons (Carter et al., 2010). 4 weeks after stroke (Figure 3-2C), an increase in connectivity between hemispheres was observed compared to 1 week after stroke, although there was still a loss of signal and connectivity in the lesioned area. Finally, at week 12 (Figure 3-2D), similar levels of connectivity to baseline are seen bilaterally in S1 and S2 in the cortex and in subcortical structures such as the striatum. This could coincide with spontaneous recovery seen previously (Figure 2-8).

Statistical maps of functional connectivity of the less-dominant sensorimotor cortex with the rest of the brain clearly illustrated that the strong baseline interhemispheric functional connectivity between the dominant and less-dominant sensorimotor cortices was lost after stroke and recovered over time (Figure 3-3). Similar connectivity was observed to the lesioned hemisphere when the seed region was placed in the S1 region corresponding to the less-dominant forepaw. At baseline (Figure 3-3A), the seeded S1 region showed the baseline connectivity to and from the hemisphere corresponding to the less-dominant forepaw. 1 week after stroke (Figure 3-3B), a decrease in connectivity was seen to the lesioned areas (on the right side of the image), corresponding with the loss of connectivity *via* transcallosal axons, although the extent of this loss is less than seen in the seeded S1 regions of the lesioned hemisphere. By week 4 (Figure 3-3C), an increased correlation between S1 and local cortical and subcortical regions was observed. By 12 weeks after stroke (Figure 3-3D), there is evidence of interhemispheric

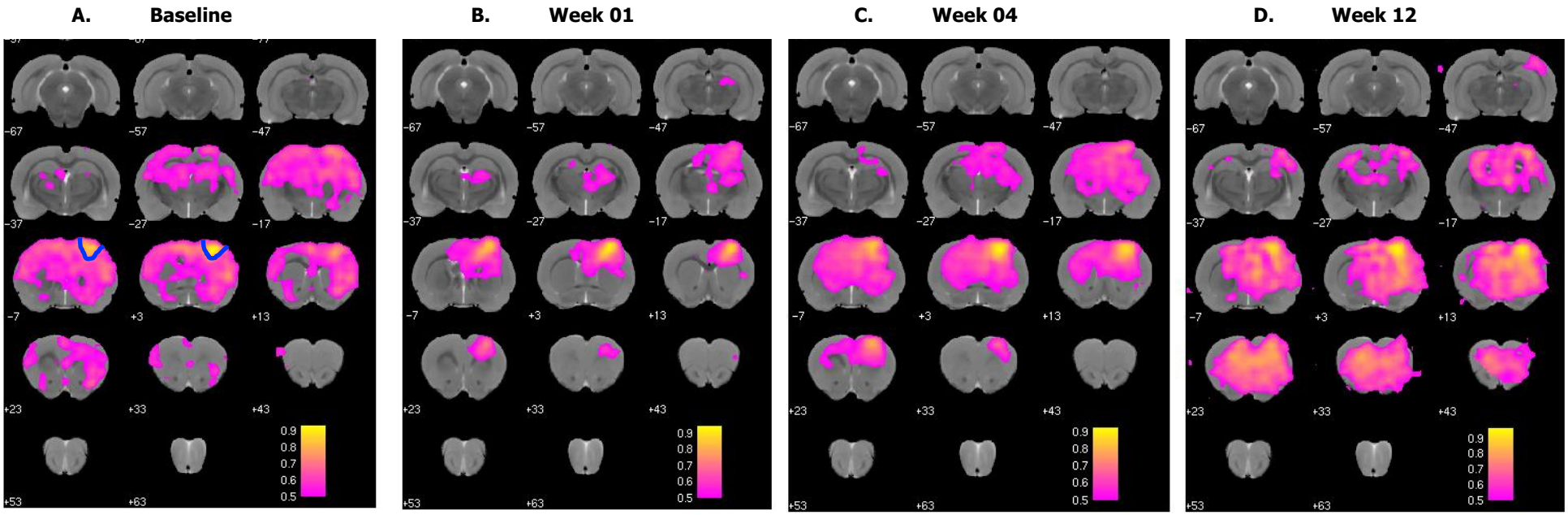


Figure 3-2 : Resting state MRI images show connectivity changes after stroke in the hemisphere corresponding to the dominant paw, which is subsequently lesioned after baseline.

Functional connectivity clusters in rat brain. Maps of functional connectivity clusters calculated by correlation analysis of signal timeseries from the dominant (lesioned) S1 region with all other voxels timeseries in stroke animals at baseline (A, n=10), 1 week (B, n=14), 4 weeks (C, n=17), and 12 weeks (D, n=8), after stroke respectively. Maps are overlaid on an anatomical T_2-weighted rat brain MRI template. The S1 seeded region in the dominant (lesioned) hemisphere is on the right (outlined in blue in A), and the connectivity with other regions is shown, thresholded at $r^2>0.5$. Numerical values represent the correlation coefficient, r^2, indicating the positive correlation between the seeded region and the rest of the brain. Note the distinct decrease in connectivity in the brain at 1 week after stroke compared with other timepoints.

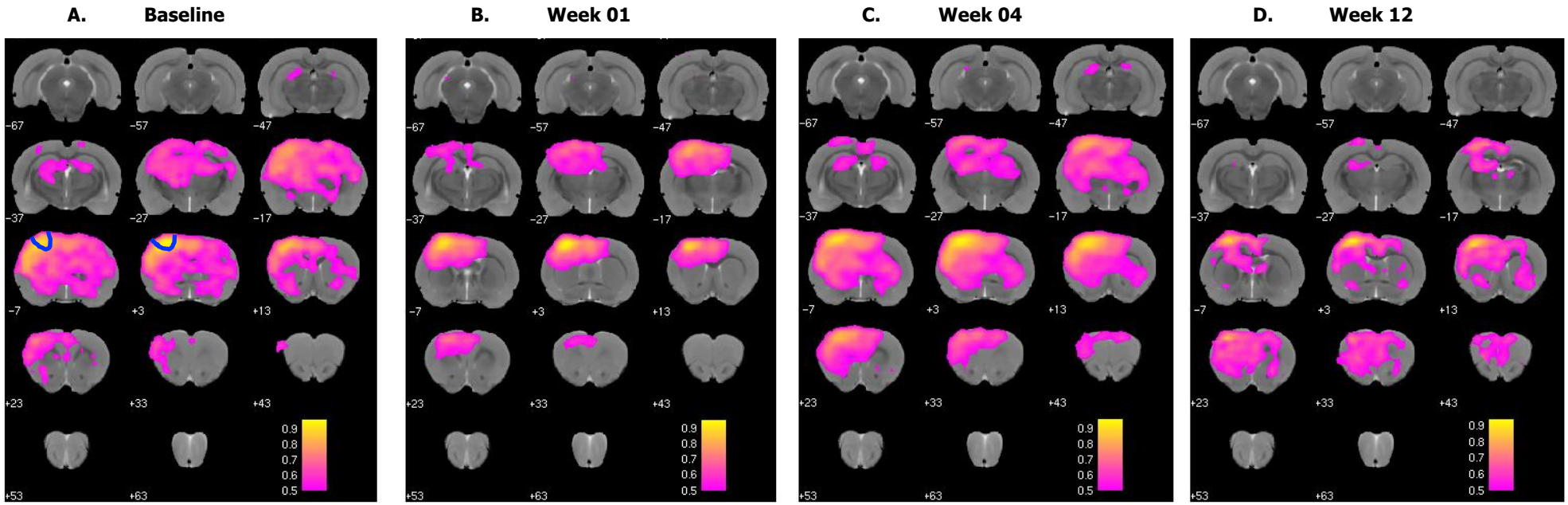


Figure 3-3 : Resting state MRI images show connectivity changes after stroke in the hemisphere corresponding to the less-dominant paw.

Functional connectivity clusters in rat brain. Maps of functional connectivity clusters calculated by correlation analysis of signal timeseries from the dominant (lesioned) S1 region with all other voxels timeseries at baseline (A, n=10), 1 week (B, n=14), 4 weeks (C, n=17) and 12 weeks (D, n=8), after stroke respectively. Maps are overlaid on an anatomical T_2-weighted rat brain MRI template. The S1 seeded region in the less-dominant (unlesioned) hemisphere is on the left (and is outlined in blue in A), and the connectivity with other regions is shown, thresholded at $r^2>0.5$. Numerical values represent the correlation coefficient, r^2, indicating the positive correlation between the seeded region and the rest of the brain. There is a distinct decrease in connectivity in the brain at 1 week after stroke compared with other timepoints.

A. Week 01 (Stroke – Sham normalised to baseline) **B. Week 04 (Stroke –Sham normalised to baseline)** **C. Week 12 (Stroke – Sham normalised to baseline)**

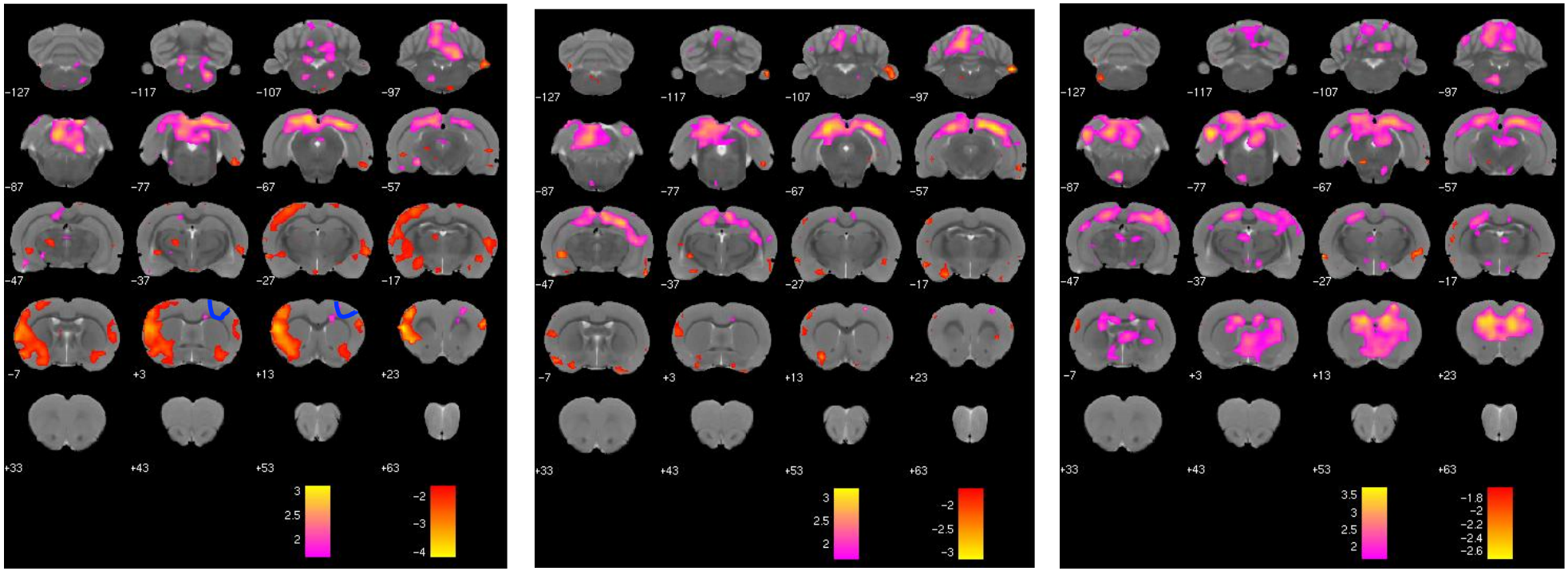


Figure 3-4 : Changes in connectivity in stroke animals from sham levels and compared with baseline when seeding the forelimb regions of S1 cortex region of the lesioned (previously dominant) forepaw

Increases (pink) and decreases (orange) in connectivity in stroke animals compared with sham animals at each timepoint are indicated; the contrast indicating (stroke animals at week X–baseline)–(sham at week X–baseline). This contrast is the mathematical subtractions of correlated voxels, expressed as T values. Areas in pink denote areas that show increased connectivity in the stroke group compared to sham, while orange areas show the decrease in connectivity in stroke animals compared to sham. The blue region shows the seeded S1FL. Baseline (stroke group=10, sham group n=9); Week 1 (stroke n=14, sham n=9); Week 4 (stroke n=14, sham n=10); Week 12 (stroke n=8, sham n=9).

A. Week 01 (Stroke – Sham normalised to baseline) **B. Week 04 (Stroke – Sham normalised to baseline)** **C. Week 12 (Stroke – Sham normalised to baseline)**

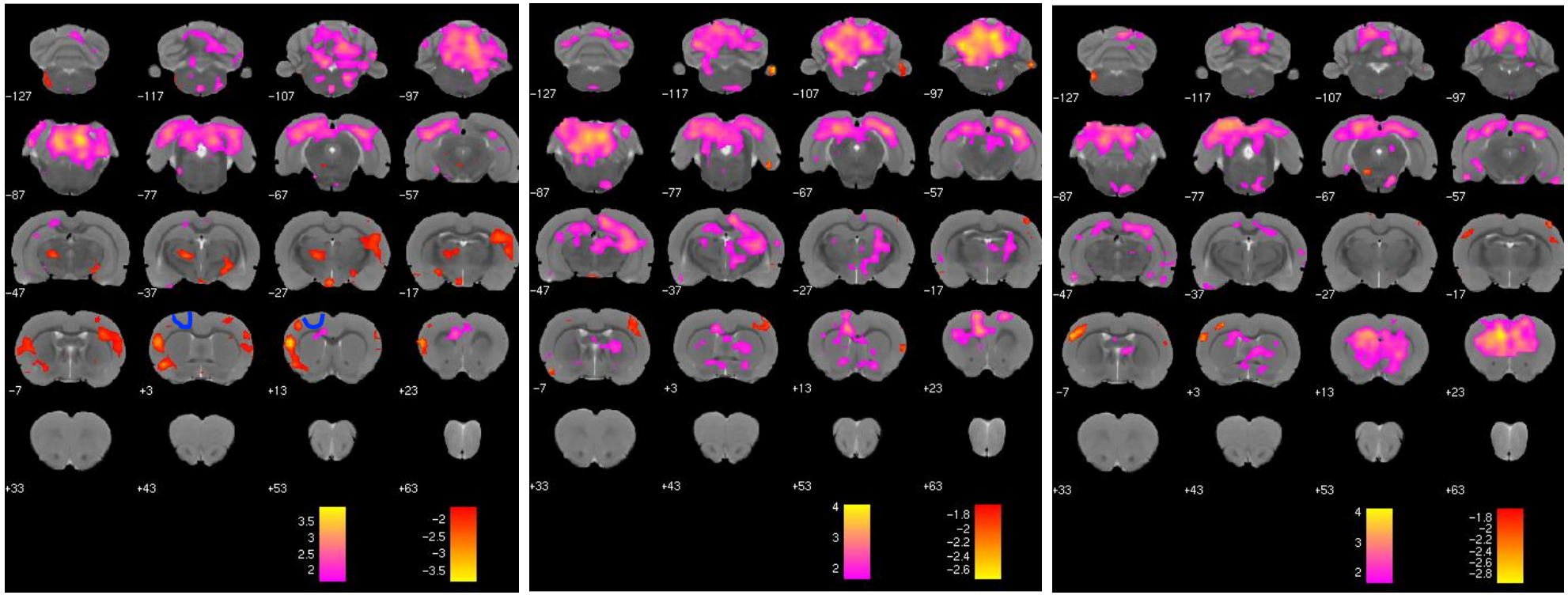


Figure 3-5 : Changes in connectivity in stroke animals from sham levels and compared with baseline connectivity when seeding the forelimb regions of the S1 cortex region of the less-dominant, unlesioned forepaw

Increases (pink) and decreases (orange) in connectivity in stroke animals compared to sham animals at each timepoint are indicated; the contrast indicating (stroke animals at week X–baseline)–(sham at week X–baseline). This contrast is the mathematical subtractions of correlated voxels, expressed as T values. Areas in pink denote areas that show more connectivity in the stroke group compared to sham, while orange areas show the decrease in connectivity in stroke animals compared to sham. The blue region shows the seeded S1FL. Baseline (stroke group=10, sham group n=9); Week 1 (stroke n=14, sham n=9); Week 4 (stroke n=14, sham n=10); Week 12 (stroke n=8, sham n=9).

connectivity because of increasing correlations observed between hemispheres, although not to the extent observed at baseline, and additionally, a reduction in connectivity is noted between week 4 and week 12, perhaps because of the lesioned hemisphere once again being the dominant paw. This coincides with the recovery seen in sensory behavioural tasks (Figure 2-8).

To identify the pattern of functional connectivity when animals recovered from stroke, areas where functional connectivity significantly changed were calculated by using the contrasts function in SPM to produce complex maps where voxels from group maps of sham animals was subtracted from group maps from stroke animals and displayed these as a probability map after comparing to baseline connectivity (Figure 3-4 and Figure 3-5). Using this type of contrast, it is possible to observe the regions of connectivity after that are lost after stroke surgery. When seeding the dominant forepaw as in Figure 3-2, a comparison at one week after stroke between stroke animals and sham (Figure 3-4A) showed a little increase in connectivity in stroke animals compared with sham in the hindbrain regions, corresponding to hindlimb and some cerebellar regions. Decreased connectivity was shown at this timepoint in the secondary somatosensory cortex of the less-dominant hemisphere (contralateral to the lesion). 4 weeks after stroke (Figure 3-4B), connectivity between stroke animals and sham shows little difference, with only a small increase in hindbrain connectivity observed. By 12 weeks (Figure 3-4C), increases in connectivity in stroke animals is observed compared to sham in the hindbrain, and some subcortical areas such as the bilateral striatum. By this timepoint in Chapter 2, these animals had shown spontaneous improvement in the behavioural tasks.

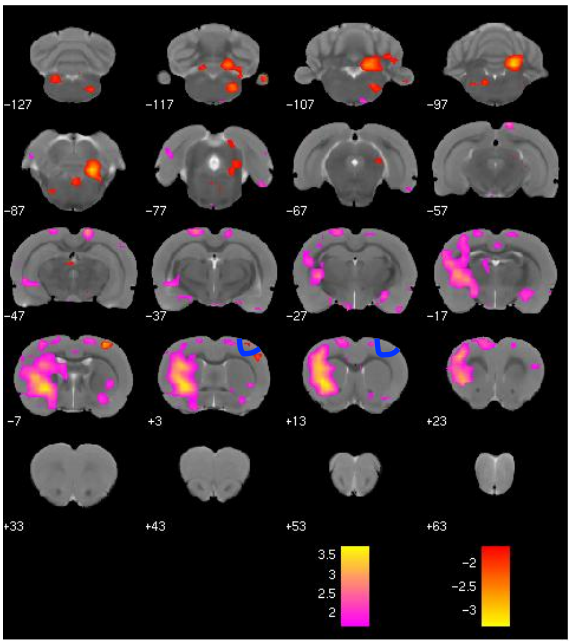
Comparing the seeded less-dominant S1FL region (as in Figure 3-3) in both stroke animals and sham at 1 week after stroke (Figure 3-5A) shows a little loss of connectivity in secondary somatosensory cortex in stroke animals compared to sham, although not to the same extent as observed in the dominant (lesioned) hemisphere. An increase in connectivity is seen in the hindbrain, particularly in the cerebellum and caudal hindlimb regions. By 4 weeks (Figure 3-5B), changes observed between stroke animals and sham are in the region of the cerebellum, with the loss in connectivity observed at 1 week having been ameliorated. Finally, at 12 weeks (Figure 3-5C), increased hindbrain (cerebellar) connectivity is still observed, with the addition of bilateral striatal connectivity, similar to the seeding of the lesioned hemisphere.

To further identify changed connectivity profiles after stroke, a final contrast was used to observe differences from the week 1 time point to identify regions that might contribute

to the functional recovery observed in the previous chapter (Figure 2-8). Compared with animals at 1 week after stroke, animals at the 4 week timepoint indicate a recruitment of the secondary somatosensory cortex contralateral to the lesion, as well as the dorsal and lateral striatum contralateral to the lesion, and the insular cortex when the S1 of the lesioned cortex is seeded (Figure 3-6A), although a very small decrease in ipsilateral ventral cerebellar connectivity is lost at week 4 compared with week 1 in these stroke animals, in addition to possible loss in the cuneate nucleus and medial longitudinal fasciculus, which both carry sensory and proprioceptive inputs. By week 12 (Figure 3-6B), seeding of the S1 of the lesioned cortex shows an increase in connectivity of primary somatosensory regions adjacent to the seeded region compared with week 1 in addition to increases in secondary somatosensory cortices bilaterally, which is most likely to produce the recovery seen. When doing the same analysis at 4 weeks after stroke with the seeding of the less-dominant S1 contralateral to the lesion (Figure 3-6C), the secondary somatosensory cortex ipsilateral to the seeded region (contralateral to the lesion) shows increased connectivity compared with the same animals at 4 weeks. By 12 weeks (Figure 3-6D), this increase in connectivity is not so well defined, although cerebellar regions show a decreased connectivity at this timepoint compared with animals 1 week after stroke.

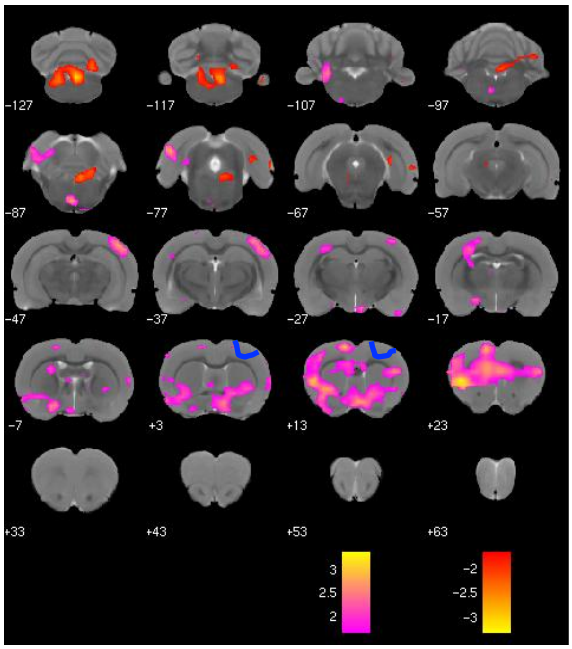
A. Week 04 -Week 01

(Dominant [lesioned] S1 seeded region)



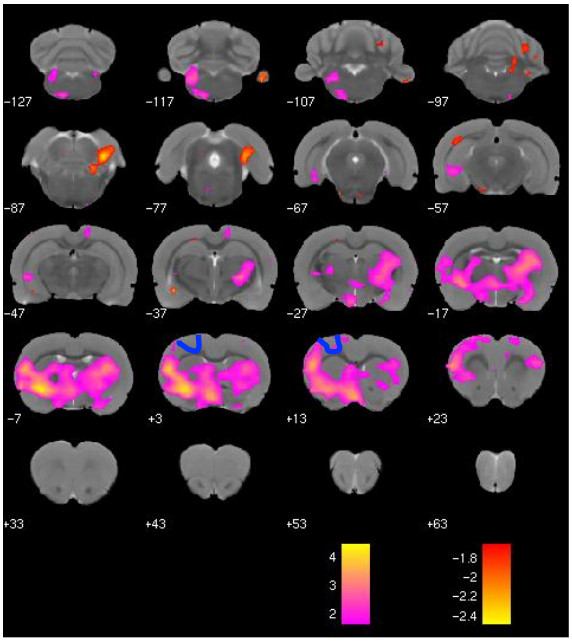
B. Week 12 - Week 01

(Dominant [lesioned] S1 seeded region)



C. Week 04 -Week 01

(Less-dominant S1 seeded region)



D. Week 12 - Week 01

(Less-dominant S1 seeded region)

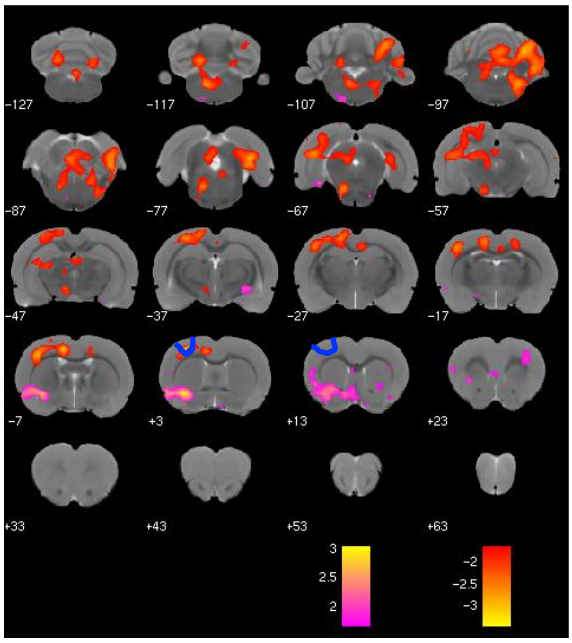


Figure 3-6 : Changes in network connectivity in stroke animals during spontaneous recovery

Changes in connectivity with resting state fMRI at week 4 (n=17) and week 12 (n=8) from week 1 (n=14). Increases (pink) or decreases (orange) in connections are observed during this phase of recovery, and recovery seen in behavioural tasks in Chapter 2 could be attributed to the changes in functional networks over the period of this experiment. The region in blue shows the seeded S1FL.

3.4.2 fMRI during forepaw stimulation indicates diminished functional activation after stroke, which does not recover over the 12 week study

Statistical parametric maps of the BOLD response are shown as heat maps in Figure 3-7. Red colour on these maps indicates an increased probability of activity, defined by a consistent increase in BOLD signal and therefore metabolism during stimulation. These overlays are indicative of increased neural activity. Yellow colour on the images indicates a decreased BOLD signal during stimulation, which could be related to a combination of factors including vascular steal, neural inhibition, or decreased blood flow. As stated above (3.3.1), statistical parametric maps for some timepoints were generated with varying rat numbers: baseline had n=36, week 1 had n=20, week 4 had n=18 and week 12 had n=16, which might have introduced error. Rats were previously tested for a paw preference (*via* the cylinder test) so that stroke lesions were always placed on the contralateral side to their preferred forelimb. Therefore the activation maps show activation in the less-dominant, dominant (*ie,* affected) or both hemispheres.

Before stroke surgery, animals underwent a baseline scan to ascertain a response in a healthy rat. During bilateral forepaw stimulation at baseline (Figure 3-7A), a clear symmetrical activation corresponding with a positive BOLD response is seen in the sensorimotor cortex of the rats (in red). In blue, a symmetrical negative BOLD response is seen primarily in some subcortical regions such as the sensory thalamus, premotor cortex, and striatum. When the dominant paw was stimulated (Figure 3-7B), a single red region corresponding to a positive BOLD response was seen, with little negative BOLD response identified, although there is some negative BOLD activity in the rostral hindlimb region of the sensorimotor cortex. Stimulation of the less-dominant paw at baseline (Figure 3-7C) resulted in unilateral activation of the contralateral hemisphere, and a negative BOLD activation in similar regions as observed when both paws were stimulated simultaneously, with areas such as the surrounding sensorimotor cortex, rostral hindlimb areas, and premotor cortex being affected. Therefore, stimulation of the dominant paw led to a more restricted pattern of changes in activation than stimulation of the less-dominant paw. 1 week after stroke, simultaneous stimulation of both forepaws (Figure 3-7D) showed a unilateral BOLD activation in the unlesioned hemisphere, but no response in the lesioned cortex, and a similar negative BOLD profile to that seen at baseline. This response of the dominant paw is further shown when the dominant (lesioned) paw is stimulated (Figure 3-7E); no BOLD activation is seen in

either hemisphere during stimulation of the affected paw. However, compared with baseline, there was an increase in negative BOLD activity involving the contralateral (unlesioned) secondary somatosensory cortex (but not in contralateral S1) and bilateral regions of the striatum. When the less-dominant paw corresponding with the unlesioned hemisphere was stimulated 1 week after stroke (Figure 3-7F), a positive BOLD activation is seen in a similar intensity and location to that observed at baseline; however, although there had previously been negative BOLD activity at baseline, this response was ablated 1 week after stroke. At 4 weeks, a similar response was seen in the response from stimulation of both paws simultaneously (Figure 3-7G); a single positive BOLD response was observed in the hemisphere corresponding to the less dominant paw, with no response seen in the lesioned sensorimotor cortex. The negative BOLD response had a similar activation profile, with bilateral areas of the striatum and secondary sensory areas being recruited into the response. Stimulation of the impaired paw 4 weeks after stroke (Figure 3-7H) again showed no positive BOLD activation response, although a large negative BOLD response was seen bilaterally in cortical and subcortical areas as previously described. Moreover, stimulation of the less-dominant forepaw at week 4 showed a unilateral rise in BOLD signal corresponding to increased activation (Figure 3-7I), while negative BOLD responses showed a small increase compared with those seen previously 1 week after stroke. By 12 weeks, the lesioned sensorimotor cortex still had not recovered the positive BOLD activation of the lesioned hemisphere in response to stimulation of the dominant paw, and less negative BOLD response was observed under all 3 conditions. During the simultaneous stimulation of both paws (Figure 3-7J), where again, only a unilateral positive BOLD response was seen, a smaller negative BOLD response was observed. Similarly, activation of the dominant paw (corresponding to the lesioned hemisphere) did not result in a positive BOLD response (Figure 3-7K), although the negative BOLD response seen at previous timepoints is reduced, and could correlate with recovery. Finally, the stimulation of the less-dominant forepaw resulted in a significant activation-induced response in the unlesioned sensorimotor cortex, with almost no negative BOLD response observed at this timepoint.

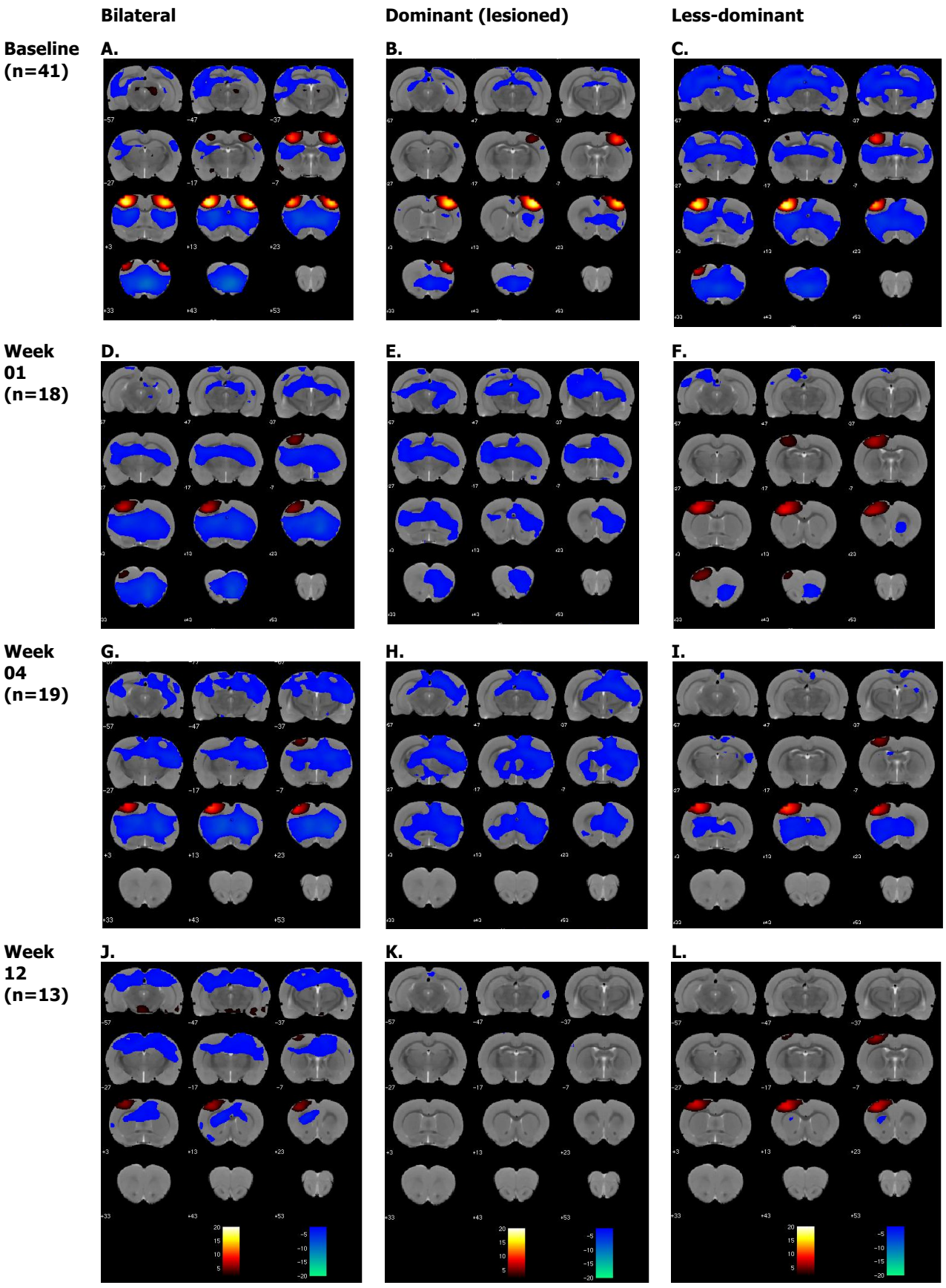

Bilateral
Dominant (lesioned)
Less-dominant
Baseline (n=41)
A.
B.
C.
Week 01 (n=18)
D.
E.
F.
Week 04 (n=19)
G.
H.
I.
Week 12 (n=13)
J.
K.
L.

Figure 3-7 : Forelimb stimulation fMRI indicates no change in activity in the less-affected hemisphere BOLD activation, while the lesioned hemisphere fails to recover functional connectivity

Increases (red) and decreases (blue) in BOLD during fMRI acquisition during bilateral forepaw stimulation, or the stimulation of the dominant (corresponding to lesioned) forepaw, or less-dominant paw are overlayed onto a high resolution structural template aligned with Bregma. These scans were performed at baseline (n=41; A-C), and at 1 (n=18; D-F), 4 (n=19; G-I), and 12 (n=13; J-L) weeks after stroke. Statistical maps use t values to indicate the probability of the BOLD activation occurrence between all rats used. Data are thresholded at p<0.001 (uncorrected for multiple comparisons).

3.4.3 ROI analysis reveals decreased activity during forepaw stimulation

To further elucidate the fMRI results an ROI analysis was done from the EPI time-series. MR signal was extracted from the forelimb regions of S1 (S1FL) ROI timeseries from all 900 volumes, from both hemispheres. Mean traces from ROI analysis of the S1FL region during forepaw stimulation are shown in Figure 3-8 (corresponding to the stimulation paradigm in Figure 3-1C) and Figure 3-9 (corresponding to the stimulation paradigm in Figure 3-1D). At baseline, a transient rise in BOLD signal was seen in S1 representing the dominant paw (orange trace) when the dominant paw was stimulated alone (brown vertical bars) or when both paws were stimulated simultaneously (blue vertical bars). Similarly, a transient rise in BOLD signal was seen in the S1 representing the less-dominant paw (purple trace) when the less-dominant paw was stimulated by itself (purple vertical bars) or when both paws were stimulated (green vertical bars). This is shown in the subset of animals that were stimulated with the less-affected paw first (Figure 3-8A); several animals did not pass the quality control for the same measure in the paradigm with the dominant paw stimulated first (Figure 3-9A; Table 3-1), resulting in fewer animals contributing to this graph, and a larger signal-to-noise ratio. One week after stroke, activity in the lesioned S1FL is lower during electrical activity than when stimulation is turned off, in contrast with the less-affected forepaw (Figure 3-8B, Figure 3-9B, quantified in Figure 3-8E and Figure 3-9E), which could possibly be due to local blood flow changes after injury. Another observation is that the signal appears to drop over the course of the 900 volumes of forepaw stimulation fMRI scan acquisition. There is some suggestion is that this could be related to diaschisis (Slater et al., 1977, Dobkin et al., 1989, Garbuzova-Davis et al., 2013, Garbuzova-Davis et al., 2016), although more research must be done to support this claim. A small improvement is observed by week 4, when there is reduced depression in activity during lesioned forepaw stimulation compared to 1 week after stroke (Figure 3-8C and Figure 3-9C, quantified in Figure 3-8E

and Figure 3-9E). Responses to forepaw stimulation at week 12 are unchanged compared to week 4 (Figure 3-8D and Figure 3-9D; quantified in Figure 3-8E and Figure 3-9E); no improvement is observed to correlate with the improvement observed with behavioural tasks in the previous chapter.

In the discussion, the possibility of compensatory activation of other areas will be discussed, and the role of these regions for the recovery of sensory function in this model of stroke observed previously.

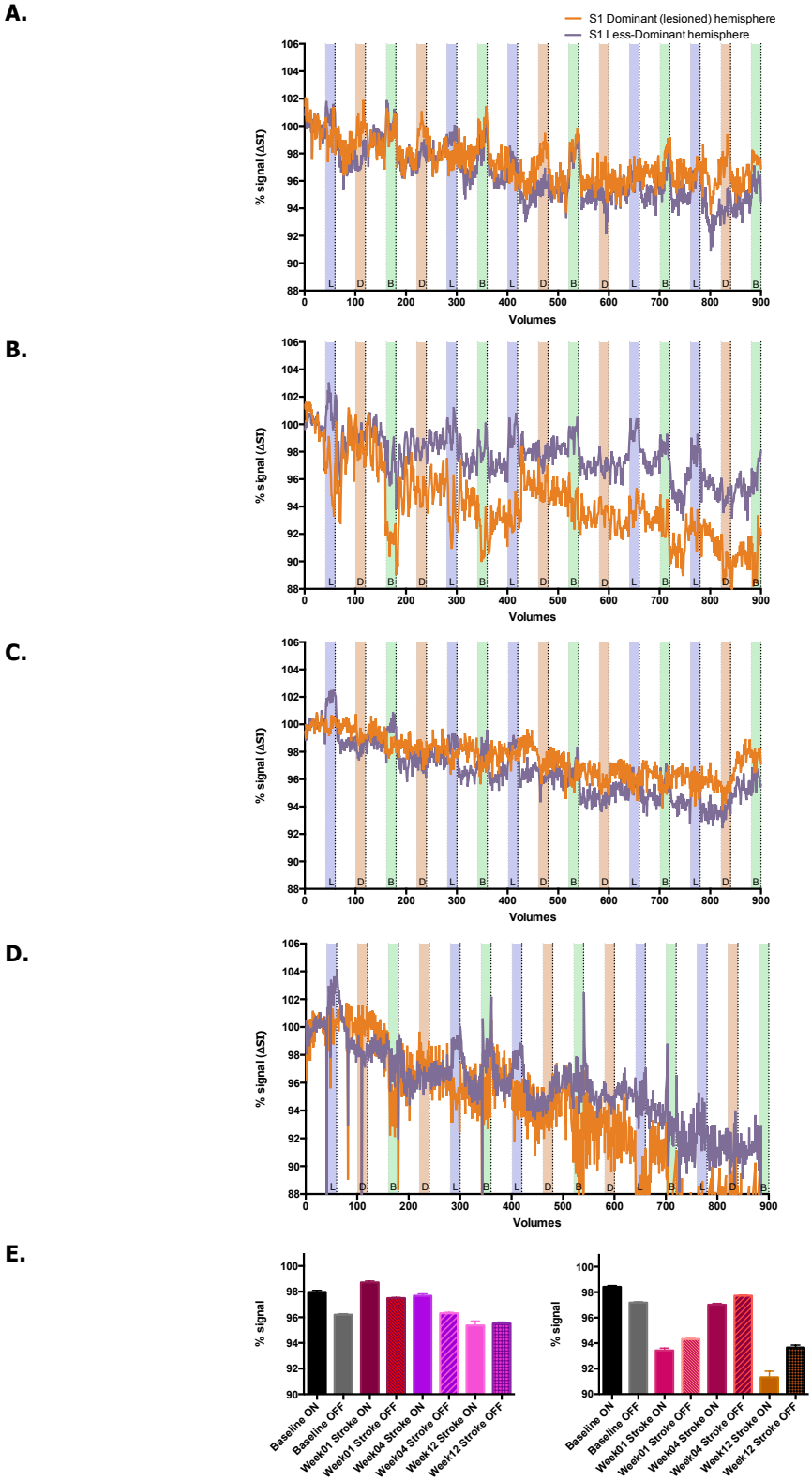


Figure 3-8 : Region-of-interest analysis indicates diminished activity of the dominant hemisphere during stimulation of the dominant forepaw in the paradigm beginning with the less-dominant forepaw stimulation

A Baseline (n=36); B week 1 (n=18); C week 4 (n=19); D week 12 (n=13). Areas shaded in A-D indicate times when the less-dominant (L, purple), dominant (D, orange) or both (B, green) paws were stimulated. This is quantified in E, where the graphs show the amount of signal in the brain when the stimulation is either on or off in the less dominant paw (left) or the dominant (lesioned) paw (right). Bars indicate mean signal, with ±SEMs for the error over the timecourse.

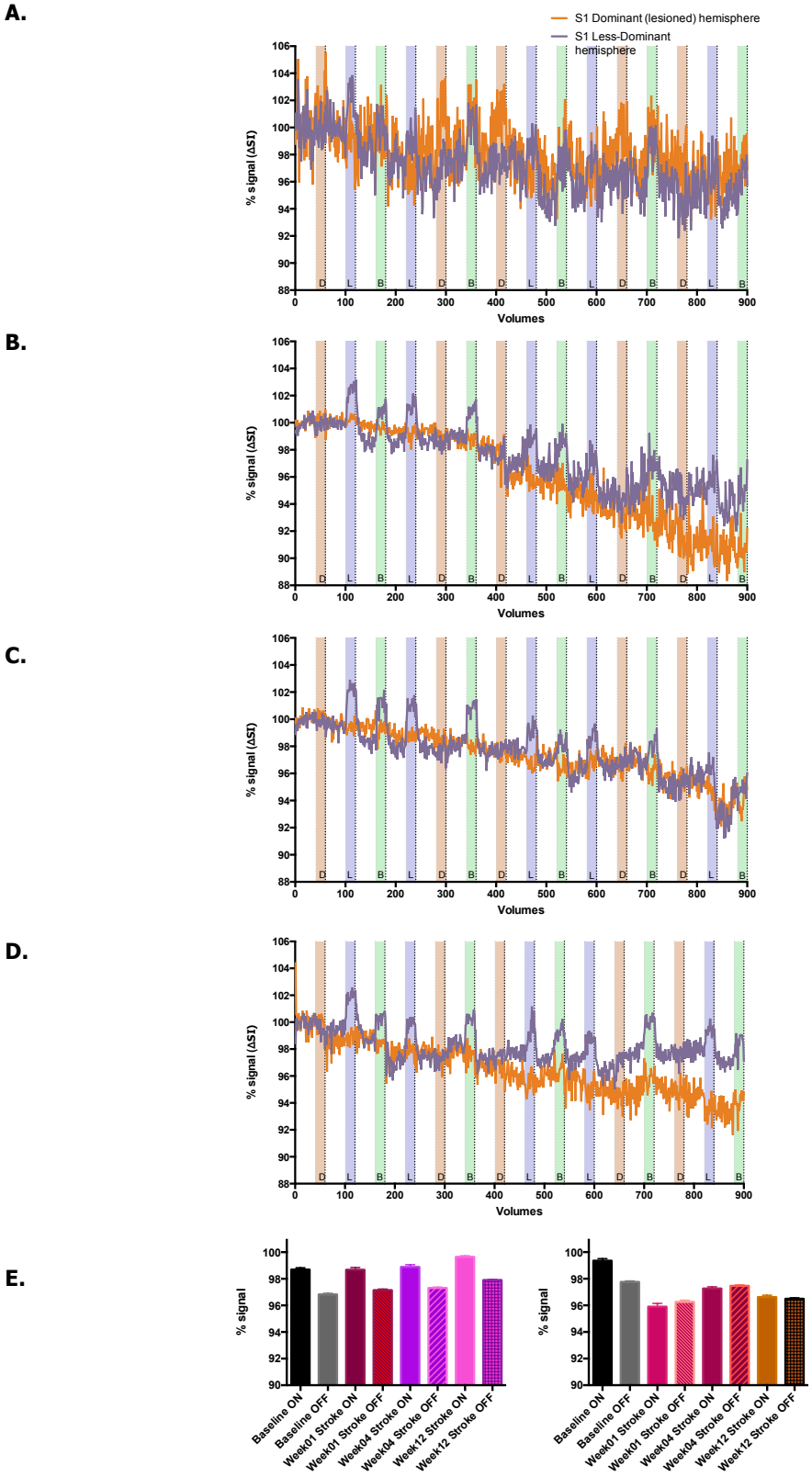


Figure 3-9 : Region-of-interest analysis indicates diminished activity of the dominant hemisphere during forepaw stimulation in the paradigm beginning with dominant forepaw stimulation

A Baseline (n=36); B week 1 (n=18); C week 4 (n=19); D week 12 (n=13). Areas shaded in A-D indicate times when the less-dominant (L, purple), dominant (D, orange) or both (B, green) paws were stimulated. This is quantified in E, where the graphs show the amount of signal in the brain when the stimulation is either on or off in the less dominant paw (left) or the dominant (lesioned) paw (right). Bars indicate mean signal, with ±SEMs for the error over the timecourse.

3.5 Discussion

In the results presented in this chapter, we have explored the use of different neuroimaging techniques to identify changes in brain connectivity and activity after stroke in the primary somatosensory cortex of young adult rats. Through the use of functional MRI, ROI analysis of the time-series during fMRI scans, and rsMRI, we have begun to identify some of the important changes during spontaneous recovery of functional improvements, such as those seen in the previous chapter.

3.5.1 Resting state MRI analysis reveals changes in connectivity over time after stroke, which could be important for behavioural recovery

The absence of increased contralateral somatosensory activity in stroke animals in this study compared with baseline is the most notable difference from previous studies (Dijkhuizen et al., 2001, Dijkhuizen et al., 2003, Baron et al., 2004, Winship and Murphy, 2008, Murphy and Corbett, 2009). The results presented here show that regions of the hindbrain, notably the cerebellum and the hindpaw regions, and bilateral areas of the striatum show different connectivity with the sensorimotor areas after stroke, eventually returning towards a pattern of connectivity observed at baseline over time. However, some, but not extensive, recovery of intracortical functional connectivity was seen by week 12 in Figure 3-2, in line with a previous study in rats; in large lesions intracortical functional connectivity remained significantly lowered up to 72 days after stroke (van Meer et al., 2012). This suggests that interhemispheric recruitment of the cortex contralateral to the lesion is not possible after larger stroke and the data presented here support the concept that recruitment of sensorimotor cortex contralateral to the lesion can only occur when the lesions are smaller and more of the neurons are spared. Additionally, this recruitment is not required for spontaneous recovery in behavioural tasks (as seen in chapter 2) but perhaps recruitment of contralateral counterparts could contribute to more functional recovery or perhaps might be a direct pathophysiological consequence due to disinhibition, thus the functional significance of enhanced activation in the unaffected hemisphere might be non-specific. Another region that may be recruited during spontaneous recovery is perilesional regions outside the seeded ROI that show increased connectivity to the lesioned somatosensory cortex over the weeks during recovery (Figure 3-6). Future analyses could include more ROIs, including regions around the lesion, for instance the secondary somatosensory and primary motor

cortices, including bilateral subcortical regions, such as the striatum, particularly substructures relating to the processing of sensory information.

The activity observed in the cerebellum in this study is of particular interest; previous studies have shown there are corticocerebellar loops involved in processing of sensory information (Proville et al., 2014); the coordination of movement (Manni and Petrosini, 2004); and learning (Doya, 2000). The bilateral and rostral signal seen on the rsMRI activation maps during recovery in stroke rats (Figure 3-4 and Figure 3-5) could therefore indicate that increased coactivation of these structures could indicate re-learning or compensatory behaviour. Adding strength to this argument is that the activation of the cerebellum appeared to occur in the 4th, 5th, and 6th cerebellar lobules, which connect to the motor cortex, and are also important for pain relay in sensorimotor areas (Diano et al., 2016), and connections between intrahemispheric motor regions (Bernard et al., 2012). The 6th lobule has been shown to be related to motor regions in human fMRI (Stoodley and Schmahmann, 2010, Stoodley et al., 2012).

3.5.2 Forelimb stimulation fMRI does not show any changes in activation profile in S1FL over time that could correlate with functional improvements observed in behavioural tasks

In this study, stimulation of the less-affected forepaw resulted in consistent, positive, BOLD activation in the corresponding S1FL, contralateral to the lesion, although stimulation of the affected forepaw resulted in loss of BOLD signal in the affected (lesioned) S1FL after the stroke lesion. Interestingly, this complete absence of positive BOLD activation responses after stimulation of the previously dominant (lesioned) paw contrasts with previous studies that have shown recruitment of areas contralateral to the lesion that are normally not involved with sensorimotor function of the forelimb such as barrel field, hindlimb, and visual regions of the somatosensory cortex (Dijkhuizen et al., 2001).

Similar to human studies, fMRI experiments in preclinical stroke models have reported diminished BOLD activation in the lesioned sensorimotor cortex (Reese et al., 2000, Abo et al., 2001, Dijkhuizen et al., 2001, Dijkhuizen et al., 2003, Weber et al., 2008). In longitudinal studies, animals have been shown to re-gain the activation of the peri-lesional somatosensory cortex, which corresponded to a restoration of electrical activity (Weber et al., 2008). In addition to this restoration of activity, enhanced activity is observed bilaterally in motor regions following stroke, which normally subsides and returns to an activation state that is more normal in the sensorimotor cortices (Abo et

al., 2001, Dijkhuizen et al., 2001, Dijkhuizen et al., 2003), resulting in substantial functional recovery; however, this pattern of activity was not observed in this study. More precise cortical mapping of sensorimotor response after focal sensorimotor strokes shows compensatory rewiring resulting in a shift of cortical sensorimotor responses to the peri-infarct area (Dijkhuizen et al., 2001, Dijkhuizen et al., 2003, Weber et al., 2008), and also redistribution of function of forelimb responses to other regions, including hindlimb regions (Winship and Murphy, 2008, Sigler et al., 2009), although these findings were not observed in this study. Intracortical stimulation of the sensorimotor cortex can also demonstrate CST plasticity and shifts in cortical representation following SCI. For instance in a study by Fouad et al. (2001), 4 weeks after a dorsal bilateral CST transection in the thoracic spinal cord, stimulation of the hindlimb cortex was found to evoke novel forelimb EMG responses which were associated with collateral sprouting of hindlimb CST fibres into cervical segments.

The bilateral negative BOLD activity in the caudate putamen (CPu) of the striatum at 1 and 4 weeks are intriguing and warrant further investigation. A previous study has shown that negative BOLD in healthy rats is seen in the striatum after noxious stimulation of the forepaw, and that this negative BOLD is also observed in rats following stroke (Shih et al., 2009, Shih et al., 2014), which is thought to be mediated by dopamine D2 receptors. In this study, the non-noxious electrical stimulation was applied to both paws in a pseudorandom order, and the stimulation must have activated the ipsilateral sensory neurons in the dorsal root ganglia. The projections of the spinal neurons directly or indirectly target central regions such as the thalamus (Willis and Westlund, 1997, Willis, 2007) and the substantia nigra (SN) (Nieoullon and Dusticier, 1982). The projections to the thalamus cross the midline whereas those to many other regions bifurcate as they ascend. The positive BOLD response observed in the intact S1FL is mainly attributed to increases neuronal activity and regional cerebral blood flow during forepaw stimulation. This mechanism is central to BOLD fMRI techniques and has been used to interpret most fMRI data; however, the negative BOLD response in the CPu is difficult to interpret, and indeed, is a source of debate in the neuroimaging community; particularly as some studies have suggested that a negative BOLD response arises from inhibition of blood flow (Northoff et al., 2007, Schäfer et al., 2012), although others suggest that inhibition through glycine and GABA release are at work (Shih et al., 2009, Shih et al., 2014), and also that vascular steal might occur (Hayes and Huxtable, 2012). The signal sources have different origins and the level of neurotransmission-induced changes might be lower, which could result in a large difference in the signal

amplitude between the positive BOLD response in the S1FL and the negative BOLD response observed in the CPu.

In another aspect, the effects of dopamine receptors in the cortex cannot be ignored since there is still a small amount of these receptors in the cortex (Chen et al., 2005, Choi et al., 2006). When dopaminergic neurotransmission occurs, a negative fMRI response might also be detected depending on the receptor distribution, as pharmacological inhibition are also thought to play a role in the negative BOLD through inhibition (Shih et al., 2009). Perhaps further research into dopaminergic transmission during stroke recovery is warranted. One explanation for negative fMRI observed here is the dominance of the paw; negative BOLD responses are observed at baseline in the less-dominant paw, but after stroke the negative BOLD is observed predominantly in the lesioned hemisphere. This occurs at the same time as paw preference in behavioural tasks transfers to the less-affected side.

From these data, paw stimulation fMRI could be able to inform more about regions of recovery if correlation analyses were done on S2 and bilateral striatal regions to find out more about the importance of these regions during recovery.

3.5.3 Other considerations

Non-human primate studies have characterised cortical map changes after focal ischaemic injury, which has greatly enhanced the knowledge of the effects of rehabilitation, axonal projections and infarct size in stroke research (Nudo, 2007, Cramer, 2008). A study by Nudo and Milliken (1996) used intracortical microstimulation to map forelimb movement representations in the primary motor cortex, before and a few months after a focal ischaemic lesion. They found that the infarcts caused a marked but transient deficit in use of the contralateral forelimb to the lesion, with a corresponding increase in the use of the ipsilateral forelimb for retrieval tasks. Functional reorganisation has been shown by a number of studies, which show substantial reorganisation within the motor cortex after a focal ischaemic stroke. An MCAO lesion placed in the forepaw representation region of the brain resulted in a larger forepaw representation area that expanded into regions formerly occupied by representations of the elbow and shoulder (Nudo et al., 1996a). Functional organisation in the undamaged motor cortex correlates with behavioural recovery of skilled hand function. These results suggest that after damage to the motor cortex, rehabilitative training shapes subsequent reorganisation in adjacent intact cortex and this could play an important role in motor recovery.

As opposed to the human studies that allow for deeper understanding at a systems level, animal studies using functional imaging after focal brain damage are able to show changes at the molecular and cellular level in addition to the systems level. Many of the techniques used in animal studies have also been used to gather an insight into how the human brain responds to focal injury and how researchers can manipulate this to enhance functional outcome (Baron et al., 2004).

3.5.3.1 The selection of anaesthesia method during fMRI

A point to consider when using fMRI in experimental stroke models, is that BOLD response relies on neurovascular coupling and cerebrovascular reactivity, both of which can be significantly affected by the cerebral ischaemia itself. Furthermore, anaesthetics also affect neurovascular coupling (Lindauer et al., 1993), and most fMRI studies in experimental animals are done under anaesthesia to minimise stress, movement, and stabilise physiology. The most common anaesthetics used for fMRI are isoflurane and alpha-chloralose. Several laboratories have used isoflurane but others have shown that brain activations with this anaesthetic result in smaller blood-oxygen-level-dependent (BOLD) signal, which could be related to the reduction of the neuronal activity or disruptions in vascular coupling. Isoflurane is a vasodilator and has direct effects on cerebral vascular smooth muscle (Jensen et al., 1992, Zhao et al., 2007) and also reduces neural activity in the cerebral cortex (Jansson et al., 2004, Duong, 2007). Conversely alpha-chloralose is an injectable anaesthetic that largely preserves the functional-metabolic (neurovascular) coupling, which in turn provides stronger fMRI BOLD activations (Ueki et al., 1992). However, alpha-chloralose has slow pharmacokinetics, which can produce metabolic acidosis and a reduction in body temperature, and the fact that it is used as a rodent pesticide has meant that this anaesthetic has mostly been used as a non-recoverable anaesthetic, and can be quite damaging to the animals.

One important point of concern is whether the anaesthetic might interfere with our behavioural results. We always made sure that the rats were scanned the day after their behavioural testing for that week and so they would have a full 7 days to recover from any effects of the alpha chloralose before behavioural testing was repeated. de Celis Alonso et al. (2011) found that rats need a minimum of 72 hours before behaviour returns to pre anaesthetic levels, therefore leaving our rats for one week should have fully allowed for this. Examination of a subset of imaged animals (under anaesthetic) showed no apparent difference in their behaviour compared to the rats that were not

imaged and anaesthetised (data not shown). We also showed that rats can survive (95% survival rate) three separate fMRI scans (of about 2 hours long) under alpha-chloralose and that the low dose regime used produces consistent and reproducible results with strong BOLD signals. The rats that died in this fMRI study did so in recovery. It is important to carefully monitor the rats after alpha-chloralose anaesthesia. The use of a heating blanket until the animals were conscious was important as we found that it was difficult for the rats to maintain their temperature, and administration of subcutaneous saline was also given for a faster recovery. Rats also exhibited difficulty in breathing which was the main symptom of the rats that died. In these cases of severe breathing difficulties 50 µl of Dopram (respiratory stimulant) was administered sub-lingually, and excess fluid removed by syringe. We will also consider using atropine in the future to minimise tracheal secretions.

3.5.3.2 Rat handedness might have distinctive characteristics using BOLD fMRI

Interestingly, we saw a difference between the activation patterns at baseline (pre-stroke) in the handedness of the rats: the hemisphere which represented the dominant paw was stronger in activation compared to the less dominant hemisphere (Figure 3-7). Additionally, the less-dominant paw had a negative BOLD profile that was not seen in the dominant paw, until after stroke, when the animals switched the preferential use of the paw to the less-dominant paw in behavioural tasks shown in the previous chapter.

This suggests that there could be a baseline difference in somatosensory activations in rats determined by their paw preference. A study by Volkov et al. (2011) showed increased functional recovery of rats following stroke when the stroke was given in their dominant hemisphere compared to their less dominant hemisphere. This is important as it highlights the importance of indicating paw preference before a study begins. This means one can make sure that it is the dominant hemisphere that is targeted in all cases to ensure functional recovery can be monitored without paw preference becoming a confounding variable. It also improves power to detect an effect.

3.5.4 Conclusions

In conclusion, this study has shown that permanent distal MCA occlusion with bilateral carotid occlusion results in large functional deficits, causing modification to the local

circuitry and recovering of functional ability in these animals. Using various neuroimaging techniques, we have shown the changes in connectivity in this model of stroke, and that regions of the brain such as the secondary somatosensory cortex, bilateral striatum, and the 4th to 6th lobules of the cerebellum are regions that could be facilitating this recovery that could be explored further in future studies. Potential methods to explore this further in the future will be discussed in Chapter 6.

Chapter 4 Delayed subcutaneous NT3 treatment provides a benefit in elderly female rats after MCAO with carotid occlusion, but the evidence is less clear in elderly male rats

4.1 Abstract

The three experiments presented in this chapter aimed to translate the three-vessel occlusion model of stroke into elderly rats. Age is the most important non-modifiable risk factor for stroke, and thus the ability to use an aged animal model is important for translating studies from the human condition for the purpose of trialling new, relevant treatments. In the first study, young and elderly animals were used in a pilot study to determine whether 60- or 90-minute occlusion was of the appropriate duration for transient common carotid occlusion. A 90-minute occlusion conferred no additional benefit over a 60-minute occlusions and resulted in more mortality, so the 60-minute occlusions were used for the rest of the studies. In the second experiment, an elderly female cohort underwent the three-vessel occlusion model of stroke; animals were treated at 24 hours either with subcutaneous NT3 or vehicle delivered by osmotic pump, for 4 weeks, and behavioural tasks were measured for 12 weeks. This experiment showed that the use of NT3 resulted in functional benefits in sensorimotor behavioural tasks, which was supported by histological assessment of crossed fibres from the intact corticospinal tract in the cervical spinal cord, which showed increased sprouting with NT3 treatment. Finally, an aged, male and female cohort of rats was used, since stroke affects both sexes, and after large cortical stroke, NT3 protein was infused subcutaneously for 4 weeks starting 24 hours after stroke. Recovery was assessed for 12 weeks using various sensorimotor tasks, resulting in no overall differences between groups. However, some functional recovery was observed by use of H-reflex electrophysiology, which indicated that NT3 ameliorated hyper-reflexia associated with stroke lesions.

4.2 Introduction

Age is the most important risk factor for stroke—1 in 5 women and 1 in 6 men will have had a stroke by the age of 75 (Seshadri et al., 2006), yet few preclinical studies include aged cohorts to test for stroke therapies (Markus et al., 2005). This is surprising as elderly stroke survivors are more likely to be disabled than younger survivors, with approximately half of elderly survivors possessing moderate to severe neurological deficits (Kelly-Hayes et al., 2003). This finding could also apply to animal models; in Chapter 2, an initial deficit was observed in young animals following MCAO stroke, but these deficits were not sustained and spontaneous recovery was observed in behavioural tasks. A preclinical study using an elderly cohort of animals could, therefore, produce more sustained deficits from which a benefit of NT3 can be measured.

Some studies have shown that following CNS injury younger animals recover faster and to a greater extent than elderly animals (Zhang et al., 2005); this is also reflected in the human condition (Hankey et al., 2002), although there are also studies that show no difference in lesion volume with varying age (Wasserman et al., 2008). However, there still is the capability to enhance neuroplasticity and functional outcome in aged animals and so understanding these mechanisms in aged cohorts might enhance translational therapies (Badan et al., 2003, Ward, 2005, Buga et al., 2008, Soleman et al., 2012). Some laboratories are now including cohorts of elderly animals into their stroke studies, however, this is limited because of the high price and high mortality rates associated with some aged stroke models (Futrell et al., 1991). The relative lack of testing in relevant models may be contributing to the widespread failure in translation of stroke therapies (Fisher and Ratan, 2003). Accordingly, the STAIR committee, which was formed in 1999, recommends that aged animals should be used in preclinical testing (Fisher and STAIR, 1999).

Sensorimotor impairment is a sign of ageing involving a decline in perception, locomotion and motor control in both rodents and humans (Fundin et al., 1997a, Bergman et al., 1999, Ulfhake et al., 2000, McGibbon and Krebs, 2004, Seidler et al., 2010). It was first thought that a decline in sensorimotor skill was due to a decrease in the number of sensory and spinal motor neurons, possibly due to age-related brain atrophy during normal aging (Lupien et al., 1998); however numbers of sensory or spinal motor neurons are not significantly decreased with age in rodents; with cell death being far less extensive than previously thought (Bergman and Ulfhake, 1998). However, peripheral innervation and the central termination of sensory axons are markedly

reduced (Kaisho et al., 1994, Fundin et al., 1997b, Bergman et al., 1999, Verdú et al., 2000). The main high affinity receptor for NT3 is TrkC, which is expressed throughout the CNS (Merlio et al., 1992) and it is now known that ageing results in significant decreases in the mRNA and protein levels of both TrkC and NT3, in spinal motor neurons (Johnson et al., 1996, 1999, Ming et al., 1999, Ulfhake et al., 2000). In the mature nervous system, neurotrophins have been suggested to play a role in the control of growth and retraction of terminal nerve fibres. Therefore, in ageing, a decrease in TrkC within sensory neurons or a failure of targets to synthesise NT3 could indicate a reduced capacity for these neurons to bind and transport NT3 (Cowen and Gavazzi, 1998, Bergman et al., 1999, Ming et al., 1999). Exogenous NT3 can protect conductance of large myelinated axons after injury (Munson et al., 1997) and, subsequently, a reduced level of NT3 in ageing could cause decreased conduction properties in the elderly. Interestingly, $p75^{NTR}$, which is associated with apoptotic mechanisms, does not decline in motoneurons with age or following injury (Johnson et al., 1999) and might suggest a changed role for $p75^{NTR}$ in aged motoneurons (Hess et al., 2007). Because of this decrease in NT3 concentrations during ageing, along with a downregulation of TrkC receptors in sensory and motor neurons, it is important to assess the role of NT3 in an aged model of stroke as the association between NT3 and its receptors may be altered.

Stroke and spinal cord injury reduce mobility and also often impair sensory and motor processing in the spinal cord (Dietz and Sinkjaer, 2007a, Thibaut et al., 2013). Signs of sensorimotor abnormalities include spasm, changes in muscle tone, involuntary movements such as clonus, and excessive reflexes—eg, the monosynaptic stretch reflex and increased polysynaptic flexor reflexes (Adams and Hicks, 2005). These can be caused by several mechanisms, which include improved activity and connectivity between proprioceptive muscle afferents and motor neurons (Kakinohana et al., 2012, Toda et al., 2014a), increased excitatory input from supraspinal pathways, and intrinsic changes in motor neurons (Murray et al., 2010). Current drug therapies only transiently treat these symptoms rather than the underlying causes (Thibaut et al., 2013).

4.2.1 Subcutaneous treatment with NT3 may improve recovery after stroke

In chapter 2, AAV-NT3 was used for intramuscular delivery of NT3 directly to the muscle. However, the nature of AAV transgene delivery means that translated protein levels increased steadily until 5 to 7 weeks after injection, and can be maintained for up to

6 months (Herzog et al., 1997). Although this can be an attractive therapy for the long-term treatments, a desirable component for maximising stroke therapy is the immediate availability of treatment, and in our case, NT3. Two human clinical trials have shown that systemic, high doses of recombinant NT3 are well-tolerated, which make the delivery of recombinant NT3 an attractive treatment option (Parkman et al., 2003, Sahenk, 2007). Subcutaneous delivery of recombinant NT3 (synthesised from *E Coli*) will be used in this chapter as it may have translational relevance. High subcutaneous doses of recombinant NT3 have been shown to be safe and well tolerated in Phase 1 and 2 clinical trials for other disorders (Chaudhry et al., 2000, Coulie et al., 2000, Parkman et al., 2003, Sahenk, 2007). Peripherally administered neurotrophins have also been shown to accumulate in sensory ganglia where gene transcription, axon growth of proprioceptive afferents, and synapse strengthening within locomotor circuits has been shown (DiStefano et al., 1992, Yan et al., 1993, Taylor et al., 2001, Chen et al., 2002a, Patel et al., 2003, Lee et al., 2012b, Wang et al., 2012).

Route of administration is important; the use of recombinant NT3 *via* a clinically-relevant method will result in more patients maintaining a regimen if either a simple method of administration (oral, patch etc), or a method that can be administered by a health professional is used while the patient is in hospital following a stoke (intravenous infusion). Osmotic pumps could become a clinically-relevant route of administration as the pumps could be inserted while in hospital and treatment can be on-going for the duration that the pump lasts. A study for endostatin delivery *via* osmotic pump as an antiangiogenic therapy for tumours was marked as a potential therapy for clinical trials, but it did not move forward past early stage trials (Kisker et al., 2001). In previous studies in our lab, intramuscular infusions of NT3 were used after small, focal cortical strokes produced by the vasoconstrictor endothelin-1 (Duricki et al., For submission), and produced detectable improvements in behavioural tasks, and increased sprouting of corticospinal tract axons from the intact to impaired side of the spinal cord. However, in that study, some of the intramuscular cannulae became dislodged. When doing a retrospective analysis on these data, it was found that animals receiving NT3 at least partly *subcutaneously* were still receiving the benefits of treatment with NT3 compared with animals that completed the full intended intramuscular dose, although it is unknown at which stage of treatment that these cannula became dislodged. As a result of this study, treatment by subcutaneous infusion could be a viable therapy for treatment of stroke animals with NT3, possibly conferring more widespread benefits than *via* intramuscular delivery.

Others have shown that AAV-NT3 gene transfer into muscle tissue (as in Chapter 2) results in NT3 secretion into circulation reaching therapeutic blood levels, sufficient to provide functional, histopathological, and electrophysiological improvements in peripheral nerves in animal models of Charcot-Marie-Tooth neuropathy (Sahenk et al., 2005). It has been shown previously that radiolabelled human NT3 rapidly crosses the intact blood–brain barrier and accumulates stably in the cortex, striatum, brainstem, cerebellum, and sciatic nerve (and other regions of the nervous system) when administered to the brachial vein (Poduslo and Curran, 1996). Because the brachial vein is partly supplied by capillary beds from the triceps brachii, it is possible that subcutaneous recombinant NT3 infusion into these muscles leads to accumulation of NT3 in the brain and spinal cord after stroke (especially as the blood–brain barrier is compromised after stroke). This suggests that a peripheral route of treatment using NT3 might be therapeutically viable. NT3 does not need to act as a neuroprotective agent to produce its beneficial effects (Zhou and Shine, 2003, Chen et al., 2006, Duricki et al., 2016b); therefore it is currently unknown whether NT3 needs to enter the CNS to produce its effects, or if acting in the PNS and retrograde transport is enough to give improvements. Recombinant NT3 is reasonably stable in the bloodstream and is delivered quickly from blood into the cervical spinal cord (relative to other regions such as the brain and thoracic or lumbar spinal cord) (Pan et al., 1998). If NT3 is being delivered into the bloodstream, a less-targeted therapy could produce an effective treatment strategy for after stroke.

More than 90% of strokes occur in people over 65 years old (Truelsen et al., 2006), and many of these will have comorbidities including carotid arterial stenosis, which led to our use of the model involving distal middle cerebral artery occlusion with common carotid occlusion (Wayman et al., 2016) to evaluate subcutaneous administration of delayed recombinant NT3.

This chapter will discuss the use of the MCAO with bilateral common carotid artery occlusion model of stroke in elderly rats, and will assess recovery with subcutaneous recombinant NT3 infused starting at 24 hours for 4 weeks. Behavioural, histological, and electrophysiological outcomes will be measured to determine functional benefit.

4.3 Methods and Materials

4.3.1 Experimental design

All procedures, treatment, behavioural testing, and analyses were done using a randomised block design (Altman and Bland, 1999b), and procedures remained blinded until the end of the study. All procedures were done in accordance with UK Home Office guidelines and Animals (Scientific Procedures) Act of 1986.

The three-vessel MCAO model was used in the three experiments presented in this chapter. In the first experiment, a comparison was made between the use of varying occlusion times: 60 minute (n=8) or 90 minute occlusion (n=12) for the creation of a large lesion volume in elderly female rats compared with 90 minute occlusions (n=10) in young female adult rats, without an associated increase in mortality. Schematics for the model (Figure 4-1A) and the experimental design (Figure 4-1C) are shown. The lesion was applied to the hemisphere corresponding to the dominant forepaw, as measured by overall handedness behavioural tasks (section 4.3.7). At 24 hours after stroke, a structural MRI scan was obtained for each rat to determine the volume of the lesion. Behaviour was done at baseline and weekly following lesions as noted previously (section 2.3.6) and structural and resting state (rsMRI) was done at 1 and 8 weeks after stroke (data not shown). Eight animals died or were humanely killed within 24 hours of stroke surgeries (Figure 4-1). Animals were humanely killed if they made choking movements or rales, since our previous experience showed that it was unlikely that animals would be able to make a recovery with these symptoms.

	90 minute occlusion young animals (n=10)	90 minute occlusion elderly animals (n=10)	60 minute occlusion elderly animals (n=10)
Animals undergoing surgery	10	12*	8*
Successful surgery	7	9	6[#]
Animals in 24 hour structural MRI	7	8	5
Animals in 1 week structural MRI	7	8	6
Animals in 12 week structural MRI	4	6	5

Table 4-1 : Animals included in lesion volume analysis from Experiment 1

**Animals from the group with 90-minute occlusion in elderly animals died in the first few surgeries, so the decision was made to take over 2 animals from the 60-minute occlusion group to make up the numbers. At the same time, we were able to refine our technique and were able to have a much better survival rate following changes to saline flow and the use of atropine, which we used as a matter of course in future studies. [#]*Data from these animals were published in (Wayman et al., 2016).*

In the second experiment, out of 53 female animals used in this study, sham animals (n=10) underwent procedures up to, but not including craniotomy. Of the animals with stroke, 20 died (47%). 24 hours after stroke, structural MRI was performed for analysis of lesion volume and rats were randomised to treatment. An osmotic pump containing NT3 protein (n=13) or vehicle (n=10) was surgically inserted at 24 hours for 4-week delivery of treatment or vehicle (Figure 4-1D). Behavioural tests were done at baseline and weekly after stroke lesion for 12 weeks. At 8 weeks, animals were cortically injected with BDA for corticospinal tract tracing, and tissues were collected after the final behavioural test at week 12.

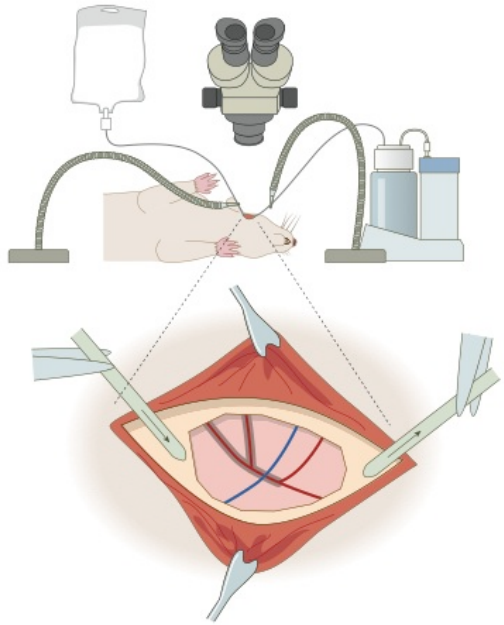
	Elderly, NT3-treated stroke animals (n=25)	Elderly, vehicle-treated stroke animals (n=25)	Elderly, sham (n=10)
Animals humanely killed before surgery[#]	3	4	0
Animals underwent surgery	22	21	10
Successful surgery	13	10	10
Animals included in behavioural tasks up until week 12	13	10	10
Animals in histological tracing data	13	10	0

Table 4-2 : Outcomes of animals in Experiment 2 and numbers of animals included in behavioural and histological analyses

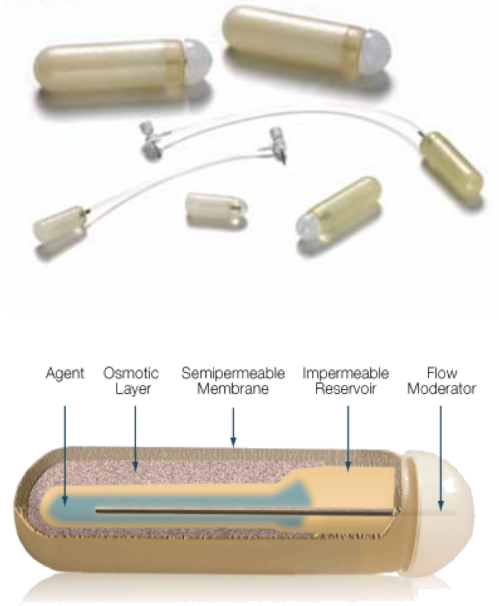
7 animals were humanely killed before surgery because of pituitary tumours (2), which are untreatable, and benign subcutaneous tumours at the request of our Home Office inspector (5), which we have found quite common in elderly female rats. We have since discussed this with our Home Office inspector, and have added onto our project licence, stating that we can remove these types of benign tumours if they are located in a region of the body suitable for surgical removal.

In the final experiment of this chapter, 50 female and 50 male elderly rats underwent sham (n=10 for both sexes) or stroke surgery. One of the female sham rats was humanely killed after discovering a tumour related to old age. Of the remaining 32 female rats, 11 (34%) rats died from stroke surgery. At 24 hours, animals were assigned to NT3 (n=10) or vehicle (n=11) treatment groups. Of the 44 male rats, 10 animals were assigned to sham, and of the remaining 34, 16 (47%) died during stroke surgery. The surviving animals were assigned to either NT3 (n=9) or vehicle (n=9) treatment groups at 24 hours. Animals underwent structural MRI for lesion volume, and behavioural assessment as described previously. Additionally, blood was taken from animals at 4 weeks for ELISA and electrophysiology done at the end of the study.

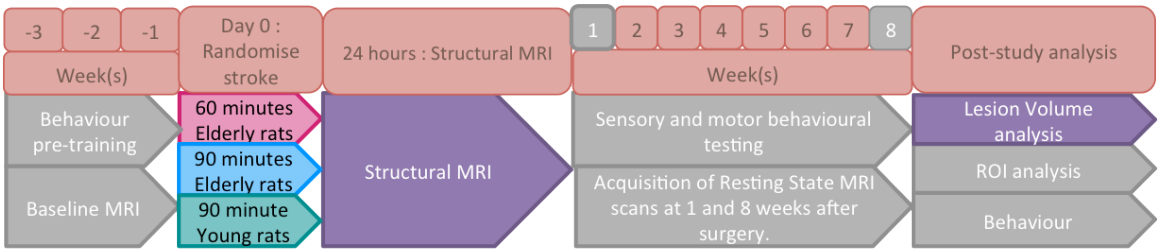
A.



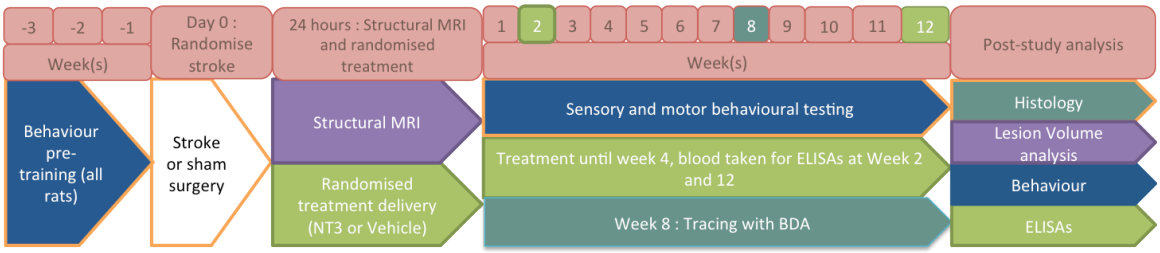
B.



C. Experiment 1, involving young and elderly female rats



D. Experiment 2, involving elderly female rats



E. Experiment 3, involving elderly male and female rats

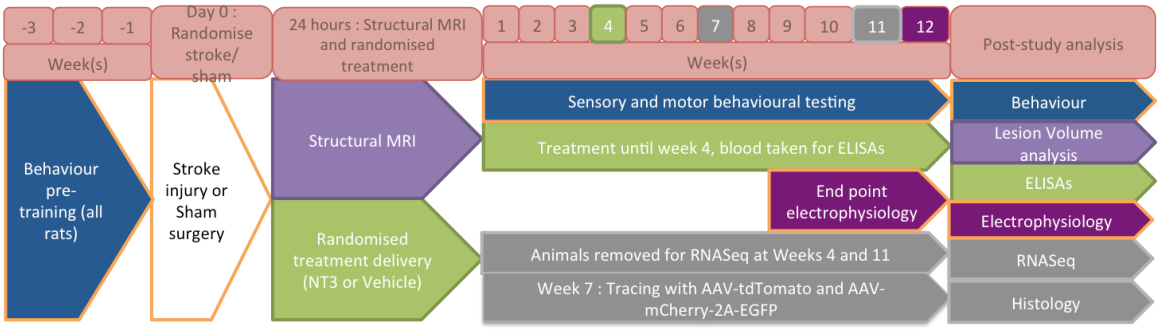


Figure 4-1 : Study design using subcutaneous osmotic pumps delivering NT3 protein in elderly rats.

A) Schematic with addition of saline infusion to improve the outcomes after surgery. In B, Alzet osmotic pumps were used for the delivery of NT3 or vehicle to animals over the course of 2 weeks. In C-E, design of the 3 experiments has been shown, where greyed portions of the flowcharts indicate data that has not been presented in this thesis. In the first experiment (C), elderly female animals were given stroke for varying time periods, as illustrated in Table 4-2 and 4-3, and structural MRI used to measure lesion volumes. In the second experiment (D) elderly female rats were lesioned and randomly assigned to stroke or sham groups. 24 hours after stroke, animals had structural MRI and surgical implantation of osmotic mumps containing NT3 or vehicle for 4 weeks, and blood taken at 2 and 12 weeks for ELISA. At week 8, animals were traced with BDA for histological assessment, and behavioural testing occurred weekly over the 12 weeks of the study. Finally in (E), animals underwent stroke surgery, behavioural assessment, and treatment as in D., with blood being taken at 4 weeks for ELISA. End-point electrophysiology was done at 12 weeks after stroke surgery.

At the end of all experiments, animals were euthanised and dissected for tissues. All surgeries and treatments were done using a randomised block design and the experimenter was fully blinded to allocation of groups until the end of analysis.

Mortality following stroke surgery was high in this study. We found one pitfall in surgery, particularly with elderly rats, was accidental interference with the vagus nerve, which resulted in gasping behaviour during recovery from surgery.

	Elderly, female stroke animals (n=35)	Elderly, male stroke animals (n=35)	Elderly, sham (n=10 male n=10 female)
Animals humanely killed before surgery	3	1	1 [female]
Animals underwent surgery	32	34	19
Successful surgery	21	16*	19
Assigned to NT3 group (GFP)	10 (11)	8 (8)	NA
Animals remaining in NT3 behaviour group after removing animals for RNASeq at week 4 (GFP)	6 (8)	6 (6)	14 (6 female, 8 male)
Animals humanely killed over the course of the study[#]	1 (1)	2 (2)	1 (male)
Animals in NT3 group for electrophysiology after week 11 behaviour (GFP)	3 (4)	2 (2)	8 (4 female, 4 male)
Animals remaining in NT3 group for week 12 behaviour	2 (3)	2 (2)	5 (2 female, 3 male)

Table 4-3: Numbers of animals included in Experiment 3, with numbers of animals in each analysis

** in this first experiment with elderly male animals, we found that 60 minute occlusions were resulting in high mortality rates in male animals. After 2 days of surgery, we shortened the occlusion time to 45 minutes to improve survival. [#]animals were humanely culled between weeks 4 and 11.*

It was noted that Experiments 1 and 2 in this chapter used long occlusion times, which were reduced in Experiment 3 (Figure 4-1E). A different strategy in future experiments would be to begin with lower occlusion times and to gradually increase times, if necessary, in the stroke cohort (Wayman et al., 2016). Humane endpoints in this study included weight loss of more than 30% (nb, elderly rats are heavy before surgery) despite efforts to combat dehydration with regular saline injections), any barrel rolling, problems with breathing that exceeded what is normal after stroke (eg, rasping), reduced responses to stimuli beyond what is considered normal after stroke in combination with piloerection, and inability to maintain core temperature.

Animals were removed from the study at weeks 4 and 11 for RNASeq and electrophysiology, respectively. As remaining animals were continuing with behavioural tasks, it was important to maintain a range of abilities in the study, to avoid any artificial changes caused by reducing the power of the study. To do this, animals were stratified according to gender, and how successfully rats were doing each task (categories; consistently achieving well in all tasks, reaching criteria [75%] in one or two tasks, almost reaching criteria in one task, unmotivated, and others). Animals were graded from the first 4 weeks of behaviour without unblinding, and were subsequently allocated for removal taking into consideration an equal balance of rats from all the stratified criteria by a second party who was otherwise not involved in the study until the electrophysiological recordings in weeks 10 and 11 (Dr C Kathe).

4.3.2 Animal subjects

In this chapter, 115 elderly female, 44 elderly male, and 10 young adult outbred Lister Hooded rats were used in three different experiments; the first comparing young and elderly female rats in a pilot experiment, the second performing the three-vessel occlusion model in elderly female rats, and the third experiment using the three-vessel occlusion model in elderly male and elderly female rats. Young rats were approximately 6 weeks old and weighed between 150–200 g, elderly rats were between 18–20 months old with weights ranging from 250–350 g in female and 500–650 g in male elderly animals. These were split into the groups mentioned previously (Tables 4-1 to 4-3).

Occlusion times in experiment 3 were reduced in male rats, potentially producing a confound between gender and time. 60-minute occlusion in male rats resulted in higher mortality at the beginning of the experiment, and as a result, the transient occlusion

time of the common carotid artery contralateral to the lesion was a pragmatic attempt to make lesion volumes comparable between males and females while reducing mortality.

These rats were obtained from Charles River, UK. Elderly rats were kept in-house until approximately 18–20 months old before commencing the study. Animals were housed in groups of 4 or 5 on a 12:12 light-dark cycle and had *ad libitum* access to water. Subjects were maintained on a restricted food regimen in which each animal had access to a reduced diet of approximately 20 g per rat per day (normal daily consumption ranges from 18 to 25g), as *ad libitum* access to food is a debateable model for obesity (Martin et al., 2010), and it was suspected that particularly the male animals would grow too large for *in vivo* MRI scanning. Rats were familiarised with veterinary recovery gel and soft chow for at least 48 hours prior to stroke surgery. As animals can find difficulty with eating and drinking after the procedure, animals were introduced to these items before surgery, to minimise neophobia. Additionally, animals were given prophylactic antibiotics, which was recommended by our Named Veterinary Surgeon given the tendency of wounds from Lister Hooded rats to become infected combined with concerns about respiratory infections after stroke. We gave Baytril 2.25% solution (enrofloxacin, 0.2 mL/kg, once every 2 days) in the 2 days before surgery, and one dose alongside pain relief, and then again as required.

4.3.3 Surgical procedures

4.3.3.1 Stroke Surgery

Lesions were induced on the hemisphere contralateral to the preferred paw, identified by the average pre-operative preference in the Montoya staircase test, bilateral tactile extinction test, and horizontal ladder tasks (See Section 4.3.7).

Rats were anaesthetised using 5% isoflurane in 1.5 L/min O_2. Following induction of anaesthesia, the level of isoflurane was reduced and maintained at an adequate but minimised depth for surgery (eg, 1–2%). Lidocaine cream was applied topically to the ventral neck and temporal regions 10 minutes before the first incision, and pain relief (Carprieve, 0.25 mg/kg, *s.cut*) given peri-operatively for pain relief. Animals were maintained at a temperature of 36.5–37.5 °C throughout surgery with the use of a rectal probe and homeothermic system.

Stroke rats were placed in the supine position and a ventral midline cervical incision was made to allow isolation of both common carotid arteries using a silk suture (5/0, non-

absorbable sutures), taking care not to damage the muscle or the vagus nerves. Animals were then placed in a lateral position and the MCA exposed and occluded as described in Section 2.3.3.1, while a constant infusion of saline (flow rate approximately 2 mL/min) and aspirator was set up over the craniotomy site to remove debris and clear minor bleeding during this stage of the surgery (Figure 4-1A). The rat was turned back to a supine position and the carotid artery ligated on the same side as the occluded MCA permanently by tying a knot in the silk suture around the carotid, while the left carotid artery was occluded transiently for 90, 60, or 45 minutes (Figure 4-1, 4-3) using a 13 mm stainless steel artery clip with 125 g pressure.

For sham animals, the ventral part of the neck and temporal regions were incised but no artery occlusion was used. Sham animals had procedures up to but not including craniotomy in this protocol, as this procedure can produce behavioural deficits (Adams et al., 1994).

4.3.3.2 Post-surgical procedures

After surgery, animals were placed in a 31 °C incubator for up to 4 hours to recover from anaesthesia. Fluids were given daily for the first 3 days to prevent dehydration. Carprieve (0.25 mg/kg) was given for pain relief at 24 hours. Because all animals receive the same dose of Carprieve, this factor is controlled for systematically. Any neuroprotective effect of Carprieve is likely to be negligible. Soft food and rehydration gels were made available to the animals in a cage lined with an absorbable pad rather than loose bedding for the first 24 hours.

A temporary weight loss of up to 20% was permitted compared with baseline and in some cases 30% was permitted in consultation with our NACWO and/or Named Veterinary Surgeon in accordance with our approved Home Office Project Licence protocol. In all cases of weight loss, we undertook special measures to care for our rats. For example, postoperative care to prevent and counter dehydration is described below.

4.3.4 Confirmation of infarct using structural MRI.

24 hours after the induction of MCAO, rats were anaesthetised with isoflurane (5% for induction, 1–1.5% for maintenance) in 0.9 L/min Medical Air and 1 L/min O_2, secured in a quadrature birdcage magnetic resonance coil (43 mm diameter) and placed in a 7T horizontal bore scanner. T2 weighted scans were obtained using a fast-spin echo

sequence: echo time (TE) 60 ms, repetition time (TR) 4000 ms, field of view (FOV) 40 x 40 mm, acquisition matrix 128 x 128, acquiring 40 x 0.5 mm-thick slices in approximately 8 minutes. Subsequently, these 40 slices were converted into 20 x 1 mm-thick slices by using the resampling function in SPM8 software. Lesion volumes were measured in Jim, a medical image display package (Xinapse) by measuring the cross-sectional area of infarct in 20 slices. The total volume was obtained by multiplying the sum of these areas by the thickness (1 mm). The group mean volumes and standard deviations were calculated.

4.3.5 Treatment with NT3 or vehicle through subcutaneous osmotic pumps.

24 hours after stroke, and immediately after MRI, rats were allocated to treatment using a randomised block design. Rats were anaesthetised as above and an incision was made on the flank on the impaired side and, using blunt dissection, a subcutaneous pocket was formed. The osmotic pump (2ML2, Alzet, Figure 4-1B) was positioned in this subcutaneous space and contained either vehicle or vehicle containing recombinant NT3 (Genentech). The NT3 dose (47 µg/24 hours) was higher than that typically used in previous successful experiments (12 µg/24 hours) (Bradbury et al., 1998b, Ramer et al., 2000) including one of our own involving intramuscular infusion (Duricki et al, unpublished results). For a 400 g elderly rat, this dose corresponds approximately to a high dose given subcutaneously in human clinical trials (150 µg/kg/day). The original vials contained 0.586 mg/mL recombinant human NT3 in 20 mM acetate, 140 mM NaCl, pH 5.0. This was diluted using PBS pH 7.4 containing 0.32% bovine serum albumin; Sigma; A3059; 9048-46-8) to give 0.4 µg/mL NT3 in 0.1% BSA. Vehicle was prepared using the same ratio of 20 mM sodium acetate, 140 mM NaCl, pH 5.0 to PBS pH 7.4 containing 0.32% BSA. Proteomic analysis confirmed that this is the 119 amino acid mature NT3 protein obtained after proteolytic cleavage of the 258 amino acid human pro-neurotrophin-3 (Uniprot P20873). All minipumps were prepared 1 day before implantation and incubated overnight in 0.9% saline at room temperature. 1 month of treatment was achieved by providing new pumps after 2 weeks. Skin was sutured and analgesic administered as above. All rats survived this surgery. Allocation concealment was performed by an independent person coding NT3 and vehicle stocks, and all experimenters remained blinded until the end of the study.

4.3.6 Enzyme-linked immunosorbent assay (ELISA)

At 5 weeks, pumps were removed by anaesthetising the animals as described previously. During this procedure, 2 mL of blood was removed intravenously from the tail before being spun to acquire serum. The flank containing the pump was then shaved, sterilised, and incised to remove the pump, the skin sutured, and pain relief given as described previously.

12 weeks after stroke surgery, rats were terminally anaesthetised with Sodium Pentobarbital (Euthatal) and one 2 mL sample of blood taken with a syringe from the heart. This was left to coagulate at room temperature for at least 15 minutes before spinning down at 13,000 rpm for 15 minutes and the supernatant was snap frozen in liquid nitrogen prior to storage at –80°C. ELISAs were performed on diluted serum samples (1:10) with the Human NT3 DuoSet kit (DY267, R&D systems) according to manufacturer's instructions with some modifications as described in (section 2.3.7).

4.3.7 Behavioural Assessment

Rats were handled every 2 weeks for 9 months before the commencement of the study, since we weighed and checked animals to ensure there were no age-related illnesses. 5 weeks before surgeries, animals were fully trained on the horizontal ladder and the staircase test task, as described previously (see 2.3.6). Two preoperative baseline scores for all behavioural tasks were collected 1 week before surgery. Handedness was decided by average dominant paw preference in all three tests. Two baselines were taken in order to improve statistical power (Vickers 2003).

4.3.7.1 Horizontal Ladder

To assess impairments in forelimb and hindlimb function after stroke, rats were required to walk along a horizontal ladder where limb placement during skilled walking can be measured (Metz and Whishaw, 2002). Details of this behavioural task and pretraining were previously described in section 2.3.6.2.

4.3.7.2 Bilateral tactile stimulation test:

To assess the magnitude of somatosensory neglect and sensorimotor impairments in forepaw function after stroke the bilateral tactile stimulation test was conducted (Schallert et al., 1982; Schallert et al., 2000a). Details of this behavioural task were previously described in section 2.3.6.4.

4.3.7.3 Montoya Staircase

The Montoya Staircase Test is designed to measure changes in gross and fine forelimb skilled movements following motor system damage (Montoya et al., 1991). Details of this behavioural task and pretraining were previously described in section 2.3.6.3.

4.3.8 Tracing for histochemical analysis

4.3.8.1 BDA injections

To visualise the corticospinal tract axons, the descending axons were traced with biotinylated dextran amine (BDA, 10% solution in PBS, 10 kDa, Invitrogen), which was injected into the sensorimotor cortex of the uninjured hemisphere. The tracer was given 8 weeks after stroke surgery (after behavioural testing for that day was completed) and left for 4 weeks to be transported along the corticospinal tract axons. The rats were anaesthetised as previously described and placed in a stereotaxic frame. The scalp was shaved, sterilised, and an incision was made to expose the skull.

In relation to Bregma, 6 holes were drilled into the skull using a dental drill at the coordinates in Table 4-4 (Barritt et al., 2006), which correspond to areas within the somatosensory cortex. BDA was injected into each hole (0.5 µl) using a Hamilton syringe. The needle of the syringe was slowly lowered 1.5 mm below the cortex surface and BDA injected at a rate of 0.25 µl/10s with a pause of 1 min between each dose. The scalp was then sutured and analgesic given as described previously.

Site	Lateral co-ordinates	Rostral co-ordinates
1	3.5 mm	–0.5 mm
2	3.5 mm	0.5 mm
3	3.5 mm	2.0 mm
4	2.5 mm	1.5 mm
5	2.5 mm	0.5 mm
6	1.5 mm	1.0 mm

Table 4-4: BDA injection co-ordinates in relation to Bregma

4.3.8.2 AAV-mCherry-2A-EGFP and AAV-tdTomato

The following injections were done in the elderly male and female rat cohort used in experiment 3 of this chapter. Because the results seen in the behavioural and electrophysiological portions of this experiment and time constraints, this tissue has not been processed, but the use of this tissue will be discussed in the discussion portion of this chapter.

4.3.8.2.1 Injection of AAV-tdTomato

Seven weeks after stroke surgery, rats were anaesthetised using isoflurane (5% induction, 1.5% maintenance), the head shaved and placed in a stereotaxic frame. Chlorhexidine (2% in 70% alcohol) was used to clean the site before a midline incision was made to the head to expose the scalp. A dental drill was attached to the stereotaxic frame to drill holes in the skull in relation to Bregma (Table 4-5). Injections were given to the intact cortex, with the intention of tracing the CST, which predominantly projects to the less-affected side. The drill was replaced with a 10 µl Hamilton syringe filled with AAV (#701, Hamilton) with 32 gauge metal needles attached (52 mm, point style 2). Injections were made at a depth of 1.5 mm from the *dura mater*, and 10µl per site was injected at a rate of 0.5 µl/1 min and a pause of 5 min was enforced before removal of the syringe in order to minimise backflow.

Site	Lateral co-ordinates	Rostral co-ordinates
1	3 mm	0.5 mm
2	2.5 mm	3 mm

Table 4-5: Injection sites for AAV-tdTomato in relation to Bregma

4.3.8.2.2 Injection of AAV-mCherry-2A-EGFP

As in 4.3.8.2.1 above, except injections were made at the injection sites listed in Table 4-6 below for AAV-mCherry-2A-EGFP. Injections were made at a rate of 0.25 µl/10 s and a pause of 1 minute between each dose.

Site	Lateral co-ordinates	Rostral co-ordinates
1	3.5 mm	–0.5 mm
2	3.5 mm	0.5 mm
3	3.5 mm	2.0 mm
4	2.5 mm	1.5 mm
5	2.5 mm	0.5 mm
6	2.0 mm	3.5 mm
7	1.5 mm	1.0 mm

Table 4-6: Injection sites for AAV-mCherry-2A-EGFP in relation to Bregma

4.3.9 Histological analysis of BDA tracing

4.3.9.1 Tissue processing:

Twelve weeks post stroke surgery and four weeks after BDA labelling, rats were terminally anaesthetised with Pentobarbital Sodium (Euthatal) and perfused transcardially with PBS (NaCl, 137 mM; KCl, 2.7 mM; Na_2HPO_4, 4.3 mM; KH_2PO_4, 1.4 mM) for 10 minutes, followed by 500 mL of 4% paraformaldehyde (PFA) in PBS for 12 minutes. The brain, spinal cord and both arms were carefully dissected and stored in 4% paraformaldehyde in PBS for 2 hours and then transferred to 30% sucrose in PBS and stored at 4 °C. The spinal cord segments were embedded in gelatin, post-fixed in PFA for 24 hours, and then cryoprotected in 30% sucrose in PBS. 40 µm transverse slices were cut using a freezing stage microtome (Kryomat, Leitz, Germany) and transferred into TBS/azide (100mM Tris, 15 mM NaCl, 0.5mM NaN_3, pH 7.4) in 24-well plates and stored at 4 °C. 10 series of sections were cut and placed in 10 wells.

4.3.9.2 Histology

Spinal cord segments and brains were embedded in 10% gelatin and a series of 40 µm-thick transverse sections of fixed spinal cord were cut using a freezing microtome (Kryomat; Leitz, Germany) and stored in 0.03% PBS/azide as 10 series of free floating sections and immunolabelled as follows.

BDA staining of free-floating spinal sections were done through incubation with 0.3% H_2O_2 and 10% methanol (30 mins) in water. Sections were incubated in ABC reagent

(VectorLabs, UK) (30 mins) then amplified using biotinyl tyramide (1:75, PerkinElmer, USA), then left overnight at 4 °C. The sections were then incubated with extra avidin FITC (1:500; 2 hours). Sections were washed between steps in PBS.

Protein kinase C gamma (PKC-γ): free floating sections were washed three times in PBS containing 0.2% Triton-X-100, and then rabbit anti-PKC-γ antibody (Santa Cruz Biotechnology, 1:500) was applied overnight on a rocker and kept in the dark at 4 °C. Sections were then washed again in PBS containing 0.2% Triton-X-100 and the secondary antibody, donkey anti-goat IgG Alexa 488 (1:1000, Sigma) was applied for 3 hours, once again on a rocker. Sections were washed with PBS and then mounted and cover slipped with Mowiol.

Immunofluorescence was visualised under a Zeiss Imager Z1 microscope or a confocal Zeiss LSM 700 laser-scanning microscope. Photographs were taken using the Axio Cam and AxioVision LE Rel 4.2. ImageJ was used for image analysis.

CST axons were counted that crossed the midline, and at two more lateral planes (Figure 4-7A). For each rat, the number of CST axons per cord segment was calculated by counting the number of CST axons in all sections in a series and then multiplying by the total number of sections in the whole segment and then divided by the number of sections counted. PKC-γ intensity was analysed using ImageJ software, in which intensity measurements were taken from both dorsal columns and a ratio of intact:denervated dorsal columns was calculated.

4.3.10 Hoffman-reflex electrophysiology

The Hoffman reflex (H-reflex) was assessed at 12 weeks post-surgery as an endpoint assessment. Rats were anaesthetised with 30 mg/kg ketamine and 0.1 mg/kg medetomidine (intraperitoneally). Two 24-G needle electrodes were inserted across the medial plantar side of the wrist to stimulate the ulnar nerve (*via* a constant current isolated pulse stimulator, stimulus width 100 μs). Two recording electrodes were inserted into the abductor digiti quinti of the affected forepaw to record electromyograms. The signal was amplified (x 4000), filtered (300 Hz to 6 kHz), and digitised via PowerLab before being subsequently visualised and analysed with LabChart. The M-wave is evoked by excitation of motor axons. The H-wave is the monosynaptic reflex: Ia proprioceptive afferents synaptically activate motor neurons in the spinal cord (Figure 4-11A). The threshold was determined as the lowest stimulation intensity that elicited an H-wave response in at least 75% of the recordings. First, the responses to

increasing stimulus intensities were tested at 0.1 Hz up to 2 x threshold, which only activates Group I muscle afferents (Iyer et al., 2010). To allow comparisons between animals (because anaesthetic depth and electrode placement will vary), M-wave and H-wave amplitudes were expressed as a percentage of the maximum M-wave recorded at higher stimulation intensities. Next, we tested the frequency dependent depression of the H-wave. We stimulated every 10 seconds with paired stimuli at frequencies ranging from 0.1 Hz to 10 Hz. The H-wave amplitude of the test stimulus was normalised to the H-wave amplitude of the conditioning stimulus. 25 paired stimuli per frequency were averaged and plotted on a frequency-depression curve. This test was done by Dr C Kathe.

4.3.11 Statistical analysis

Statistical analyses were done using SPSS (version 18.0). Graphs show means ± SEMs (except where otherwise stated) and 'n' denotes number of rats. Asterisks (*, **, ***) indicate $p \leq 0.05$, 0.01 and 0.001 respectively. Threshold for significance was 0.05. ELISA and lesion volume data were assessed using Kruskal-Wallis (Mann-Whitney tests), or t-tests with appropriate corrections, and are defined in the text as required. Behavioural data were analysed using linear models and Restricted Maximum Likelihood estimation to accommodate data from rats with occasional missing values (Gueorguieva and Krystal, 2004, Krueger and Tian, 2004, Duricki et al., 2016a), because some rats died or were humanely killed before the end of the study. Akaike's Information Criterion (AIC) showed that the model with best fit for the behavioural data, and are indicated in the text as having unstructured (UN), first-order autoregressive (AR1), or compound symmetric (CS) covariance matrix. Baseline scores were used as covariates. Degrees of freedom are reported to nearest integer. t-tests were two-tailed.

4.4 Results

In this chapter, three experiments have been used to form the scientific argument. In the first, a time course in elderly female rats was used to inform future experiments as to how long the occlusion period should last, based on lesion volume and mortality (Figure 4-1C, Figure 4-2A, B).

The second experiment used elderly female rats to measure the effect of subcutaneous NT3 on permanent distal MCAO with bilateral carotid occlusion and assessed behavioural and histological outcomes to assess benefit on treatment (Figure4-1D, Figure 4-2C, Figure 4-3A, Figure 4-4 – Figure 4-7). Dr D Duricki was the lead researcher on these first two experiments, performing the histology and behavioural assessment and scoring, and they have been included in this thesis as they form part of the rationale behind the third experiment. Dr B Haenzi assisted with animal care following surgery, and my role was to assist in surgeries.

The final experiment in this chapter was performed in elderly male and female rats with permanent distal MCAO with bilateral carotid occlusion. Behavioural and electrophysiological assessments were used to determine if subcutaneous NT3 delivered 24 hours after stroke would elicit an effect on recovery (Figure 4-1E, Figure 4-2D, Figure 4-3B, Figure 4-8–Figure 4-11). Electrophysiology was performed and analysed by Dr C Kathe. Drs D Duricki and B Haenzi participated in surgery and post-operative care.

4.4.1 Assessing time of occlusion by lesion volume

For assessment of lesion volumes, rats in all groups underwent isoflurane anaesthesia and received structural T2-weighted MRI scans at 24 hours. Animals in Experiment 1 of this chapter (Figure 4-1C) also underwent a second structural scan at 8 weeks after stroke. In these scans, hyperintense (bright) areas represent regions containing fluid such as CSF or oedema and the volume of these areas can be measured and is correlated with the area of infarction (Gerriets et al., 2004). The 24-hour infarction volumes were compared to the 8-week lesion volumes (Figure 4-2A). Mean lesion volumes of the young animals with 90-minute occlusion were 70.5±17.7 mm^3 at 24 hours compared to 10.0±3.4 mm^3 at 8 weeks; with elderly animals with 60-minute occlusion having mean infarct volumes of 62.8±11.3 mm^3 and 22.4±2.45 mm^3 at 24 hours and 8 weeks respectively, with similar values achieved for elderly animals with 90-minute occlusions at these timepoints of 45.9±10.3 mm^3 at 24 hours and 12.7±3.84 mm^3 at the 8-week timepoint. Analysis of these groups using ANOVA with

Sidak's test for multiple comparisons indicates that there is a highly significant effect of time ($F_{1,17}$ =35.68, p<0.0001; see Figure 4-2A for *post hoc* values).

Oedema develops over the first 1-2 days and then is lost over the course of the following 1–2 weeks following stroke (Schwamm et al., 1998). Therefore, oedema present early after stroke (eg, at 24 hours) can lead to an overestimation of the final lesion volume (eg, at 8 weeks) and therefore a comparison has been made to mean infarct volumes adjusted using Gerriets' formulae (Gerriets et al., 2004). Mean data from the raw (unadjusted) lesion volume at 24 hour of elderly animals with 60-minute occlusion (red, Figure 4-2A) occupies 9.8±2.1% of the affected hemisphere. When corrected for brain swelling using Gerriets' formulae this value is reduced to 4.12±1.0% (Figure 4-2B, pink). By calculating lesion volume in this way, oedema is removed as a factor, and it can be observed that there is no statistical difference between the lesion volumes at 24 hours compared with 8 weeks (paired two-tailed t-test p=0.125).

On the basis of the results observed in experiment 1, 60-minute occlusions were selected for the elderly female rats used in experiment 2, as 90 minute occlusions resulted in a slightly higher mortality without resulting in higher mortality (Figure 4-2A) after stroke.

In Figure 4-2C and D, comparisons are made between the treatment groups occlusion in experiment 2 and 3, respectively. In experiment 2, mean lesion volumes were not significantly different at 24 hours between elderly female rats in the NT3 group (51.2±5.8 mm^3) compared to vehicle (48.1±5.5 mm^3, unpaired, two-tailed t-test p=0.906), and this was also the case when corrected for oedema (NT3: 6.20±0.70%, vehicle: 5.05±1.26%, unpaired, two-tailed t-test with Mann-Whitney *post-hoc* test p=0.510, Figure 4-2C, bottom graph). A similar pattern was observed in the mixed gender elderly study, where no difference was observed between elderly females given NT3 (109.1±16.0 mm^3) or vehicle (104.8±28.2 mm^3, p=0.895) or males in the same treatment groups (Figure 4-2D, top graph, NT3: 167.6±25.7 mm^3, vehicle: 204.9±28.9 mm^3, p=0.348). Again, once oedema had been accounted for and lesion displayed as a percentage of the ischaemic hemisphere, there was no evidence for a difference in lesion volumes between elderly female rats treated with NT3 compared with vehicle using multiple unpaired, two tailed t-tests in both elderly female rats (NT3: 11.4±1.5%, vehicle: 9.3±2.5%, p=0.485) or between elderly male rats treated with NT3 compared to vehicle (NT3: 11.7±2.2%, vehicle: 13.5±2.7%, p=0.617), indicating that the severity of stroke lesions were split equally between treatment groups. There was no effect of gender in the lesion volumes (two-way ANOVA, p=0.386).

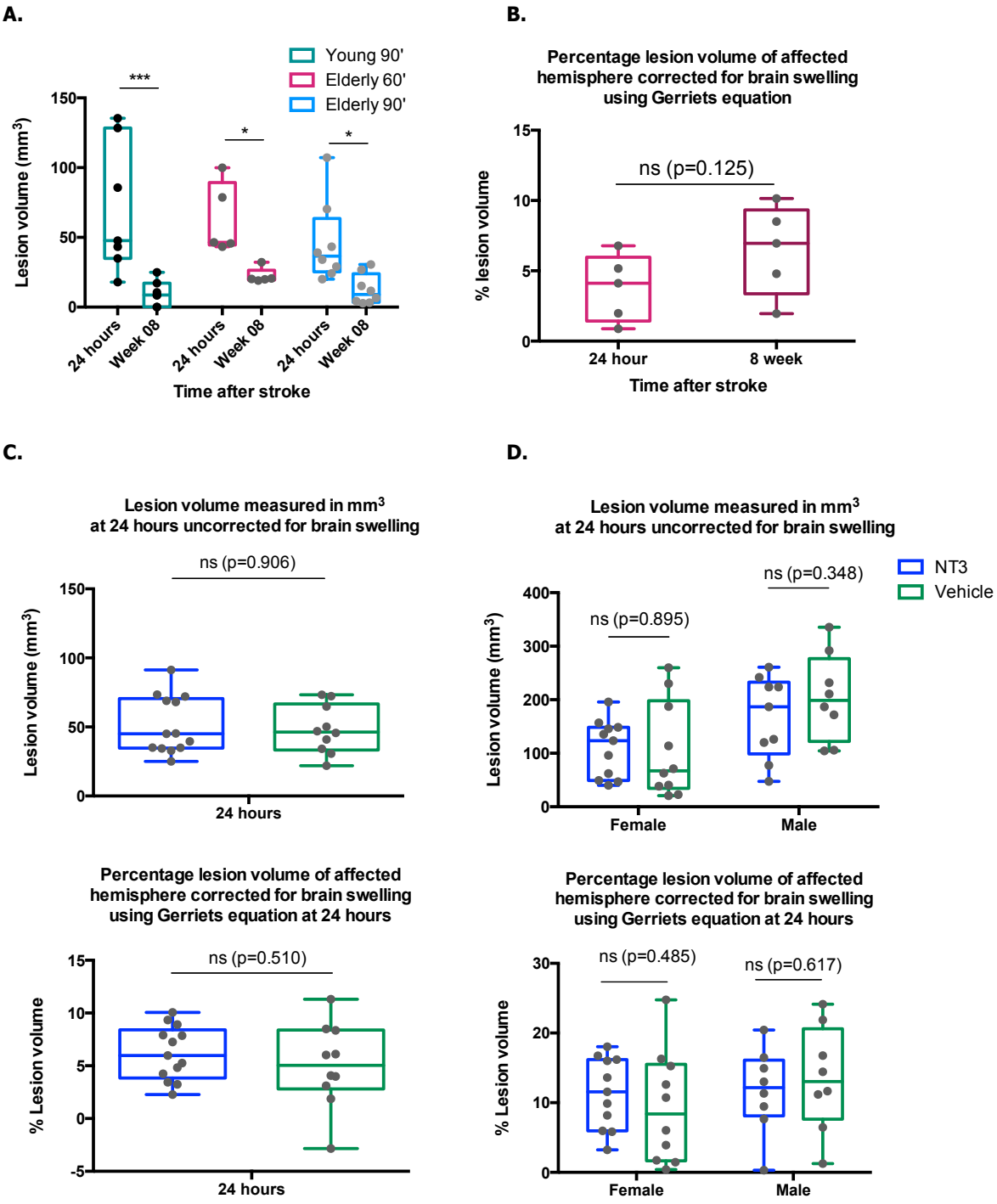


Figure 4-2 : Comparison of lesion volume between different treatment groups of this chapter.

*T2-weighted imaging with box plots showing median lesion volume and IQR, where the error bars indicate the minimum and maximum lesion volumes. Experiment 1 explored occlusion times of young (n=7) and elderly rats with 90-minute occlusion (n=8) or 60-minute occlusion (n=5) (A). At 24 hours, the lesion volume (in mm^3) is not significantly different between groups, but there is a significant effect of time on the lesion volumes within groups (ANOVA with Sidak's multiple comparison test, ***=p<0.001, *=p<0.05). When accounting for brain swelling using Gerriets' equation (Gerriets et al., 2004), this is no longer significant when calculated as a percentage of the lesioned hemisphere, and is shown in elderly animals with 60-minute occlusion (B). C compares lesion volumes between the two treatment groups from experiment 2 (top), and when normalised for brain oedema (bottom), both showing no difference in stroke severity between NT3 (n=13) and vehicle (n=10) stroke groups. In D), no difference can be observed between male (n=8 NT3 and vehicle) and female (NT3 n=10, vehicle n=11) raw lesion volumes, and accounting for oedema and representing results as a percentage of the lesioned hemisphere shows no differences between treatment groups or gender. Non-significance was identified by unpaired, two-tailed t-tests.*

4.4.2 ELISA results confirm elevated levels of NT3 in the serum after treatment with NT3, although not in as high quantities as seen with AAV-NT3

In light of results observed in section 2.4.5, one major aim of this study was to elucidate whether altering the method of NT3 delivery to protein delivered subcutaneously could be used as a method of treatment in stroke-injured rats. NT3 protein has been delivered in stroke animals previously, although *via* catheters for intramuscular delivery (Duricki et al., 2016b). To test whether the delivery of the NT3 was successful, an ELISA was performed on blood serum.

Analysis of the blood from the treated animals in the elderly female study revealed NT3 levels in the blood serum of 13.9±2.31 pg/mg compared to 0.311±0.107 pg/mg in vehicle control animals 2 weeks after stroke and treatment initiation (2-week NT3 *vs* vehicle; p=4.57e–05). At the end of the study, NT3 protein levels in treatment animals were reduced to 0.135±0.063 pg/mg and were similar to vehicle treated animals, which had NT3 concentrations of 0.318±0.154 pg/mg (12-week NT3 *vs* vehicle, p=0.236, data not shown). These statistics were calculated using multiple two-sided, unpaired t-tests.

In the final study using male and female rats, sham animals had NT3 concentrations of 1.81±0.582 pg/mg and 2.54±0.525 pg/mg in the female and male animals, respectively. Female and male vehicle animals had similarly low levels of NT3 at 2.00±0.469 pg/mg and 1.92±0.548 pg/mg, respectively. NT3-treated female animals had blood NT3 concentrations of 8.17±2.88 pg/mg and male animals had 11.1±2.83 pg/mg. Statistically, the only significant result was between male NT3 animals compared to vehicle control (p=0.0032).

In the second experiment, all but one of the NT3 treated rats had higher NT3 concentrations than the vehicle control, while in the final experiment in this chapter, a larger proportion of NT3 rats had similar NT3 concentrations to vehicle treated rats, although the reasons for this are unclear.

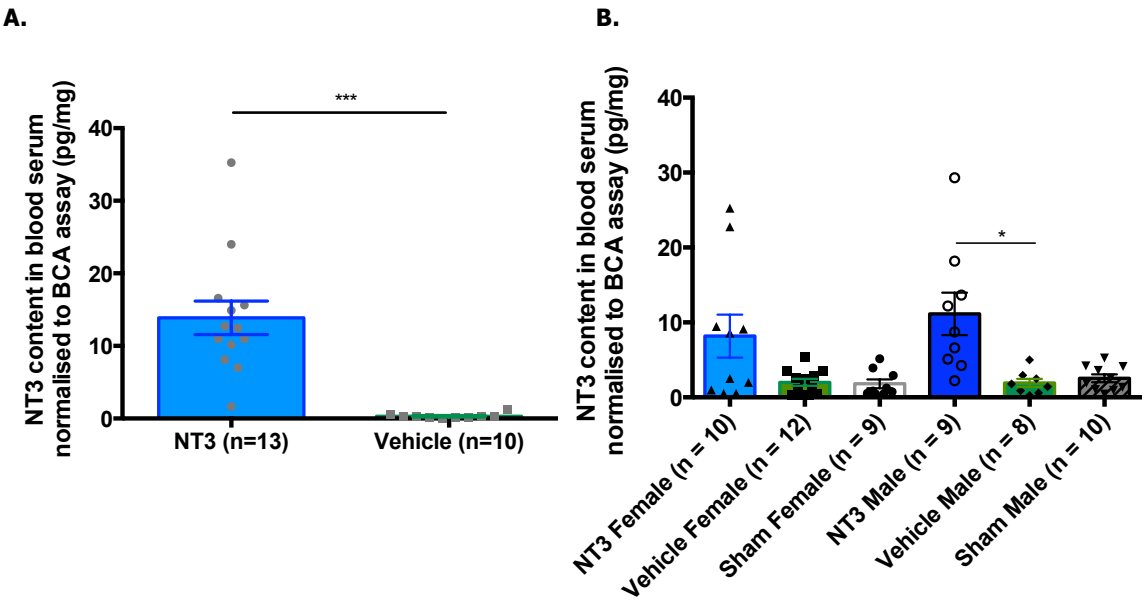


Figure 4-3 : ELISA showed a low concentration of NT3 became available in the blood after 4-week subcutaneous delivery.

Graphs show mean±SEM. (A) In the elderly female study in this chapter, blood was removed at 2 weeks after treatment with NT3 or vehicle and 2 weeks after inducing stroke. ELISA confirmed that there was a higher level of NT3 in animals treated with NT3 compared to controls (n=10–13 per group, p=4.57e-05). In (B), animals receiving stroke or sham treatment were assessed after four weeks, having had NT3 or vehicle treatment delivery via subcutaneous infusion (n=8–12/group). Protein levels analysed by ELISA concluded that there was a difference in the levels of NT3 compared with vehicle in male elderly rats (p=0.0032).

4.4.3 Elderly female animal treatment with subcutaneous NT3 results in improved functional outcome after permanent distal MCAO with tandem CCA occlusion.

4.4.3.1 Staircase test

Recovery of dexterity was assessed using the staircase test as described previously (section 2.3.6.3). Before stroke surgery, elderly rats retrieved more than 70% of pellets with their dominant paw and stroke was induced in the hemisphere representing the dominant paw. One week after stroke, both groups were impaired in reaching for pellets with their affected paw compared to baseline. Recovery was slightly greater in the group of rats treated with NT3 than those treated with vehicle (Figure 4-4A), although did not reach significance ($F_{1,19}$=2.97, p=0.101). There was an effect of time ($F_{11,17}$=11.4, p<0.001) and an interaction between time and treatment group ($F_{11,17}$=2.91, p=0.024), suggesting that NT3 treatment played a role in the recovery observed in this test (linear models with unstructured covariance matrix analysis). After stroke, the less-affected paw showed a small impairment compared to baseline but no effect of NT3 treatment group ($F_{1,31}$=2.56, p=0.120) and no interaction between group and time ($F_{11,163}$=1.55, p=0.118), although there was an effect of time ($F_{11,163}$=3.33, p<0.001), indicating recovery in function over time (linear models with first order autoregressive covariate analysis).

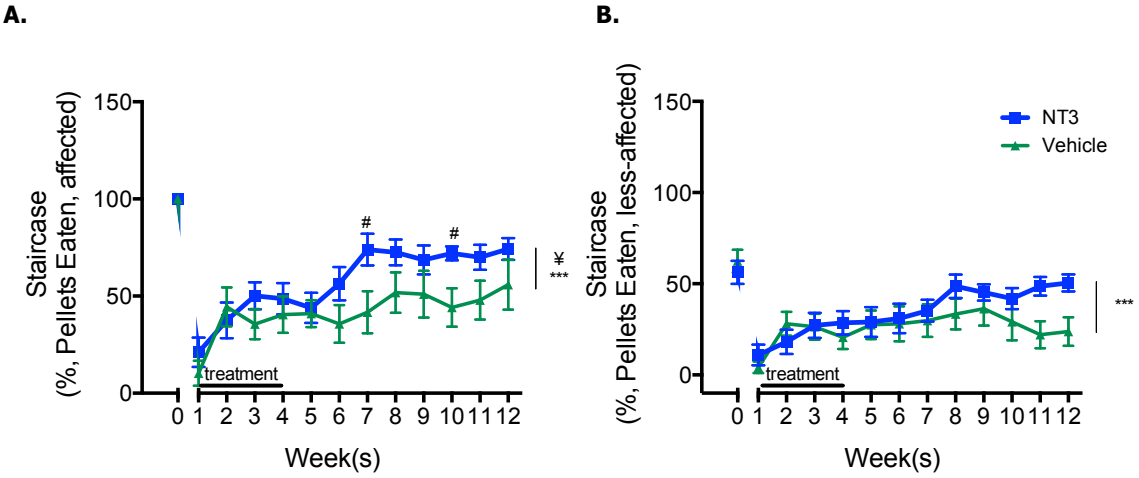


Figure 4-4 : Subcutaneous NT3 treatment following stroke surgery in elderly rats confers a significant benefit in the staircase test for skilled reaching.

*Induction of permanent distal MCAO in elderly rats caused a significant impairment in motor control of the affected (A) and less affected (B) forepaws. A sustained deficit was observed in both paws of vehicle treated rats, while rats treated with NT3 show an improvement over time in the use of the stroke-affected forepaw, and this this interaction was significant (linear models with first order autoregressive covariance matrix analysis, p=0.024). The graph shows means±SEM (n=10 in vehicle group, n=13 in NT3 group). ***=effect of time p<0.0001, ¥=interaction between group and time p<0.05, #=effect between NT3 and vehicle at time point p<0.05.*

4.4.3.2 Horizontal ladder

Accuracy of limb placement during walking was assessed using a horizontal ladder with irregularly spaced rungs as described previously (section 2.3.6.2). Accuracy of limb placement requires proprioceptive sensory feedback from the limbs especially because rung spacing was changed frequently to avoid fixed stride lengths or other pattern learning (Takeoka et al., 2014). Video recordings were assessed in slow motion to count the number of errors made with the affected forelimb (expressed as a percentage of steps). Before stroke, rats crossed the ladder making few errors. 1 week after stroke, animals treated with NT3 or vehicle made a large, similar number of errors with their affected forelimb (Figure 4-5A). The stroke group treated with NT3 recovered more fully then the stroke group treated with vehicle, although this did not reach significance ($F_{1,32}$=1.35, p=0.253). Animals recovered significantly over time to reach baseline levels ($F_{11,142}$=4.87, p<0.001) and there was no significant interaction between group and time ($F_{11,142}$=1.28, p=0.243). Errors made with the less-affected forepaw (Figure 4-5B) were fewer than the affected forepaw, and as a result, no group effect was observed ($F_{1,20}$=3.38, p=0.081). There was an effect of time ($F_{11,213}$=4.08, p<0.001) but no interaction between group and time ($F_{11,213}$=0.921, p=0.521). The hindpaw on the affected side suffered a small impairment after stroke (Figure 4-5C), but no differences were observed between groups ($F_{1,20}$=0.032, p=0.861), there was no effect of time ($F_{11,214}$=0.972, p=0.473) although an interaction was noted between treatment group and progression of the study ($F_{11,214}$=2.73, p=0.003), as the lines are seen to cross in the graph below. The less-affected hindpaw had a similar small decrease in success in the ladder task after stroke, but there was no significant effect of group ($F_{1,21}$=1.83, p=0.190) although there was a significant effect of time ($F_{11,18}$=2.39, p = 0.050) and interaction between time and group ($F_{11,18}$=3.54, p=0.009). However, post-hoc tests only revealed a difference between groups at week 8 and indicate that there was no lasting effect of NT3 treatment, and possibly that this result is a one-off false positive result (*post hoc* test NT3 *vs* vehicle at 8 weeks, p=0.042). Overall, there was no convincing effect of NT3 when delivered subcutaneously after stroke in this model, although there appears to be a small improvement with the ability with the affected paw using NT3 compared with vehicle-treated animals.

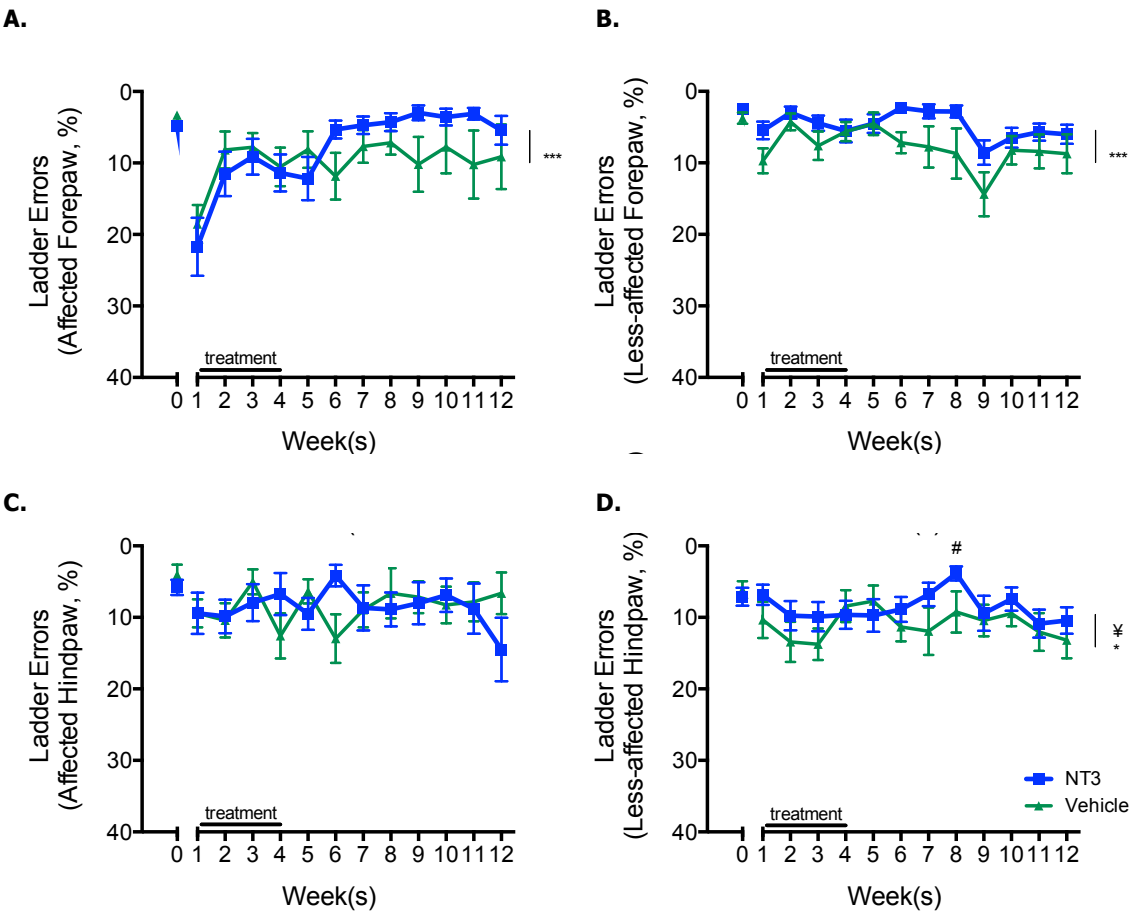


Figure 4-5 : Horizontal ladder tests shows no improvement with delayed (24 hour) NT3 treatment for 4 weeks compared with vehicle in a female elderly model of stroke.

Following MCAO, rats exhibited significant impairments in motor control of their affected forelimb (A). The two stroke groups made a similar number of foot faults one week after injury, with both NT3 and vehicle groups beginning a recovery process following the first week time-point so that they were not significantly different. The NT3 treatment in this study does not result in improved motor recovery of the affected forelimb following stroke injury compared to vehicle. The percentage of foot faults on the less-affected forelimb indicates no change from sham in either of the stroke groups (B). The hindpaws also have no effect of stroke throughout the study (C, affected hindpaw; D, less-affected hindpaw). Results displayed as mean±SEM. Statistics used are linear models (=effect of time p<0.05, ***=effect of time p<0.001, #=effect of group at timepoint p<0.05, ¥=interaction of group with time p<0.05).*

4.4.3.3 Sticky tape test

Sensory responsiveness and dexterity was measured using the sticky patch test as described earlier (section 2.3.6.4). Stickers of equal radius were attached to each wrist and the time taken to contact and then remove them was measured. Before stroke, rats contacted the first sticker rapidly and subsequently removed it rapidly. One week after stroke, both treatment groups took longer to contact the sticker on the affected forepaw (Figure 4-6A). The stroke group treated with NT3 recovered more fully than the stroke group treated with vehicle ($F_{1,21}$=7.25, p=0.014). There was a significant effect of time ($F_{11,20}$=7.46, p<0.001) and an interaction between groups ($F_{11,20}$=2.59, p=0.031). From 8 weeks, a clear-cut difference was observed between groups (post-hoc test NT3 *vs* vehicle at 8 weeks, p=0.01).

Although sensation with the less-affected paw was not affected as severely as the affected paw initially, over the course of the study the length of time needed to sense the sticker on the paw increased, and by week 5, the time taken to sense the sticker on the paw was longer than that observed at week 1 (Figure 4-6B), which could possibly be because of inappropriate somatosensory activation, as was seen during forepaw stimulation in fMRI in chapter 3. There was no overall effect of group ($F_{1,7}$=3.34, p=0.109). However, there was a significant effect of time ($F_{11,19}$=6.24, p<0.001), and an interaction between time and group ($F_{11,19}$=6.45, p<0.001, week 8 NT3 *vs* vehicle *post hoc* p=0.003).

Similarly, 1 week after stroke, both treatment groups took longer to subsequently remove the sticker from the affected paw after indicating that the sticker had been sensed (Figure 4-6C). The stroke group treated with NT3 recovered more fully than the stroke group treated with vehicle, although this effect of treatment group was not significant ($F_{1,20}$=1.89, p=0.184). Additionally, there was a significant effect on time ($F_{11,20}$=11.4, p<0.001) and a trend towards an interaction between group and time ($F_{11,20}$=2.26, p=0.055). The less-affected paw, where a small impairment was observed one week after stroke, showed no significant effect of time on recovery ($F_{11,144}$=1.75, p=0.069), no effect of treatment ($F_{1,49}$=3.22, p=0.079) and no interaction between group and time ($F_{11,144}$=1.08, p=0.378).

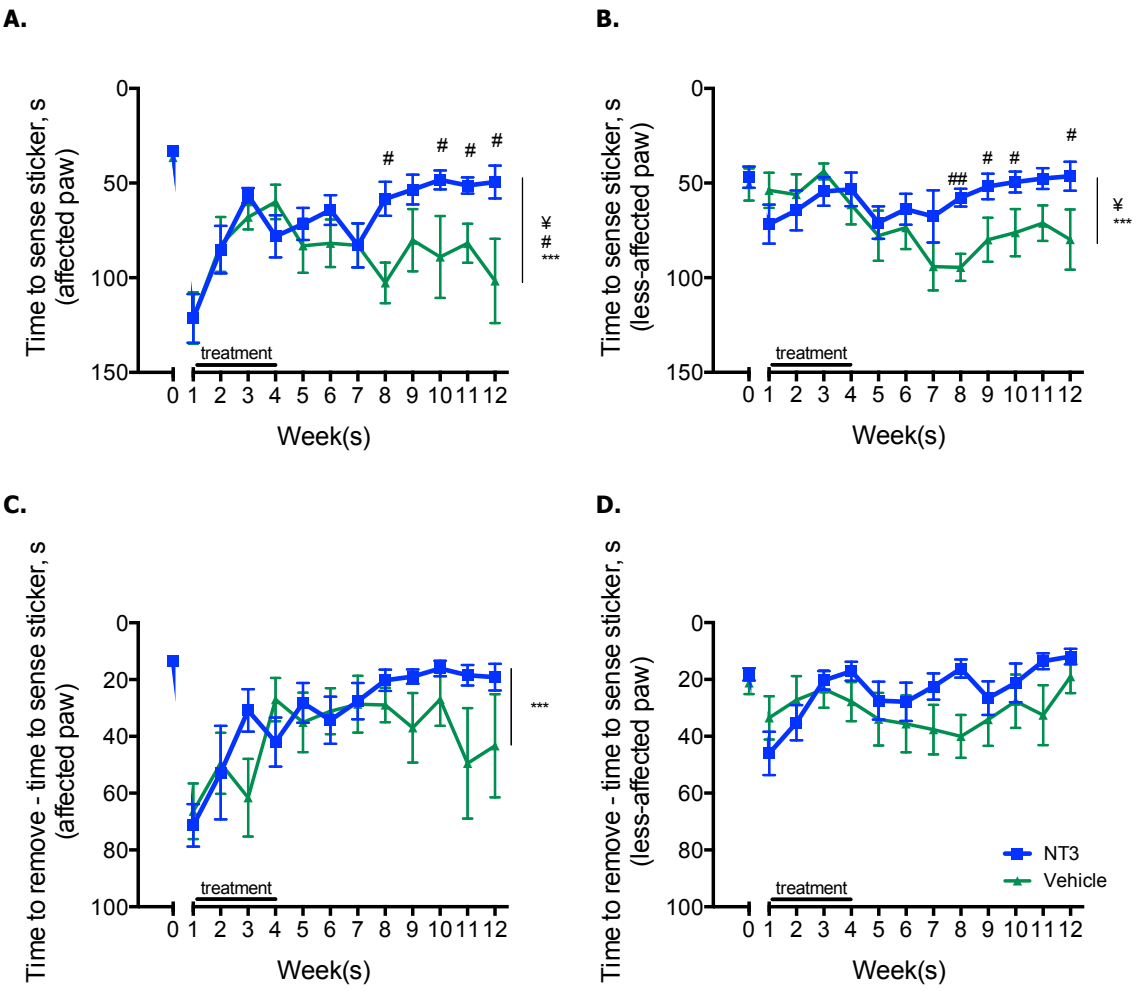


Figure 4-6 : NT3 improves functional sensory outcomes in the bilateral extinction test after distal permanent MCAO in an elderly female stroke model

In the bilateral extinction test the time taken for rats to first contact the sticker label on the affected forelimb was measured (A) and a small difference observed between NT3 treated animals after MCAO compared to vehicle treated animals, although this difference was not significant (p=0.144). This measure was also assessed in the less-affected forepaw (B), where there was an effect of treatment (p=0.049) and time (p=0.007) and specific interactions observed between NT3 and vehicle groups at weeks 7–10 and 12.

*The time taken to subsequently remove the sticker after sensing was less significant, with no effect of treatment group in either the affected forepaw (C) or the less-affected forepaw (D). ***denotes time difference compared with week 1, p<0.001; ¥ denotes interaction of group with time, p<0.05; # denotes difference between stroke groups, p<0.05; Graphs represent mean ± SEM and statistics analysed by linear models.*

4.4.4 Elderly female animal treatment with subcutaneous NT3 results in corticospinal sprouting after permanent distal MCAO.

Immunolabelling for BDA, injected 4 weeks before the end of the study, was used to assess axonal sprouting of the less-affected corticospinal tract. This tract decussates in the medullary pyramids and projects to the contralateral side of the spinal cord in the dorsal columns and in the dorsolateral columns; there is also a minor projection that does not decussate but runs in the ventral spinal cord (see Figure 4-7A). We chose to analyse CST axons in every cervical segment from C2 to C8 as the shoulder, forelimb and paw muscles are supplied by motor neurons arising from these regions (McKenna et al., 2000, Tosolini and Morris, 2012). Corticospinal axons from the less-affected hemisphere were measured at three parasagittal planes on the denervated side ("Midline", "Distance 1" and "Distance 2"; Figure 4-7A) and obliquely where ventral corticospinal axons that have projected ipsilaterally from cortex enter the grey matter ("Ipsi"; Figure 4-7A).

NT3 treatment significantly increased corticospinal tract sprouting at all levels from C2 to C8 compared with vehicle-treated animals (Figure 4-7A–H). Sprouting was frequently observed at the midline, at distance 1, distance 2, and from the ventral CST. Increased sprouting at distance 2 indicates that more CST axons extended into the territory of the forelimb motor neuron pools which are located in lateral and ventrolateral grey matter (Romanes, 1964). Figure 4-7I shows representative pictures of corticospinal axons at C7. Corticospinal tract axons (green) are shown to sprout into the dorsal horn on the affected side amongst PKCγ labelled sensory neurons (red). The axons very close to PKCγ-labelled sensory neurons were then quantified. NT3 treated animals had 107±13.8 (mean±SEM, n=13) crossed CST axons on the affected side compared with 44.4±15.5 (mean±SEM, n=10) in vehicle treated animals (unpaired two-tailed t-test, p=0.0062, Figure 4-7J).

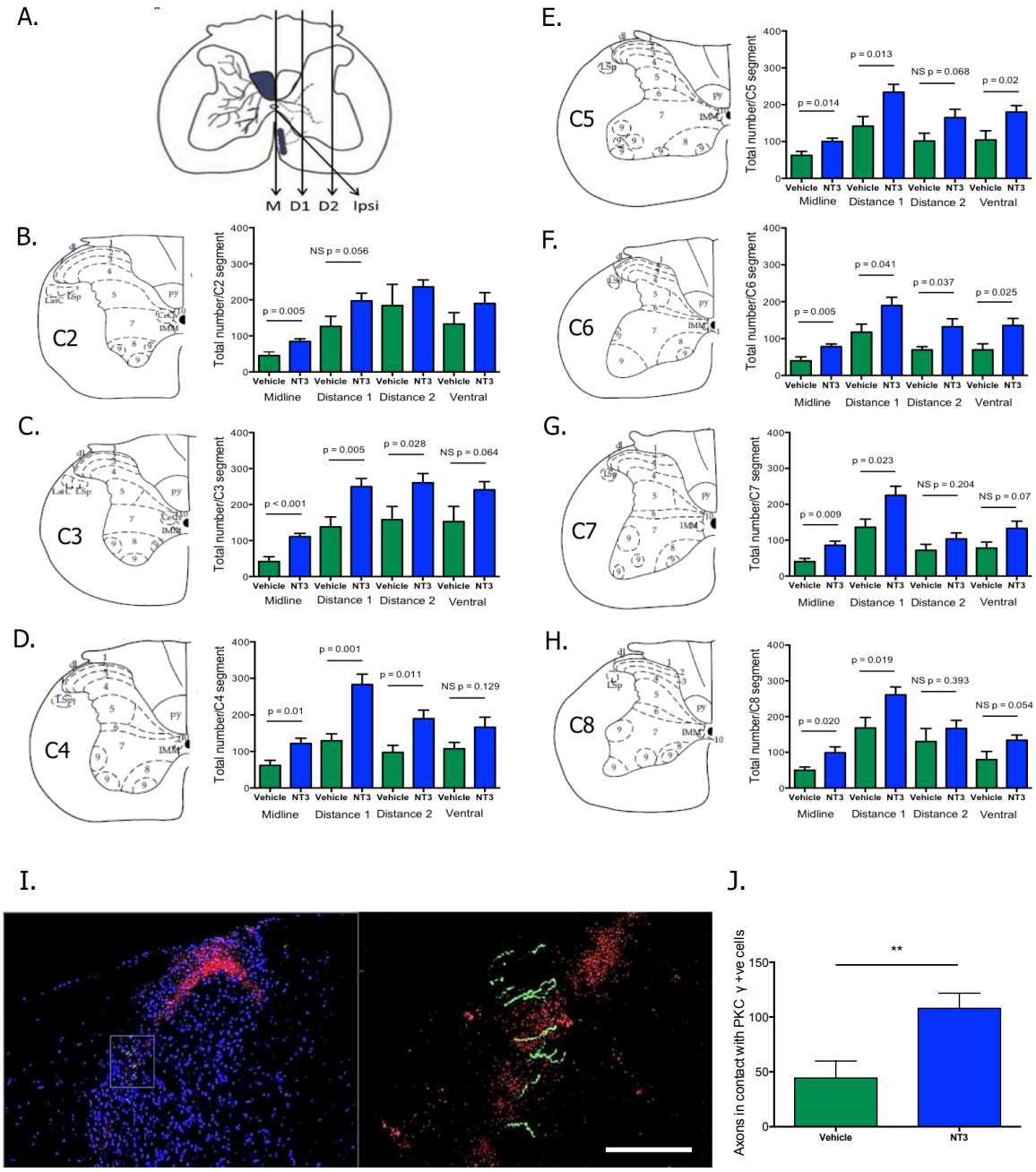


Figure 4-7 : NT3 treatment stimulates sprouting of corticospinal axons in the cervical spinal cord after stroke injury

(A-H) Anterogradely traced corticospinal axons were counted in the cervical cord segments C2 to C8 at the midline (M), at two more lateral planes (D1 and D2) and crossing into grey matter from the ipsilateral, ventral tract (Ipsi). Overall, rats treated with NT3 had an increased number of crossed fibres compared to those treated with vehicle. Anterograde tracing data were assessed using two-way ANOVA and post hoc t-tests. I) Corticospinal tract axons (green) sprouted into the dorsal horn on the affected side amongst PKC-γ labelled sensory neurons (red). Scale bar represents 250 µm. J) NT3 enhanced the number of corticospinal tract axons which sprouted into the laminae containing PKC-γ labelled sensory neurons (p=0.0062, unpaired two-tailed t-test). Adapted from Figure provided by Dr Denise Duricki.

4.4.5 Treatment with subcutaneous NT3 in male and female rats results in no observed effect of treatment in behavioural tests.

In the final study of in this chapter, male and female animals were assessed after three-vessel MCAO and treatment at 24 hours for 4 weeks using either NT3 or vehicle delivered *via* subcutaneous pumps. Behavioural assessment occurred weekly after 2 baseline measurements for each test before surgery as recommended (Vickers, 2003).

4.4.5.1 Staircase

Recovery of dexterity was assessed using the staircase test as described previously (section 2.3.6.3). Before stroke surgery, elderly rats that retrieved more than 75% of pellets with their dominant paw were allocated to a stroke or sham group. Stroke was induced in the hemisphere representing the dominant paw. 1 week after stroke, both groups were impaired in reaching for pellets with their affected paw compared with baseline. Stroke surgery in both groups resulted in large, sustained deficits compared with sham in the affected paw (Figure 4-8, top left), although there was no effect of group ($F_{2,54}$=2.89, p=0.064). There was an effect of time ($F_{11,39}$=5.57, p<0.001) although interaction between time and treatment group was not significant ($F_{22,41}$=0.828, p=0.676). When considering gender as a factor in the analysis, female rats (Figure 4-8, middle left) overall had less severe impairments in the affected paw (effect of gender, $F_{1,24}$=21.0, p<0.001) following stroke compared to the male stroke groups (Figure 4-8, bottom left). When including this difference in behavioural outcome, there was an effect of group, but no effect of treatment after stroke ($F_{2,54}$=4.53, p=0.015, *post hoc* NT3 *vs* vehicle p=0.774). There was also an effect of time ($F_{11,37}$=4.78, p<0.001), although there was no interaction between group and time ($F_{22,37}$=0.77, p=0.737).

The less-affected paw (Figure 4-8, top right) showed a small impairment after stroke injury compared to sham. There was a significant effect of time ($F_{11,35}$=2.36, p=0.027) and no effect of group, ($F_{2,49}$=0.131, p=0.877), and no interaction between group and time ($F_{22,35}$=1.33, p=0.224). When gender was included as a factor in the analysis, there was no effect of treatment ($F_{2,47}$=0.449, p = 0.641), no interaction between group and time ($F_{22,36}$=1.24, p=0.277), but an effect of time ($F_{11,35}$=2.41, p=0.024). An effect of gender was also observed, suggesting that male rats performed significantly more poorly than female rats ($F_{1,46}$=25.5, p<0.001).

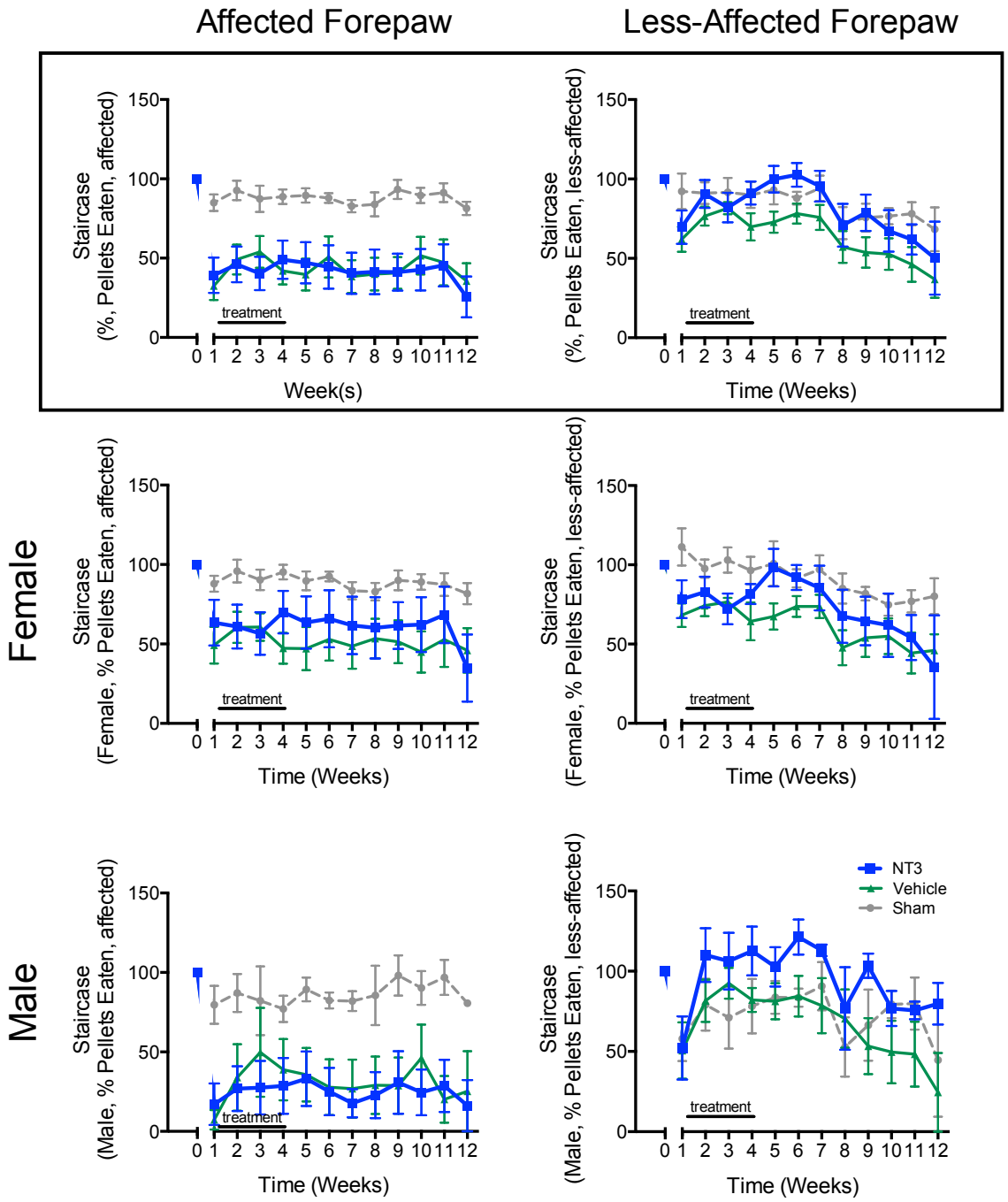


Figure 4-8 : Skilled forelimb use assessment using the staircase test in elderly male and female rats after MCAO or sham surgery and treatment with NT3 or vehicle.

Induction of permanent distal MCAO in elderly rats exhibited a significant impairment in motor control of the affected (top left) and less affected forelimb (top right). A sustained deficit was observed between sham and stroke groups but there was no difference between treatment groups. When accounting for gender differences, stroke resulted in less severe deficits in the affected forepaw in female rats (middle left; NT3 n=7–10, vehicle n=6–11) compared with male rats (bottom left; NT3 n=6–8, vehicle n=6–8; p=0.025), although even accounting for these differences, there was no effect of treatment with NT3. Graphs represent mean±SEM and statistics analysed by linear models with a unstructured covariance matrix.

4.4.5.2 Ladder

Accuracy of limb placement during walking was assessed using a horizontal ladder with irregularly spaced rungs as described previously (section 2.3.6.2). Video recordings were assessed in slow motion to count the number of errors made with the affected forelimb (expressed as a percentage of steps). Before stroke, rats crossed the ladder making very few errors. 1 week after stroke, rats treated with NT3 or vehicle made a large, similar number of errors with their affected forelimb compared with sham, which were sustained throughout the study (Figure 4-9, top left). Regarding the affected forelimb, there was an overall effect of group, but no effect of NT3 treatment compared with vehicle ($F_{2,53}$=8.49, p=0.001, *post hoc* NT3 *vs* vehicle p=0.433). There was also an effect of time ($F_{11,24}$=3.40, p=0.005) although interaction between time and treatment group was not significant ($F_{22,24}$=1.56, p=0.146). When gender was included as a factor in the model, a less severe impairment was seen in female rats (Figure 4-9, middle left) compared with male rats (Figure 4-9, bottom left; effect of gender, $F_{1,36}$=5.53, p=0.024), there was an effect of group, with an effect observed between the stroke treatment groups ($F_{2,53}$=8.91, p<0.001, *post hoc* NT3 *vs* vehicle p=0.399) and time ($F_{11,37}$=3.14, p=0.004) with an interaction between group and time ($F_{22,38}$=1.88, p=0.043).

Errors made with the less-affected forepaw (Figure 4-9, second left on the top row) were fewer than the affected forepaw, and as a result, no group effect was observed ($F_{2,25}$=3.29, p=0.051; *post hoc* NT3 *vs* vehicle p=0.946). There was an effect of time ($F_{11,311}$=10.6, p<0.001), but no interaction between group and time ($F_{22,30}$=1.79, p=0.070). On separating out effects due to gender, these same observations were made, albeit that the male animals (Figure 4-9, second left on the middle row) had worsened outcome in the less-affected forepaw (effect of gender $F_{1,24}$=5.72, p=0.025) compared with female rats (Figure 4-9, second left on the bottom row). There was a difference between treatment groups when including gender as a factor, but no difference between stroke groups ($F_{2,28}$=3.43, p=0.046, *post hoc* NT3 *vs* vehicle p=0.883), and an effect of time ($F_{11,31}$=12.5, p<0.001) but only a trend of interaction between time and group ($F_{22,32}$=1.79, p=0.066). This could be as a result of inappropriate brain activity in the less-dominant, unlesioned hemisphere.

The hindpaw on the affected side suffered a small impairment after stroke (Figure 4-9, second right on the top row), with a significant effect observed between groups, although this was not seen between stroke treatment groups ($F_{2,53}$=4.12, p=0.022; *post hoc* NT3 *vs* vehicle p=0.155). There was also an effect of time ($F_{11,437}$=2.81, p=0.002)

although an there was no interaction between treatment group and progression of the study ($F_{22,437}$=0.778, p=0.754). When gender is included as a factor in the analysis, female stroke rats (Figure 4-9, second right on the middle row) show no noticeable impairment in this task compared with male rats (Figure 4-9, second right on the bottom row; effect of gender, $F_{1,51}$=1.72, p=0.195). There was an effect of group, but no effect of stroke treatment on outcome ($F_{2,52}$=4.01, p=0.024; *post hoc* NT3 *vs* vehicle p=0.168), an effect of time ($F_{11,437}$=2.79, p=0.002), although there was no interaction between group and time ($F_{22,437}$=0.778, p=0.754). The less-affected hindpaw (Figure 4-9, top right) had no decrease in success in the ladder task after stroke, and no significant effect of group ($F_{2,38}$=1.33, p=0.276), but there was an effect of time ($F_{11,36}$=10.4, p<0.001), although no interaction between time and group ($F_{22,36}$=1.12, p=0.376). This pattern was also observed in the female (Figure 4-9, middle right) and male (Figure 4-9, bottom right) rats when analysed with gender as a factor ($F_{1,42}$=0.378, p=0.542). There was no effect of group ($F_{2,37}$=1.32, p=0.280), and no interaction between time and group ($F_{22,36}$=1.12, p=0.377), although an effect of time was seen ($F_{11,36}$=10.7, p<0.001).

Overall, this behavioural test was an appropriate measure of the deficit produced by MCAO and in elderly rats, this model produced a large, sustained deficit in this task. However, treatment had no effect on recovery of these animals. This will be discussed further in Section 4.5.4.

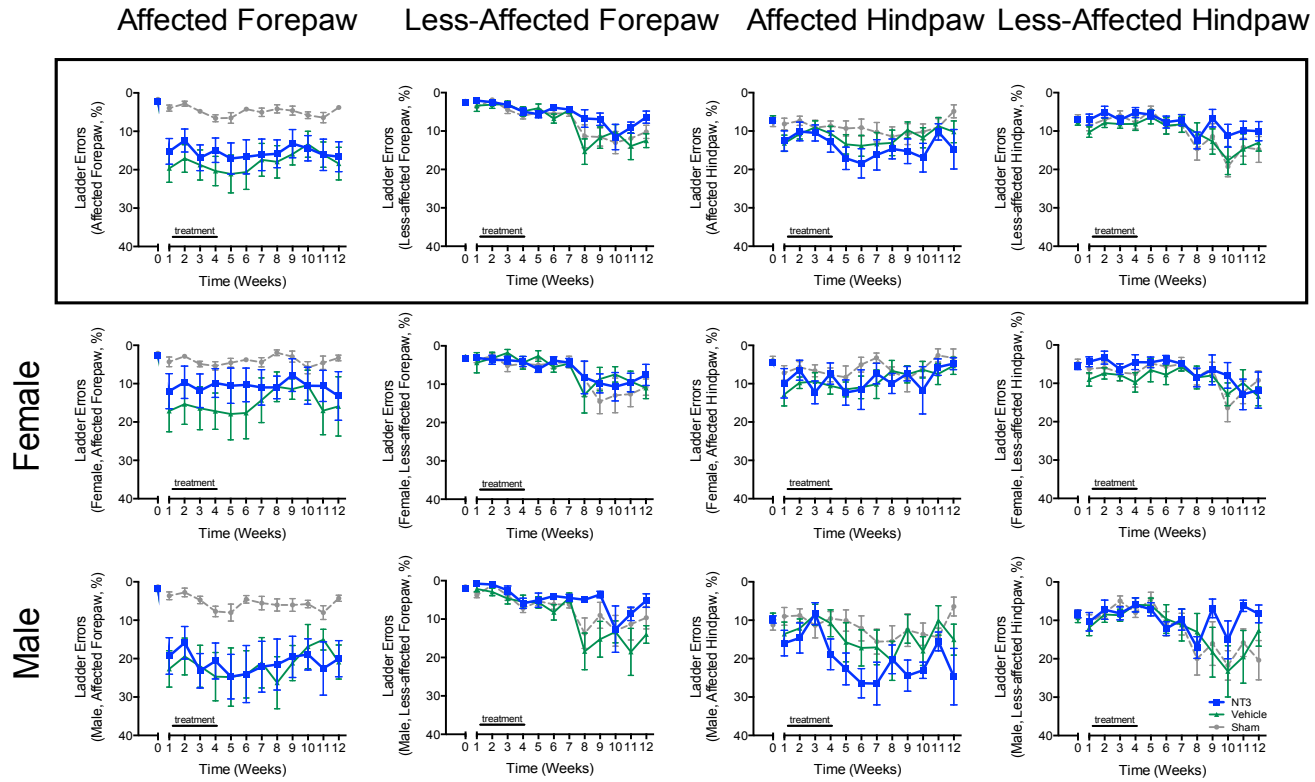


Figure 4-9 : Horizontal ladder assessment of locomotion after stroke injury in elderly male and female rats indicates no effect of NT3 treatment

Following MCAO, rats showed substantial impairments in motor control of their affected forelimb (top left). The two stroke groups made a similar number of foot faults 1 week after injury, with both the NT3 and vehicle groups beginning recovery. The NT3 treatment in this study does not result in motor recovery of the affected forelimb following stroke injury. The percentage of foot faults on the less-affected forelimb indicates no difference from sham in either of the stroke groups. Measuring the error rate when using the hindpaws in the horizontal ladder task, there was no observed difference of outcome between treatment groups throughout the study. Results displayed as mean±SEM. Statistics used are linear models. See Table 4-3 for details about numbers of animals throughout the study.

4.4.5.3 Sticky tape

Sensory responsiveness and dexterity was measured using the sticky patch test as described earlier (section 2.3.6.4). Stickers of equal radius were attached to each wrist and the time taken to contact and then remove them was measured. Before stroke, rats contacted the first sticker rapidly and subsequently removed it rapidly. 1 week after stroke, a deficit was observed in both stroke groups compared with sham (Figure 4-10, top left). The deficits from stroke injury were not sustained, shown by the effect of time observed that was statistically significant ($F_{11,36}$=3.13, p=0.005); the graphs show an initial deficit, followed by a spontaneous recovery and a plateau in performance over the 12-week study. There was also an overall effect of group, but no effect of NT3 treatment on these animals ($F_{2,55}$=3.87, p=0.027; *post hoc* NT3 *vs* vehicle p=0.574), and no interaction between group and time was observed ($F_{22,37}$=1.24, p=0.278). When accounting for differences in gender in the analysis, the recovery over the course of the study is significantly different between male (Figure 4-10, bottom left), and female rats (Figure 4-10, middle left; effect of gender $F_{1,25}$=4.43, p=0.046). There is an effect of group, but no difference between stroke treatment groups when this difference in gender outcomes is taken into consideration ($F_{2,54}$=4.21, p=0.020; *post hoc* NT3 *vs* vehicle p=0.585) and no interaction of group with time ($F_{22,37}$=1.23, p=0.286), although there is an effect of time ($F_{11,35}$=3.15, p=0.005). Lesioned animals showed little impairment in this task when using the less affected forepaw when compared with sham (Figure 4-10, second left on the top row). There was no effect of group ($F_{2,8.23}$=0.765, p=0.496), but there was an effect of time ($F_{11,18}$=3.10, p=0.018) and an interaction between group and time ($F_{22,17}$=2.93, p=0.014); indicated by the worsening in the task with this paw towards the end of the 12 week study. When accounting for differences in gender in the analysis, the recovery over the course of the study is significantly different between female (Figure 4-10, second from the left on the middle row) and male rats (Figure 4-10, second left on the bottom row; effect of gender $F_{1,11}$=11.6, p=0.006) groups separately. There is no effect of group when gender is included as a factor in the analysis ($F_{1,7.2}$=0.750, p=0.506), although there is an effect of time ($F_{11,17}$=3.28, p=0.014), and a significant interaction between group and time ($F_{22,17}$=2.87, p=0.014).

The time taken to subsequently remove the sticker from the paw is a measure of motor ability, and the processing ability from the sensing of a stimulus to planning and producing a motor response (Schallert et al., 2000). The time taken to sense the sticker was subtracted from the time to remove the sticker to obtain these values. The affected forelimb showed a small, sustained deficit (Figure 4-10, second from right on the top

row) after stroke injury compared to sham. There was no effect of group ($F_{2,55}$=2.68, p=0.078), although there was an effect of time ($F_{11,41}$=4.16, p<0.001) and an interaction between group and time ($F_{22,41}$=2.97, p=0.001). When accounting for gender as a factor in the analysis, female (Figure 4-10, second from right on the middle row) and male (Figure 4-10, second from right on the bottom row) animals showed no difference in performance based on gender ($F_{1,35}$=3.32, p=0.077). There was no effect of group ($F_{2,54}$=2.78, p=0.072), although there was an effect of time ($F_{11,41}$=3.85, p=0.001) and an interaction between group and time ($F_{22,41}$=2.93, p=0.001). This suggests that both stroke treatment groups improve behaviour to baseline levels over the course of the study. The subsequent removal of the sticker in the less-affected paw showed no effect of time ($F_{11,4.16}$=5.36, p=0.055), no effect of group ($F_{2,4.26}$=0.655, p=0.565), and no interaction between group and time ($F_{22,4.17}$=3.21, p=0.126). When accounting for gender in the analysis, female (Figure 4-10, middle row, far right) and male (Figure 4-10, bottom right) animals show an effect of gender in recovery ($F_{1,11}$=15.2, p=0.002). However, there was no effect of group ($F_{2,3.02}$=0.590, p=0.608), no effect of time ($F_{11,2.18}$=5.44, p=0.149), and no interaction between group and time ($F_{22,2.05}$=3.03, p=0.272).

Overall, the lesion to the dominant hemisphere did not produce deficits in sticky-tape test performance required to assess a treatment. Separating out the male and female groups resulted in more variable and difficult to interpret results throughout the behavioural tasks. An increased statistical power could have been reached through the inclusion of more animals in the experiments.

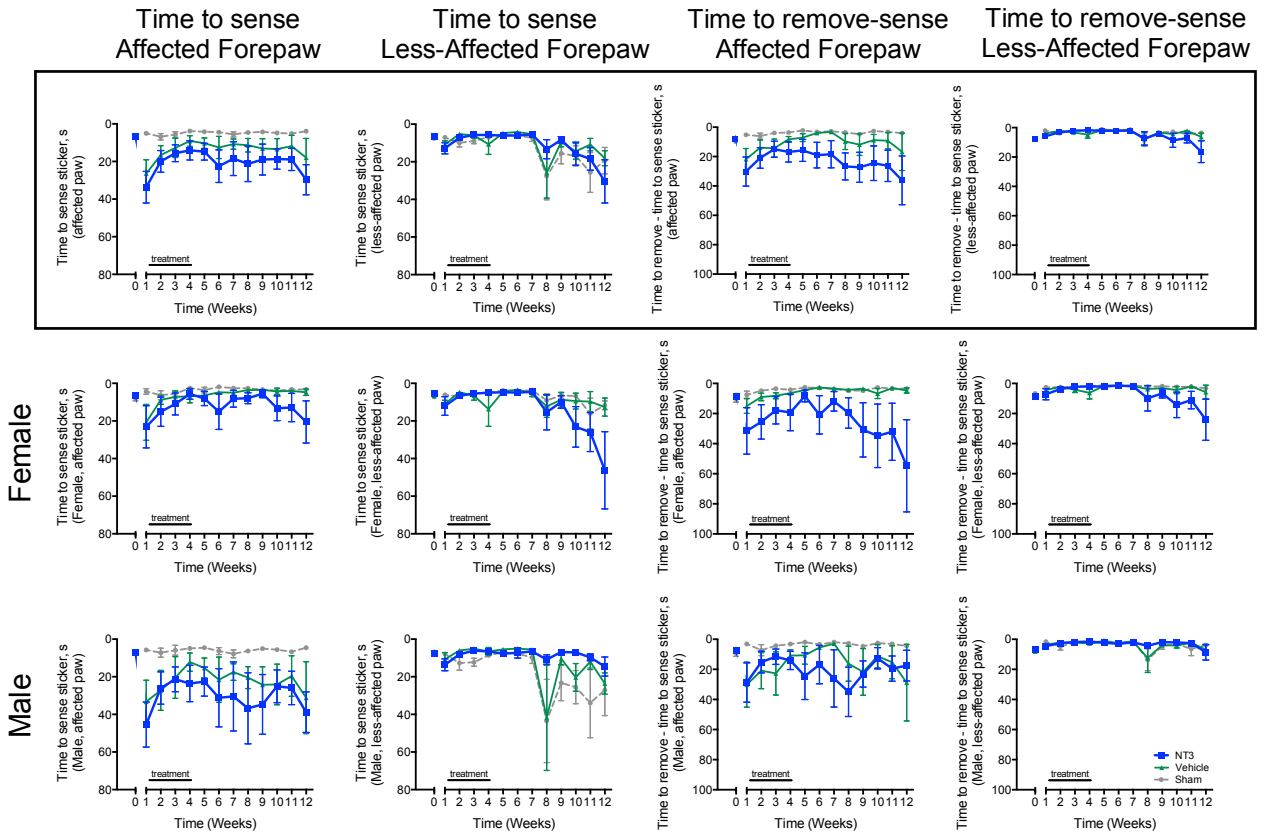


Figure 4-10 : Sensory feedback is impaired transiently in the bilateral extinction test after stroke injury, and no effect of NT3 is observed during recovery.

In the bilateral extinction test the time taken for rats to first contact the sticker label on the affected forelimb was measured (top left). This measure was also assessed in the less-affected forepaw (second left on the top row), in which there was no effect of treatment or time. This was split into female (middle row), and male (bottom row) data. The time taken to subsequently remove the sticker after sensing was less significant, with no substantial effect of treatment group in either the affected forepaw (second right on the top row) or the less-affected forepaw (top right). Female (middle row) and male (bottom row) split data emphasises the lack of effect from the individual gender baselines. Graphs represent mean±SEM and statistics analysed by linear models. Numbers of animals in each graph is presented in Table 4-3 since number varied between weeks

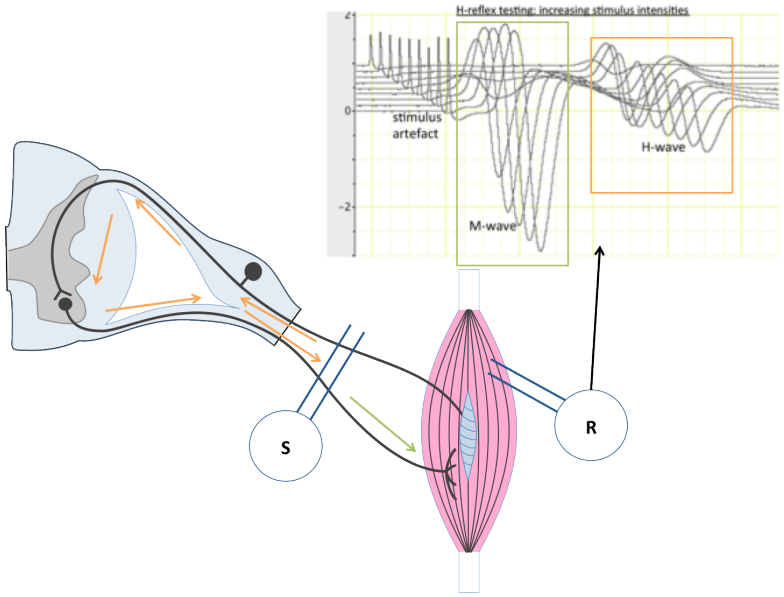
4.4.6 Hyper-reflexia is reduced with NT3 treatment in both elderly male and female rats after permanent distal MCAO.

In the third experiment, to measure the monosynaptic Hoffmann (H) reflex from the affected flexor hand muscle (the *abductor digiti quinti*) (Grey et al., 2008, Toda et al., 2014b) electromyograms were recorded 12 weeks after MCAO stroke during stimulation of the ulnar nerve through electrodes placed across the medial plantar surface of the wrist of the rat. The M-wave, the first short-latency EMG wave, occurs early after stimulation and is evoked by rapid motor axon excitation, and the H-wave is evoked by excitation of primary sensory afferents, which make synapses on motor neurons (Figure 4-11A). Data from the averaged M-wave (Figure 4-11B, middle column) and H-wave (Figure 4-11B, right column) have been included to indicate that there are no differences between groups or gender in the amplitudes of the M- or H-waves at each stimulus ($p>0.05$).

In sham rats, the H-wave was depressed by inter-stimulation intervals less than 1 s. After unilateral stroke, the H-wave was less depressed at intervals less than 0.5 s (ie, hyper-excitability), which suggests rats with MCAOs were more hyperreflexic (Figure 4-11B, top left). NT3 treatment restored the H-wave depression at these inter-stimulation intervals, indicated by an interaction between group and inter-stimulus interval ($F_{12,96}=2.47$, $p=0.007$). When comparing between groups, *post hoc* results show that NT3 is no different to shams at 0.1 s ($p=0.963$), but NT3 is significantly different from vehicle-treated stroke animals at the same inter-stimulus interval ($p=0.023$; two-way repeated measures ANOVA. There was an overall effect of inter-stimulus interval ($F_{6,96}=33.8$, $p<0.001$) consistent with the depression at short inter-stimulus intervals and no overall effect of group ($F_{2,16}=6.36$, $p=0.691$), which is likely to be because we included in our analysis inter-stimulus intervals at which depression is known not to occur (ie, 10 s, 5 s, 2 s, 1 s) and intervals at which depression is known to occur (0.5 s, 0.2 s, 0.1 s) (Toda et al., 2014b). In future it would be sufficient to analyse H reflexes only at the shortest inter-stimulus intervals (at which depression is known to occur). There was no effect of gender on the frequency-dependent depression ($p>0.05$), although the graph suggests that male animals had a reduced depression in the vehicle treated group compared with the same group in females; perhaps a higher powered study will support these findings.

Overall, these data indicate the NT3 may play a role in reversing the hyper-excitability and will be discussed further in section 4.5.6.

A.



B.

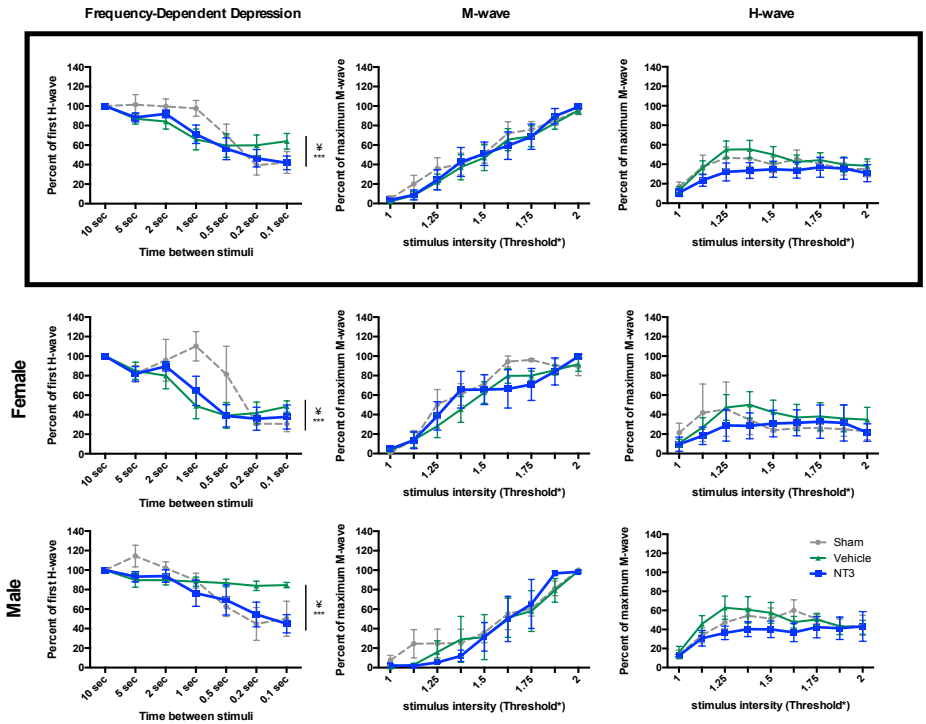


Figure 4-11 : Electrophysiological assessment of the H-reflex indicates rats with strokes develop hyperreflexia compared to sham, and NT3 ameliorates this effect.

Stimulation of the ulnar nerve (stimulating electrode, S) and recording of the M and H wave (by recording electrode, R) from the distal flexor muscles of the wrist (A). The effect of decreasing the inter-stimulus interval between two stimuli are assessed (B, left column). Sham animals (n=8) show no depression of the H-wave at inter-stimulus intervals of 1 s or more, but at less than one second, a depression in the response is seen. Stroke animals (n=8 per treatment group) experience hyper-excitability at shorter latencies between stimuli. An overall effect of stimulus (***) and interaction between stimulus and group was observed (¥), both p<0.001. M- (central column) and H-wave (right column) have been included to show no difference between groups. Graphs show mean±SEM and statistics are repeated measures two-way ANOVA.

4.5 Discussion

4.5.1 Study overview

This chapter shows that permanent, distal MCAO with bilateral common carotid artery occlusion is a feasible model of cortical stroke in elderly rats and that human recombinant NT3 can be infused subcutaneously as a clinically relevant administration route. Treatment with NT3 resulted in some behavioural recovery in elderly female rats in this in the second experiment in this chapter; benefits were observed in bilateral tactile extinction with the sticky tape test, and also with fine motor control in the staircase test. These benefits resulted from changes in spinal and brain locomotor circuits as assessed by tract tracing and proprioceptive reflex assessment by electrophysiology, which showed some significant enhanced plasticity and sensorimotor recovery in the NT3 treated groups. Delayed subcutaneous infusion of recombinant human NT3 is a viable method of drug delivery in which treatment initiated in a clinically-relevant time frame induced spinal neuroplasticity.

4.5.2 Lesion volumes

In large population studies, human stroke lesions range from 5–14% of the ipsilateral hemisphere (Brott et al., 1989, NINDS Stroke rt-PA Trial, 1995); malignant stroke accounts for approximately 10% of strokes and gives rise to larger infarcts, often requiring a hemicraniectomy to reduce intracranial pressure. Patients with smaller lesions are more likely to survive (Lövblad et al., 1997), and have a less severe symptom profile. We have shown in the studies in this chapter, a reproducible model that produces lesions that occupy a similar proportion of the hemisphere as many human strokes.

In the first experiment in this Chapter, large strokes were produced in young and elderly female rats through permanent distal MCAO with bilateral carotid artery occlusion in the hemisphere corresponding to the dominant paw (Figure 4-2A). In this pilot study, where young and elderly animals were compared, and young animals showed a broader range of lesion volumes at 24 hours compared with elderly rats, irrespective of the length of transient carotid artery occlusion duration, although the mean lesion volumes were similar. These findings are in line with some studies that state that there is no effect of age on the lesion volume (Duverger and MacKenzie, 1988, Wasserman et al., 2008), but not with others that have found an effect of age after focal ischaemia using the MCAO filament model of stroke (Davis et al., 1995), and this has also been shown in human

studies (de Leeuw et al., 2001). At 8 weeks after stroke lesions, the lesion volumes appear to reduce in volume because of resolution of the oedematous tissue; however, elderly animals have less resolution of the size of the lesion compared with their young animal counterparts. This is evident when taking a single dataset (elderly animals with 60 minute transient occlusion of the carotid artery contralateral to the lesion) and applying the Gerriets' equation; a result that had been previously significant when comparing 24 hour data to 8 week data is non-significant when accounting for the transient effect of oedema (Figure 4-2B), allowing comparisons to be made between lesion volumes in the first 24 hours after stroke with later time-points (Wayman et al., 2016).

One additional benefit to using the Gerriets' formulae over traditional calculations of lesion volume is to not only account for oedema, but also the difference in total volume of the rat brain in young animals compared with larger, elderly animals.

Experiment 1 was used as a pilot study to determine the length of occlusion required to produce a sustained deficit in elderly rats without high mortality rates. 60-minute occlusions were found to be suitable for this purpose, and resulted in a lower mortality rate than 90 minute occlusion of the common carotid artery.

Additionally, the larger sizes of male rats resulted in larger lesion volumes in the brain, which may have accounted for larger mortality rates observed in these animals (Figure 4-2D); surviving male animals with 45 minute transient occlusions of the common carotid artery had similar lesion volumes to female rats that had 60 minute occlusions. However, male animals died more frequently when being occluded for 60 minutes, which is why occlusion time was reduced further into the study; this was on account of larger, malignant stroke formation, which was confirmed by structural MRI (data not shown). Atropine sulphate was given (0.05 mL of 600 µg/mL solution, *s.cut*) to reduce tracheal secretions (Ryan et al., 2006, Wayman et al., 2016).

4.5.3 Treatment with NT3 using subcutaneous infusion.

Subcutaneous treatment with NT3, initiated 24 hours after stroke, caused some changes in spinal locomotor circuits, and promoted some recovery of sensory and motor function in elderly female rats in the second study. The fact that NT3 can reverse disability even when initiated 24 hours after stroke is exciting because the vast majority of stroke victims are diagnosed within this time frame (Evenson et al., 2009). Thus, NT3 could potentially be used to treat a substantial number of patients.

NT3 itself has good clinical potential. There is good conservation from rodents to primates and humans in expression of NT3 receptors in the locomotor system (Arvidsson et al., 1994, Yamamoto et al., 1996, Duberley et al., 1997, Brock et al., 2010). In line with this, it could provide an effective and simple treatment for stroke patients when NT3 is delivered *via* subcutaneous or intravenous infusion following hospitalisation. Phase 2 clinical trials show that subcutaneous doses of NT3 up to 150 µg/kg/day are well tolerated and safe in healthy humans and in humans with other conditions (Chaudhry et al., 2000, Coulie et al., 2000, Parkman et al., 2003, Sahenk et al., 2005, Sahenk, 2007), which provide encouraging information for the use of NT3 as a therapy for stroke. Importantly, it has been shown previously that radiolabelled human NT3 rapidly crosses the intact blood–brain barrier and accumulates stably in the cortex, striatum, and other regions of the nervous system when infused into the bloodstream (Poduslo and Curran, 1996). Recombinant human NT3 is relatively stable in the bloodstream and is delivered mostly quickly from blood into the cervical spinal cord compared with brain and other regions of spinal cord (Pan et al., 1998).

We attempted to use a high dose of NT3 in the experiments in this chapter: however, the levels of NT3 available in the blood were low, and at times, difficult to discern from the vehicle control animals. In the elderly female study, ELISA data indicated the presence of 13.9±2.31 pg/mg in the blood normalised to the BCA assay for protein content (Figure 4-3A), which was similar to concentrations observed in elderly male and female rats in the final study in this chapter (11.1±2.83 pg/mg and 8.17±2.88 pg/mg respectively, Figure 4-3B). However, in the second experiment, all but one rat in the NT3 treatment group had concentrations that were higher than the vehicle treated rats whereas in the final study of this chapter far fewer NT3 trested rats had higher levels than control animals. This could explain why the mixed gender study resulted in a smaller mean increase in NT3 concentration than in the second study. Furthermore, when compared with the concentration of NT3 observed in Chapter 2, where concentrations of NT3 reached 56.6±4.34 pg/mg 8 weeks after AAV-NT3 treatment, the concentrations achieved in the experiments presented in this chapter resulted in much less available NT3 in comparison, although recombinant protein would be present in the bloodstream at a much earlier point after stroke than would be expected when using viral vectors, which typically begin expressing protein after 1 week, and begins to plateau at 3–4 weeks (Murphy et al., 1997). Increasing the dose of NT3 given could counteract this issue. An additional factor was the quality of the recombinant protein used in these studies. The age of the NT3 used in these experiments was approximately 10 years old. When stored correctly, this should not be a large issue, but there could still

be issues related to aggregation or degradation. Aggregation can cause errors in protein measurement, and could have contributed to the relatively low amounts of available protein in this study as performed in the ELISA.

In Chapter 5, we will use an alternative newly-synthesised preparation of recombinant NT3 and increase duration of treatment to maximise the potential effect of NT3 and promote additional recovery after stroke, which might be especially likely if combined with appropriate rehabilitation (Wahl et al., 2014), although this is beyond the remit of this thesis. Other members in our lab are considering the use of rehabilitation and investigating the combined effect with NT3.

4.5.4 Behavioural analysis indicates some significant improvement of motor tasks following treatment with NT3

Following a human stroke, the most common impairments are hemiparesis of the contralateral upper limb, deficits in sensory function (Cramer et al., 1997, Langhorne et al., 2009), and often involve interruption of the CST (Whishaw et al., 1993, Whishaw and Metz, 2002, Starkey et al., 2005). This model of stroke incorporates these three key processes, resulting in a sustained deficit in all of our motor and sensory tests when used in elderly rats. One benefit of the behavioural tasks used in this chapter was that they were suitable for measuring a sustained deficit in elderly rats, particularly on the horizontal ladder task (Figure 4-5 and Figure 4-9). This provides an excellent point from which to measure the functional effect of NT3.

Subcutaneous infusion of NT3 resulted in significant recovery on the horizontal ladder (Figure 4-4) and Montoya staircase (Figure 4-5) tasks in elderly female rats, but conferred no benefit in the mixed gender study (Figure 4-8–Figure 4-9). This could be due to insufficient sample sizes, as elderly male animals were less motivated to complete the behavioural tasks and, therefore, provided an insufficient baseline for scoring in this factorial-designed study. Even though male rats were on a restricted diet throughout the study period, many were not able to be incentivised to perform in behavioural tasks; on this basis, it would be suggested to avoid their use in behavioural-related functional assessment, although their larger size might make them more ideal for histological or electrophysiological outcomes.

As discussed in the introduction of this chapter, sensorimotor impairment is a sign of ageing involving a decline in perception, locomotion, and motor control in both rodents and humans (Bergman et al., 1999, Ulfhake et al., 2000). The receptor for NT3 is TrkC,

which is expressed throughout the CNS (Merlio et al., 1992) and ageing results in significant decreases in both mRNA and protein levels of TrkC and NT3 (Johnson et al., 1996, 1999, Ming et al., 1999, Ulfhake et al., 2000). In the mature nervous system, neurotrophins have been implicated in the control of the growth and the retraction of terminal nerve fibres. Therefore, in ageing, a decrease in TrkC within sensory neurons or a failure of targets to synthesise NT3 indicates a reduced capacity for these neurons to bind and transport target derived, as well as locally produced, NT3 (Bergman et al., 1999, Ming et al., 1999). The decrease in TrkC mRNA in aged rats could be due to axonal dystrophy of proprioceptive sensory fibres, which would lead to decreased amounts of NT3 observed with age (Fundin et al., 1997a, Fundin et al., 1997b, Ulfhake et al., 2000). Following nerve injury, TrkC receptors are downregulated even further (Bergman et al., 1999). Because of this decrease in muscle NT3 levels in elderly rats, it is important to assess the role of NT3 in an aged model of stroke as NT3 receptor synthesis may be altered. However, a higher dose of NT3 may be necessary to counteract decreased levels of TrkC receptors.

Perhaps using more challenging studies, such as the single pellet retrieval task, would be better suited to measuring the deficits from this model (Whishaw et al., 1993, Gonzalez and Kolb, 2003), in addition to the use of an increased dose of NT3. These will be considered in more detail in Chapter 5.

4.5.5 Tract tracing and Histology

The results in this chapter are relevant to other studies shedding light on the mechanisms whereby sensory cues enhance recovery after CNS injury in human beings and other animals (Harkema et al., 2011, Takeoka et al., 2014). It has been shown previously that proprioceptive signals can cause sprouting of descending locomotor pathways resulting in an enhanced recovery after unilateral spinal cord injury as genetic depletion of muscle spindles diminishes central plasticity and impairs recovery (Takeoka et al., 2014). It has also been shown that corticospinal axons express NT3 receptors in adult rodents and primates (Brock et al., 2010) and these may be modulated after spinal cord injury.

We now extend these findings by showing that NT3 can cause sprouting of descending locomotor pathways and enhance locomotor recovery after unilateral stroke when onset of treatment is delayed by 24 hours. Thus, to some extent, subcutaneously-infused NT3 might mimic proprioceptive signalling after neurological injury. This idea is also consistent with work from postnatal mice showing that a single injection of NT3 can

restore monosynaptic connectivity between proprioceptive afferents and spinal motor neurons with genetically depleted muscle spindles (Wang et al., 2012). From these data, the mechanisms whereby NT3 promotes recovery can be explored in more detail. How NT3 enables the sprouting of the spared CST is exciting, and one hypothesis is that NT3 enters the DRG and causes changes in local gene expression in proprioceptive neurons in adult rats after stroke, as has been shown in development, which then drives central changes (Lee et al., 2012b). Central sprouting and some spontaneous recovery has been observed after SCI in mice, but when genes encoding muscle spindles are knocked out, this observation is no longer seen (Takeoka et al., 2014). This suggests that a peripheral signal drives central plasticity, and that changes in DRG could be relevant to improved functional ability after stroke. It has been shown previously that neurotrophins accumulate in sensory or sympathetic ganglia after subcutaneous, intravenous, intramuscular, or intraneural administration following transport in the bloodstream or retrograde transport in nerves (DiStefano et al., 1992, Tria et al., 1994, Helgren et al., 1997, Curtis et al., 1998). Many groups have shown that the dorsal root ganglia is highly vascularised and contain fenestrated endothelial cells which make its blood-nerve interface leaky to proteins as big as albumin, which suggests that NT3 might also be transported (Jimenez-Andrade et al., 2008). We had hoped to perform RNASeq to identify transcriptional changes in the DRG that might cause the observed sprouting in this study, but because of the low serum levels of NT3 and the failure to observe behavioural recovery in Experiment 3, possibly due to protein aggregation, this shall be explored further in Chapter 5.

In Experiment 3, the goal was to look at trans-synaptic connections of the CST (Hutson et al 2016) but in hindsight we used too great a volume of AAV-tdTomato at a single site and diffusion was greater than desired. This, combined with the time available, has meant that this tissue has not yet been processed.

4.5.6 NT3 improved proprioceptive reflex arc hyperexcitibility after stroke

NT3 modifies the strength of connections between proprioceptive afferents and motor neurons in development and newborn rats (Mendell and Arvanian, 2002). There is some data indicating that NT3 cannot modify a proprioceptive reflex in intact mammals after the second postnatal week unless complex combination treatments are administered (Arvanian and Mendell, 2001, Mendell et al., 2001, Arvanian et al., 2006a, Arvanian et al., 2006b, Hunanyan et al., 2013). However, our data indicate that NT3 can, in fact, by

itself, modify a proprioceptive reflex in the adult mammal after CNS injury. When looking at spinal reflexes involved in movement, the M-wave is the first activity wave when recording EMGs from muscles, and is a measure of motor axon excitation. The recruitment of the motor axons and M-wave at different stimulation intensities remains unchanged in animals after stroke (Figure 4-11B, middle column). The H-wave, with approximately 5 ms delayed latency, assesses the strength of synapses between proprioceptive afferents to motor neurons in the spinal cord. The recruitment of the H-wave at different stimulus intensities remained unchanged in this study (Figure 4-11B, right column), suggesting that the axons excitability remains unchanged and that there is no difference in conduction properties. Normally, muscle spindles mediate excitatory and inhibitory signals *via* proprioceptive neurons; indeed, mice lacking functional muscle spindles (lacking proprioceptive feedback and retrograde signalling by NT3) show impaired re-organisation of spinal circuitry and restricted locomotor recovery after CNS injury compared to animals receiving NT3 (Akay et al., 2014, Takeoka et al., 2014). In this study, we assessed the frequency-dependent depression, where a second stimulus is initiated at varying delayed intervals after the control stimulus (Figure 4-11B, left column). The H-wave was reduced in sham animals when there is a small latency between stimuli (less than 0.5 ms). This effect was reduced in the vehicle-treated rats, indicating that these rats developed hyper-reflexia. The NT3-treated animals restored the H-wave depression and were more similar to the sham animals at shorter latencies, indicating that small concentrations of NT3 in the blood stream is still able to mediate a significantly beneficial effect. This will be explored further in Chapter 5, when a larger dose of NT3 will be delivered to animals to identify whether an additional benefit of NT3 can be observed.

4.5.7 Conclusions

This study was novel as we have now shown that delayed subcutaneous treatment of disabled forelimbs with recombinant human neurotrophin-3 (NT3) causes functional recovery. Structural magnetic resonance imaging at 24 hours and 8 weeks after stroke showed that NT3 did not induce neuroprotection, and the use of the Gerriet's equation allows estimation of lesion volume without oedema. Furthermore, anatomical anterograde tracing from the less-affected hemisphere showed that CST axons sprouted from the unlesioned to the lesioned side of all levels of the cervical spinal cord. Electrophysiological recording of the spinal reflex showed that hyper-reflexia seen in lesioned animals was attenuated following treatment with NT3. However, little

behavioural benefit was observed using NT3 in this study—perhaps because of the low availability of NT3 combined with the decreased availablility of TrkC as observed in other studies (Fundin et al., 1997b).

In summary, delayed treatment with NT3 induces anatomical and electrophysiological plasticity, but an increased dose of NT3 may help to further enhance functional benefit at a behavioural level. The next chapter will also look into RNASeq to begin to elucidate the mechanism behind these benefits observed using NT3.

Chapter 5 Delayed infusion of human NT3 subcutaneously after stroke improves hyperreflexia in elderly female rats.

5.1 Abstract

In the final study reported in this thesis, subcutaneous infusion of recombinant NT3 protein or vehicle was delivered to elderly female rats after large cortical stroke. Owing to the low blood serum concentrations of NT3 observed at 4 weeks in the previous chapter when NT3 was delivered subcutaneously for 4 weeks, a new preparation was produced for use in this study. The three-vessel occlusion model was used, and treatment initiated at 24 hours. Although sensorimotor behavioural tasks did not show significant improvements in functional outcome in the NT3 treated animals, the use of H-reflex electrophysiology showed normalisation of proprioceptive reflexes. An exploratory RNASeq analysis on cervical DRG at 5 and 11 weeks after stroke revealed interesting changes in mRNA regulation, including changes to glutamate receptor, semaphorin, and macrophage genes, and including changes to FGF and apoptotic pathway signalling. This opens up questions as to what peripheral mechanisms are involved in enhanced recovery seen following delayed treatment of NT3 after stroke injury.

5.2 Introduction

In the previous studies in this thesis, we have observed a modest recovery in elderly rats when we delivered NT3 subcutaneously for 4 weeks 24 hours after permanent, distal MCAO. In this chapter, we hoped to maximise the recovery observed by using higher doses of NT3 treatment for a longer duration than used previously to maximise the potential for recovery.

In this study, NT3 was delivered subcutaneously for translational relevance. It has been shown previously that high, repeated subcutaneous doses of recombinant NT3 are safe and well-tolerated in Phase 1 and 2 clinical trials for other disorders (Chaudhry et al., 2000, Parkman et al., 2003, Sahenk, 2007, Sahenk et al., 2014). This is a clinically-viable route after ischaemic stroke in humans. Peripherally administered neurotrophins have been shown to accumulate in sensory ganglia where they induce gene transcription, primary afferent axon growth, and synapse strengthening within locomotor circuits (DiStefano et al., 1992, Taylor et al., 2001, Patel et al., 2003, Lee et al., 2012b, Wang et al., 2012, Duricki et al., 2016b). After peripheral delivery, NT3 might be accumulating in the DRG; which could be possible as Jimenez-Andrade et al. (2008) showed that DRG are highly vascularised and that they are permeable to proteins that are larger than 15 kDa NT3 (eg, Evans blue labelled albumin; 68 kDa) when given intravenously.

More than 90% of strokes occur in people over the age of 65, and these patients frequently present with comorbidities such as carotid arterial stenosis (Truelsen et al., 2006). As a result, this study measured the effectiveness of NT3 in elderly rats with large cortical strokes caused by distal middle cerebral artery occlusion and simultaneous common carotid occlusion. Treatment was initiated 24 hours after cortical ischaemia as most stroke victims are admitted and diagnosed within this timeframe (Harraf et al., 2002, Evenson et al., 2009).

In previous work from our group, NT3-induced sensorimotor recovery after stroke has been shown to increase over several months and continues even after infusion of NT3 is complete (Duricki, unpublished). We hypothesised that 5-week subcutaneous treatment with NT3 would induce changes in gene expression in DRG that persist for months.

To identify whether changes in DRG gene expression are transient or permanent, and to understand the involvement of different molecular pathways in the recovery process, RNASeq was used in this study. RNAseq can be used to identify changes in DRG gene expression after subcutaneous NT3 treatment and to provide insight into the molecular mechanisms behind functional recovery observed following ischaemic stroke treated with

NT3. It is possible that NT3 causes DRG neurons to make, centrally transport and secrete factors that induce corticospinal plasticity (eg, BDNF) or that NT3 causes proprioceptive DRG neurons to strengthen their connections with motor neurons (eg, produce synaptic proteins *via* transcription factors) and to normalise spinal reflexes. In fact, inhibitory modulation of interneurons has been shown through the synthesis and secretion of BDNF by proprioceptors into the postnatal spinal cord (Betley et al., 2009). This could have ramifications for the proprioceptive reflex arc as well as corticospinal tract adaptive plasticity, resulting in functional recovery (Jiang et al., 2016). Little is known about the molecular process of recovery in stroke patients. One working hypothesis in our laboratory is that after stroke, the adult DRG synthesise and secrete BDNF, causing spinal sprouting when provided with sufficient NT3.

Hyperreflexia, an associated feature of spasticity, is a hyper-excitability of spinal reflexes including the proprioceptive stretch reflex (Dietz, 2002, Dietz and Sinkjaer, 2007b). Proprioceptive afferents projecting from muscle spindles via the dorsal root ganglia (DRG) into the spinal cord synapse onto that muscle's motor neurons. NT3 is essential for the survival and correct patterning of the proprioceptive system in development (Patel et al., 2003). Overexpression of NT3 might be able to normalise spinal excitability *via* proprioceptive afferents leading to balanced sensorimotor muscle control, normoreflexia, and functional recovery in the adult after CNS injury with sufficiently high doses of NT3.

RNASeq allows the identification of a larger number of candidate transcripts than microarrays. Additionally, RNASeq can interrogate the whole genome regardless of prior annotations, being able to detect transcription from areas of the genome not currently annotated as exons, which is a limitation in the interpretation of the study presented in this chapter. It is hoped that the results from the analysis in this chapter could then be used to identify transcripts that are involved in the recovery process after ischaemic stroke; previous RNASeq studies have shown that only 56–58% of the transcripts have been previously aligned with annotated exons (Perkins et al., 2014).

5.3 Methods and Materials

5.3.1 Experimental Design

All procedures, behavioural testing and analyses were done using a randomised block design (Altman and Bland, 1999, Moher et al., 2010, van der Worp et al., 2010), and all procedures remained blinded until the end of the study. The experiments were designed in accordance with guidelines from the Stroke Therapy Academic Industry Roundtable (STAIR) (Fisher et al., 2005, Fisher et al., 2009) and others (Macleod et al., 2009). All procedures were performed in accordance with the UK Home Office guidelines and Animals (Scientific Procedures) Act of 1986.

The permanent distal three-vessel MCAO model was used in the experiment presented in this chapter. A schematic for the design of the experiment is shown below (Figure 5-1). The lesion was applied to the hemisphere corresponding to the dominant forepaw of the rat, as measured by overall handedness in the staircase test, bilateral tactile stimulation, and single pellet reaching tasks. At 24 hours after stroke onset, osmotic pumps were inserted subcutaneously for 5 weeks. Behaviour was done at baseline and weekly following surgery on the horizontal ladder, Montoya staircase, single pellet reaching, and bilateral tactile stimulation tests and structural was performed *ex vivo* at 12 weeks after stroke. 20 animals (33%) died or were humanely killed in total within 24 hours of the stroke surgeries. In contrast to other studies presented in this thesis, this experiment did not use MRI to confirm the lesion sizes, as the equipment was unavailable; in this case ex-vivo scans were done at the end of the study to verify the presence of a lesion (data not shown).

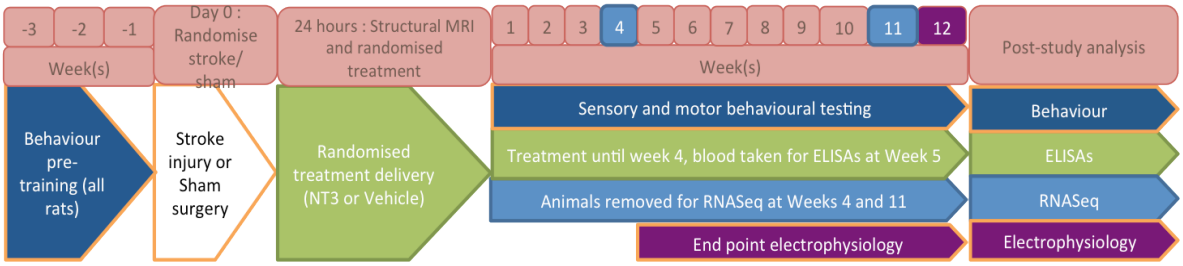


Figure 5-1 : Schematic of the study in this chapter

5.3.2 Subjects

In this chapter, 90 elderly female outbred Lister Hooded rats were used (Table 5-1). Rats were approximately 20 months old with weights ranging from 250–350 g. These animals were obtained from Charles River, UK aged 3 months and rats were kept in-house until approximately 18–20 months old before commencing the study. Animals were housed in groups of 3 or 4 on a 12:12 light-dark cycle and had *ad libitum* access to food and water. Rats were familiarised with veterinary recovery gel (ClearH20, TripleT) and soft chow for at least 48 hours before stroke surgery. As animals can find difficulty with eating and drinking after the procedure, animals were introduced to these items before surgery, to minimise neophobia. Additionally, animals were given prophylactic antibiotics, which was recommended by our Named Animal Care and Welfare Officer (NACWO) given the tendency of Lister Hooded rats to become infected. We gave Baytril 2.25% solution (enrofloxacin, 0.2 mL/kg, once every 2 days) in the 2 days before surgery, and one dose alongside pain relief, and then again as required.

	Elderly, NT3-treated stroke (n=35)	Elderly, vehicle-treated stroke (n=35)	Sham animals (n=20)
Baseline assessment*	33	28	19
Died during surgery	18	7[#]	0[#]
Animals in behavioural study	19[±]	19	17
Animals remaining after removing animals for RNASeq after week 5 (RNASeq animals)	12 (6)	13 (6)	13 (4)
Animals removed for RNASeq at week 11	6	5	6
Animals completing electrophysiology	6	8	7**

Table 5-1 : Animals included in various analyses in the experimental design presented in this chapter

**animals were humanely culled before the study, if presenting with piloerection, inoperable tumours, the inability to maintain core body temperature, or unexplained weight loss. [#]n=2 animals per group were moved to the NT3 group during surgery week to weight treatment groups appropriately. [±]1 animal was removed from the study after 1 week behavioural assessment because of a severe weightloss, which was not attributable to water loss. **1 animal was culled at week 10 because of deterioration in health, piloerection, and slow movements.*

5.3.3 Surgical procedures

5.3.3.1 Stroke Surgery

Before surgery, animals were allocated randomly to either stroke or sham surgery groups. Procedures were done in a randomised block design to minimise bias. Lesions were induced on the hemisphere contralateral to the preferred paw, determined by the average pre-operative preference in the Montoya staircase test, bilateral tactile

extinction test, single pellet reaching and horizontal ladder task. Surgeries were done alongside S Kakanos, who also acquired data from the behavioural tasks and did the behavioural analysis.

Rats were anaesthetised using 5% isoflurane in 1.5 L/min O_2. Following induction of anesthesia, the amount of isoflurane was reduced and maintained at an adequate but minimised depth for surgery (eg, 1–2%). Lidocaine cream was applied topically to the ventral neck and temporal region of the head 10 minutes before the first incision, and pain relief (Carprieve, 0.25 mg/kg, *s.cut*) given peri-operatively for pain relief. Animals were maintained at a temperature of 36.5–37.5 °C throughout surgery with the use of a rectal probe and homeothermic system. Atropine sulphate was given (0.05 mL of 600 µg/mL solution, *s.cut*) to reduce tracheal secretions (Ryan et al., 2006, Wayman et al., 2016).

Stroke rats (n=67) were placed in the supine position and a ventral midline cervical incision was made to allow isolation of both common carotid arteries using a silk suture (5/0, non-absorbable sutures), taking care not to damage the muscle or the vagus nerves. Animals were then placed in a lateral position and the MCA exposed and occluded as described in Section 2.3.3.1.1, with a constant infusion of saline (flow rate approximately 2 mL/min) and aspirator was set up over the craniotomy site to remove debris and clear minor bleeding during this stage of the surgery, as described previously (Figure 4-1A). The rat was turned back to a supine position and the carotid artery ligated on the same side as the occluded MCA permanently by tying a knot in the silk suture around the carotid, and the left carotid artery was occluded transiently for 60 minutes using a 13 mm stainless steel artery clip with 125 g pressure (2.3.3.1.2).

For sham animals, the ventral part of the neck and temporal regions were incised but no artery occlusion was used. Sham animals had procedures up to but not including craniotomy in this protocol, as this procedure can produce behavioural deficits (Adams et al., 1994).

5.3.3.2 Post-surgical care

After surgery, animals were placed in a 31 °C incubator for up to 4 hours to recover from anaesthesia. Fluids were given daily for the first 3 days to prevent dehydration. Carprieve (0.25 mg/kg) was given for pain relief at 24 hours. Because all animals receive the same dose of Carprieve, any neuroprotection is controlled for systematically and is

likely to be negligible. Soft food and rehydration gels were made available to the animals in a cage lined with an absorbable pad rather than loose bedding for the first 24 hours.

After surgery, a temporary weight loss of up to 20% was permitted compared with baseline, and in some cases 30% was permitted in consultation with our NACWO or Named Veterinary Surgeon in accordance with our approved Home Office Project Licence protocol. In all cases of weight loss, we undertook special measures to care for our rats. For example, postoperative care to prevent and counter dehydration is described below.

5.3.4 Treatment with NT3 or vehicle through subcutaneous osmotic pumps.

24 hours after stroke rats were allocated to treatment using a randomised block design. Rats were anaesthetised as above and an incision was made on the flank on the impaired side and, using blunt dissection, a subcutaneous 'pocket' was formed. The osmotic mini-pump (2004, Alzet Figure 5-1) was positioned in this subcutaneous space and contained either vehicle or vehicle containing recombinant NT3 (Peprotech). Vehicle was PBS containing 20 mM sodium acetate, 140 mM NaCl and 0.1% bovine serum albumin; Sigma; A3059; 9048-46-8) pH 7.4 (filter sterilised). NT3 was made up in vehicle to 7 mg/mL. According to the lot datasheet, mini-pump average fill volume was 238 µl (thus 1.67 mg per pump) and flow rate would be 0.23 µl/hour for 40 days (thus 42 µg/24 hours). The NT3 dose (42 µg/24 hours) was higher than that typically used in previous successful experiments (12 µg/24 hours) (Bradbury et al., 1998b, Ramer et al., 2000) including one of our own involving intramuscular infusion (Duricki et al, unpublished results), and similar to the study in the previous chapter (47 µg/24 hours). For a 400 g elderly rat, this total dose corresponds approximately to a high single dose given subcutaneously in human clinical trials when adjusted for bodyweight (150 µg/kg/day). All minipumps were prepared one day before implantation and incubated for 48 hours in 0.9% saline at room temperature. One pump implanted at 24 hours was sufficient for forty days of treatment and therefore does not need to be replaced after 14 days (as in our previous studies). This pump is smaller than we have used in previous studies and we found it was tolerated better. Skin was sutured and analgesic administered as previously. All rats survived this surgery. Allocation concealment was performed by an independent person coding NT3 and vehicle stocks, and all experimenters remained blinded until the end of the study. Pumps were removed after 5 weeks of treatment.

5.3.5 Enzyme-linked Immunosorbent Assays (ELISA)

At 5 weeks, pumps were removed by anaesthetising the animals as described previously. During this procedure, 2 mL of blood was extracted through cannulation of the tail vein. The flank containing the pump was then incised to remove the pump, the skin sutured, and pain relief given as described previously.

12 weeks after stroke surgery, rats were terminally anaesthetised with Sodium Pentobarbital (Euthatal) and a 2 mL sample of blood taken from the heart.

Blood was left to coagulate at room temperature for at least 15 minutes before spinning down at 1,800 x g on a benchtop centrifuge for 15 minutes and the supernatant was snap frozen in liquid nitrogen prior to storage at –80°C. ELISAs were done on diluted serum samples (1:10) with the Human NT-3 DuoSet kit (DY267, R&D systems) according to manufacturer's instructions with some modifications as described in (Section 2.3.7).

5.3.6 Behavioural Techniques

Rats were handled every 2 weeks for 9 months before the commencement of the study, since we weighed and checked animals to ensure there were no age-related illnesses. 5 weeks before surgeries, rats were fully trained on the horizontal ladder and both the staircase and single pellet tasks before the study began. Two preoperative baseline scores for all behavioural tasks were collected 1 week before surgery. Handedness was decided by dominant paw in all three tests. Two baselines were taken to improve statistical power (Vickers 2003).

5.3.6.1 Montoya Staircase

The Montoya Staircase Test is designed to measure changes in gross and fine forelimb skilled movements following motor system damage (Montoya et al., 1991). Details of this behavioural task and pretraining were previously described in Section 2.3.6.3. An adaptation was used in this chapter to include coloured pellets to further analyse the ability of the animals to reach more out-of-reach pellets (Kloth et al., 2006). However, the graphs are presented as in previous chapters because of time constraints.

5.3.6.2 Bilateral tactile stimulation test:

To assess the magnitude of somatosensory neglect and sensorimotor impairments in forepaw function after stroke the bilateral tactile stimulation test was done (Schallert et

al., 1982; Schallert et al., 2000a). Details of this behavioural task were previously described in Section 2.3.6.4.

5.3.6.3 Single pellet reaching task

Impairments in skilled hand tasks are seen in humans after many neurological conditions including stroke (Foroud and Whishaw, 2006, Harris-Love et al., 2011), and similar observations have been noted in rodent models of the disease (Gharbawie et al., 2005a, 2008, Whishaw et al., 1991). As such, rodent models of skilled reaching have excellent transferability (Cenci et al., 2002, Iwaniuk and Whishaw, 2000, Annett et al., 1995, Brooks and Dunnett, 2009).

One task developed to measure skilled reaching in rodents is the single-pellet reaching test (Klein and Dunnett, 2012, Whishaw et al., 2008a,b, 1991, Farr and Whishaw, 2002, Metz and Whishaw, 2000). Rats were trained to advance their preferred forelimb through the slot in the apparatus (Figure 5-5), grasp the sugar pellet, and withdraw their paw to bring the pellet to the mouth for eating. Thus, each reaching act is an individual event comprised of postural adjustments in preparation for reaching, the reach itself, grasping, and withdrawal and consumption of the food. Training lasted for 5 weeks. Animals were first habituated to sugar pellets in their home cage for 2–3 consecutive days to familiarise themselves with the food source. Next, animals were acclimatised to the single pellet reaching apparatus. The first session required the animals to spend 10 minutes in the Perspex box to habituate, with pellets placed inside the main section, and on the reaching shelf to encourage reaching. After this stage, animals would spend time in the box daily, with pellets were placed on the shelf close enough to the opening for the animals to be able to reach with their tongue. If sufficiently interested, further trials would then have multiple pellets on the shelf at a time to encourage reaching behaviour, and pellets were moved further away over time. By the middle of the second week of training, rats were familiar with the environment, and were able to identify that pellets would become available from the slit in the front of the box. Pellets were moved further away from the slit to encourage paw use. Once animals were able to reach with both paws, training was spent with one week of establish dominance: to do this, pellets were placed in the shelf recesses (See Figure 5-5A for schematic) and animals allowed to freely retrieve pellets, with pellets replaced whenever they were eaten. Finally, once rats rats were successfully retrieving pellets during the first stages of training, animals were a maximum of 20 pellets each day during the last 2 weeks of training until they achieved a success rate of 60 to 80%, and thereafter the task was done weekly after stroke injury.

The procedure can be varied to tailor the task to specific research goals (Gharbawie and Whishaw, 2006). In this study, both paws were trained at baseline, and both paws provided pellets; we hoped that this would result in a measure for compensatory mechanisms in addition to the task-related ability of the paw corresponding to the lesioned hemisphere.

The single pellet reaching task is an interesting outcome measure, as the hand shaping movements in humans strongly resembles those seen by rats reaching for pellets, although this has only been shown for the retrieval of a single pellet (Sacrey et al., 2009). Analysis of the reach can be done using various measures with different grades of sensitivity. Scoring can be achieved by counting the number of reaches, hits and misses during each trial. A reach is defined as any advance of the paw through the reaching slot. A miss is then defined as a reach that does not result in a successful transfer of the pellet to the mouth of the animal. The opposite is true for a hit, where a single reaching movement results in a successful reach and transport of the pellet to the animal's mouth. The following tailored scoring techniques can then be used depending on the sensitivity required to disseminate the outcomes from the data (Whishaw et al., 2008). The most liberal scoring method was used in this study, where success was defined as:

$$Success = \frac{Number\ of\ pellets\ reached}{Number\ of\ trials}$$

The most common of the measurement schemes is more stringent method and uses the following formula:

$$Success = \frac{Number\ of\ hits}{Number\ of\ reaches}$$

While an even more sensitive scoring can be produced with the following scoring formula, which is the most demanding of the scoring methods:

$$Success = \frac{Number\ of\ hits}{Number\ of\ trials}$$

The latter two formulae were not used in this study in the interests of time available before the submission of this thesis, but will be discussed further in the discussion.

5.3.7 Electrophysiology

The Hoffmann reflex (H-reflex) was assessed at 12 weeks post-surgery as an endpoint assessment. Rats were anaesthetised with 30mg/kg ketamine and 0.1 mg/kg

medetomidine. Two 24-G needle electrodes were inserted across the medial plantar side of the wrist to stimulate the ulnar nerve (*via* a constant current isolated pulse stimulator, stimulus width 100 μs). Two recording electrodes were inserted into the abductor digiti quinti to record electromyograms. The signal was amplified (4000-fold), filtered (300Hz to 6kHz), and digitised by PowerLab before being subsequently visualised and analysed with LabChart. The M-wave is evoked by excitation of motor axons. The H-wave is the monosynaptic reflex: Ia proprioceptive afferents synaptically activate motor neurons in the spinal cord (Figure 5-6). The threshold was determined as the lowest stimulation intensity that elicited an H-wave response in at least 75% of the recordings. First, the responses to increasing stimulus intensities were tested at 0.1 Hz up to 2 x threshold, which only activates Group I muscle afferents (Iyer et al., 2010). M-wave and H-wave amplitudes were normalised to the maximum M-wave recorded at higher stimulation intensities. Next, we tested the frequency dependent depression of the H-wave. We stimulated every 10 sec with paired stimuli at frequencies ranging from 0.1 Hz up to 10 Hz. The H-wave amplitude of the test stimulus was normalised to the H-wave amplitude of the conditioning stimulus. 25 paired stimuli per frequency were averaged and plotted on a frequency-depression curve. Electrophysiology was done by Dr C Kathe.

5.3.8 Total RNA extraction for RNA sequencing

12 weeks after stroke surgery, rats were terminally anaesthetised with Sodium Pentobarbital (Euthatal) and perfused with approximately 50 mL PBS before dissecting.

Total RNA for RNA sequencing was extracted from C4, C6, and C8 DRGs from the affected side of the rats from the study. These three DRGs were pooled per rat. Tissue was homogenised in 700 μL QIAZOL (Qiagen, 79306) using a hand-held rotor-stator and 140 μL chloroform was added. After shaking, nucleic acids were separated in Phase Locked Gel columns (5Prime, 230 2830). The aqueous phase was transferred to a fresh tube by decanting and 1.5 volume (525 μL) of 100% ethanol was added to the aqueous phase, mixed, and transferred to spin columns from the RNA extraction kit (miRNeasy kit, Qiagen, 217004) according to manufacturer's instructions. Samples were also DNase I treated on-column with double volume of the recommended amount (Qiagen, 79254). We estimated the total RNA quality and quantity by spectrophotometry (NanoDrop, ND-1000) and measured the RIN scores (Agilent RNA 6000 Nano Reagents Part I and Agilent 2100 Bioanalyzer). The integrity number for each sample was greater than 7.6, with an average of 8.0. Samples were frozen at –80 °C and then shipped on dry ice to the High-Throughput Genomics Group at the Wellcome Trust Centre for

Human Genetics at the University of Oxford. The samples were subjected to poly-A enrichment with oligo-dT beads (Illumina) and the library was prepared using the TruSeq Stranded prep kit (Illumina). Samples were run as a barcoded multiplex over 4 lanes on the Illumina HiSeq 2500 platform using the Rapid SBS kit v2 (50 bp read length, paired end).

5.3.9 RNA sequencing analysis

Read quality and potential sequencing biases were assessed using custom scripts for quality control of the raw sequencing data were done by the High-Throughput Genomics Group at the Wellcome Trust Centre for Human Genetics at the University of Oxford. The 100 bp paired-end reads were aligned to the reference genome (Rattus Norvegicus rn6) using TopHat2 (Kim et al., 2013) with default parameters (except for setting mate-inner-dist=100 and mate-std-dev=50). Just under 40 million reads per sample were obtained on average (mean±SD, 39.2±2.7 million). 98% of these could be aligned to the reference genome and over 45% mapped to Ensembl genes. Duplicate reads were identified using Picard Tools MarkDuplicates (Picard Tools by The Broad Institute) and the highest quality read at each position was retained. This reduced the dataset by approximately one-fifth. Reads mapping to each gene feature were counted using HTseq (Anders et al., 2015) to create a raw gene count table for RefSeq annotated genes with at least one mapped read. Because of a relatively large number of reads annotated as 'no feature' (ie, mapping to intronic or intergenic regions) and exclusion of duplicate reads and those mapping to multiple locations, a raw gene count table for 18,092 Ensembl genes was created. Further quality control plots and exploratory analyses including principal component analysis (PCA) were done to assess the overall behaviour of the dataset. A total of 15,308 genes were considered expressed in at least one sample and retained for differential expression analysis using the edgeR package (Robinson et al., 2010) comparing and creating following data sets: 5 week Injured Vehicle vs 5 week Sham, 5 week Injured Vehicle vs 5 week Injury NT3, 11 week Injured Vehicle vs 11 week Sham, 11 week Injury Vehicle vs 11 week Injury NT3.

The resulting datasets were imported into PANTHER, an online tool for comparing datasets from microarray or RNASeq results. Core analysis and comparison analysis was performed to reveal relevant pathways and functional groups. Next, lists of genes were created that were differentially regulated in each comparison. This revealed genes that are dysregulated after injury and then normalised spontaneously with vehicle treatment (comparison of vehicle *vs* sham at 5 and 11 weeks), dysregulated after injury and

normalised with NT3 treatment (comparison of vehicle *vs* NT3 at 5 and 11 weeks), genes that are not dysregulated with NT3 treatment, but are dysregulated after injury, (vehicle *vs* sham and vehicle *vs* NT3 at 5 weeks), and those that are not dysregulated after injury, but are dysregulated with NT3 treatment, indicating a delayed dysregulation with NT3. By comparing these datasets we can also ascertain which genes are altered after stroke (Table 5-2–Table 5-3).

5.3.10 Statistical analysis

Statistical analyses were done by use of SPSS (version 18.0). Graphs show means ± SEMs (except where otherwise stated) and 'n' denotes number of rats. Asterisks (*,**,***) indicate $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. Threshold for significance was 0.05. ELISA and lesion volume data were assessed using Kruskal-Wallis and Mann-Whitney tests, respectively. Behavioural data were analysed using linear models and Restricted Maximum Likelihood estimation to accommodate data from rats with occasional missing values (Gueorguieva and Krystal, 2004, Krueger and Tian, 2004, Duricki et al., 2016a), because some rats died before the end of the study. Akaike's Information Criterion (AIC) showed the model with best fit for the behavioural data, and are indicated in the text as having unstructured (UN), first-order autoregressive (AR1), or compound symmetric (CS) covariance matrix. Baseline scores were used as covariates. Degrees of freedom are reported to nearest integer. t-tests were two-tailed.

5.4 Results

5.4.1 Treatment of MCA-CCAO elderly rats using subcutaneous NT3 caused an increase in NT3 during treatment

To test whether the delivery of the NT3 was successful, an ELISA was done on blood serum acquired from animals at 5 and 12 weeks (12-week data not shown).

Analysis of the blood from the treated animals in the elderly female study revealed NT3 levels in the blood serum of 17.3±2.5 pg/mg compared to 3.2±0.83 pg/mg in vehicle control animals 5 weeks after stroke and treatment initiation (5-week NT3 vs vehicle, two sided, unpaired t-test; $p < 0.00001$).

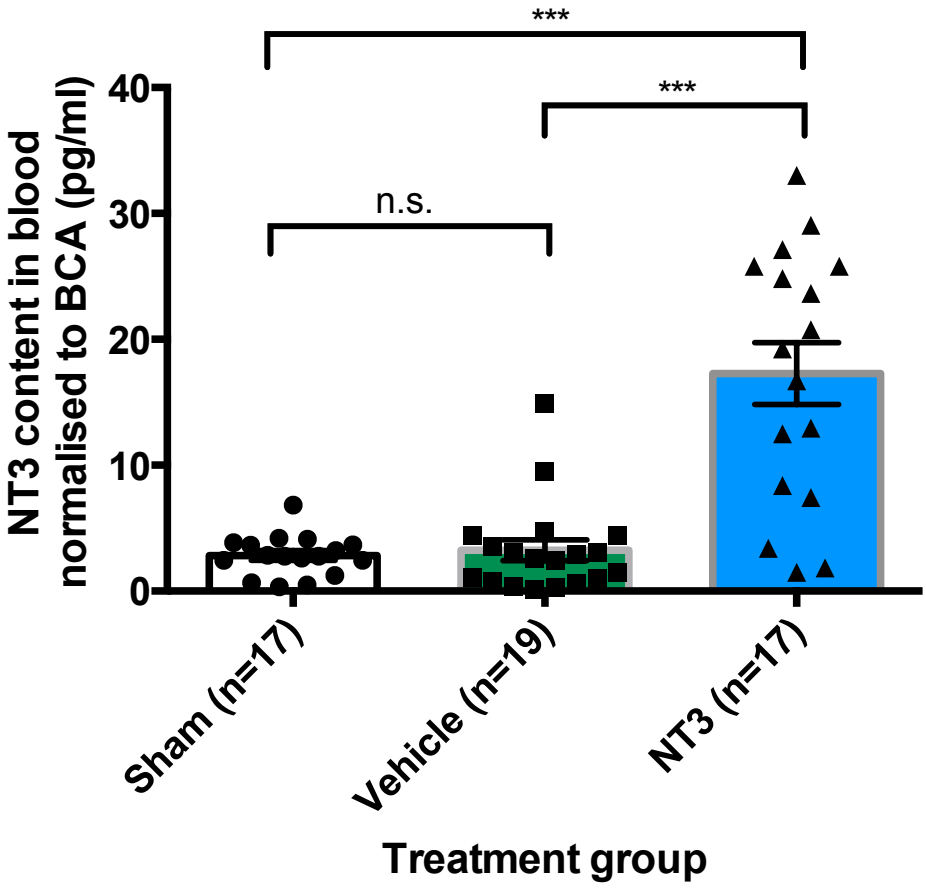


Figure 5-2 : ELISA showed significant NT3 availability in the blood after 5-week subcutaneous delivery.

*Blood was removed at 5 weeks after treatment with NT3 or vehicle. ELISA confirmed that there was a higher level of NT3 in animals treated with subcutaneous infusion of NT3 compared to controls (sham n =17, NT3 n=17, vehicle n=19; p<0.00001). Protein levels analysed by ELISA showed that there was a difference in the levels of NT3 compared with vehicle in female elderly rats. Graph shows mean±SEM; *** denoted p<0.001.*

5.4.2 Elderly female animal treatment with subcutaneous NT3 results in improved functional outcome after three vessel occlusion.

5.4.2.1 The staircase test showed no improvement with NT3 in elderly female rats compared to vehicle-treated control

Recovery of dexterity was assessed using the staircase test as described previously (Section 2.3.6.3). Before stroke surgery, elderly rats retrieved more than 70% of pellets with their dominant paw and stroke was induced in the hemisphere representing the dominant paw. After stroke, staircase test analysis indicated that retrieval of pellets was slightly greater in the group of rats treated with NT3 than those treated with vehicle (Figure 5-3A), although there was no overall effect of treatment ($F_{2,50.2}$=0.749, p=0.478). The interaction between time and treatment group was not significant ($F_{22,440}$=1.25, p=0.204), but there was an effect of time ($F_{11,440}$=0.194, p=0.032) consistent with spontaneous or learned recovery over time. The less affected paw also showed an effect in pellet retrieval over time ($F_{11,448}$=2.16, p=0.016; Figure 5-3B), but no effect of group ($F_{2,55.6}$=0.500, p=0.609), and no interaction between group and time ($F_{22,448}$=1.07, p=0.383).

Pellet displacement with the affected paw (Figure 5-3C) showed no effect of group ($F_{2,51.2}$=0.301, p = 0.742) but there was an effect of time ($F_{11,441}$=3.87, p<0.001) and an interaction between group and time ($F_{22,441}$=2.50, p<0.001), although no difference was observed between stroke groups (p>0.05). After stroke, displacement of pellets with the less-affected paw (Figure 5-3D) showed no change over time ($F_{11,447}$=1.30, p=0.219), no effect of NT3 treatment ($F_{2,55.7}$=1.35, p=0.266) although there was an interaction between group and time ($F_{22,448}$=1.64, p=0.034). Sham animals had a different trajectory in the pattern of behavioural achievement compared to stroke animals, although there was no difference between stroke groups.

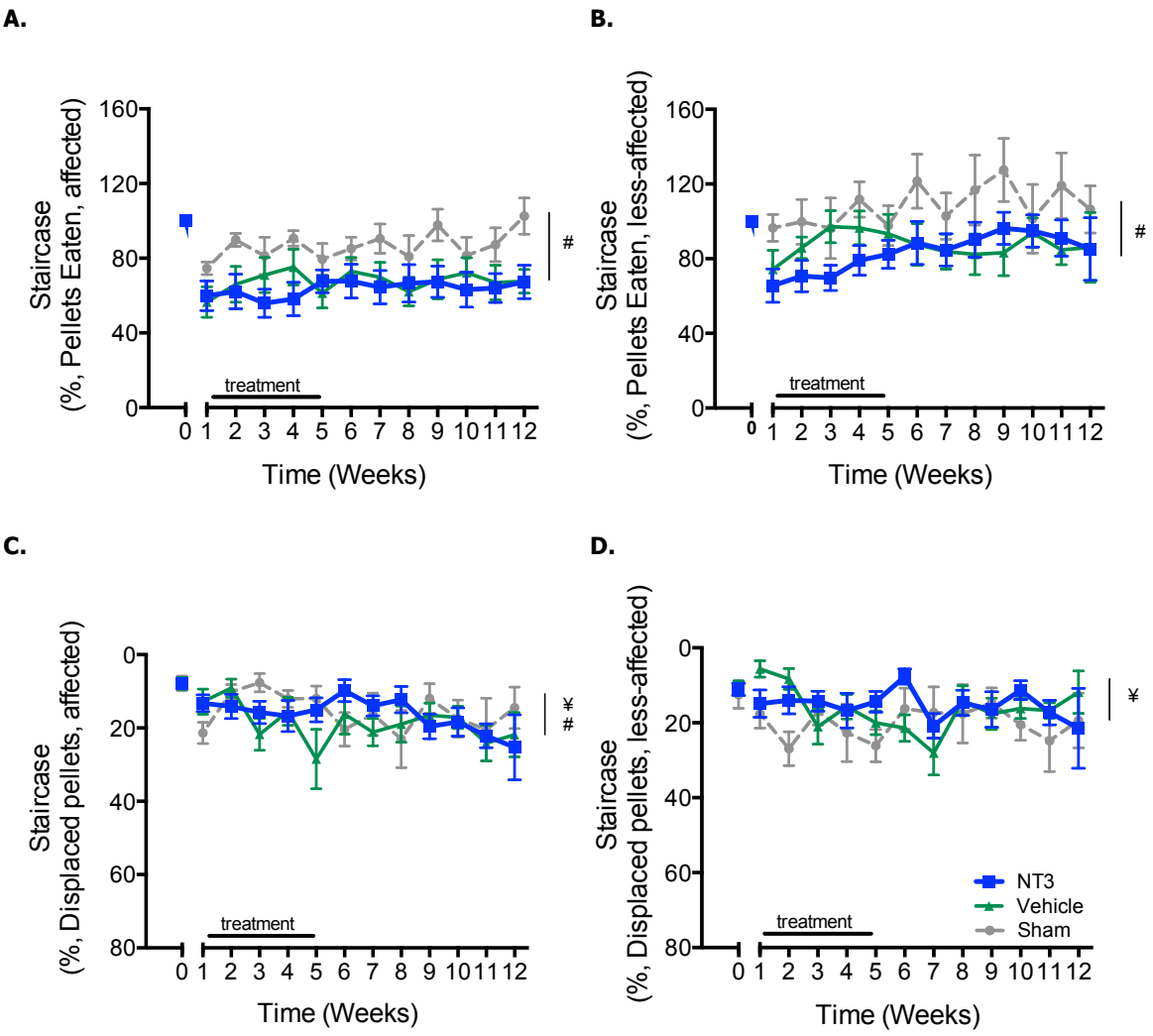


Figure 5-3 : Subcutaneous NT3 treatment following stroke surgery in elderly rats confers no significant benefit in the staircase test for skilled reaching.

Induction of permanent distal MCAO in elderly rats caused a significant impairment in motor control of the affected (A and C) and less affected forelimb (B and D) forepaws respectively, where A and B determines the ability of pellet retrieval and C and D verifies the accuracy through the use of displaced pellets during pellet retrieval. A sustained deficit was observed in the affected paw of all stroke rats compared to sham, but no improvement is observed with treatment with NT3 compared to vehicle (p=0.478; linear models with compound symmetry covariance matrix; n=17–19 per group) # effect of time, ¥ interaction between group and time, both p<0.05.

5.4.2.2 The bilateral extinction test showed both stroke groups were impaired following stroke injury, but no effect of NT3

Sensory responsiveness and dexterity was measured using the sticky patch test as described earlier (2.3.6.4). Stickers of equal radius were attached to each wrist and the time taken to contact and then remove them was measured. Before stroke, rats contacted the first sticker rapidly and subsequently removed it rapidly. One week after stroke, both treatment groups took longer to contact the sticker on the affected forepaw (Figure 5-4A). There was no effect of group ($F_{2,33.7}$=1.45, p=0.250). There was no effect of time ($F_{11,2.87}$=1.90, p=0.334) and no interaction between group and time ($F_{22,2.71}$=1.37, p=0.469). Sensation with the less-affected paw was not affected as severely as the affected paw overall (Figure 5-4B). There was no effect of group ($F_{2,33.0}$=1.19, p=0.316), though there was a significant effect of time ($F_{11,12.3}$=3.75, p=0.015), probably caused by the observed fluctuation in performance of the less-affected forepaw in week 10, although there is no discernable reason for this within the experimental design used. There was a trend towards an interaction between time and group ($F_{22,12.3}$=2.32, p=0.065), probably due to the sham animal trajectory, which is distinct from the two stroke groups until 8 weeks into the study.

The time taken to subsequently remove the sticker from the affected paw after indicating that the sticker had been sensed was not changed in stroke animals compared to sham (Figure 5-4C). There was no effect of group ($F_{2,1.81}$=1.979, p=0.500), no interaction between group and time ($F_{22,2350}$=1.46, p=0.078), which is probably because of the absence of deficit seen in sham animals and the deficit observed in the stroke groups that improved over time. There was an effect of time ($F_{11,89.0}$=4.06, p<0.001) consistent with spontaneous recovery or compensatory behaviour. A similar result was seen with the less-affected paw, where a small impairment was observed one week after stroke, with no overall effect of group ($F_{2,44.5}$=0.225, p=0.799), no interaction between group and time ($F_{22,15.8}$=1.00, p=0.509), but an effect of time was observed ($F_{11,15.2}$=3.20, p=0.019), because of the recovery seen over time in the task compared with week 1 levels.

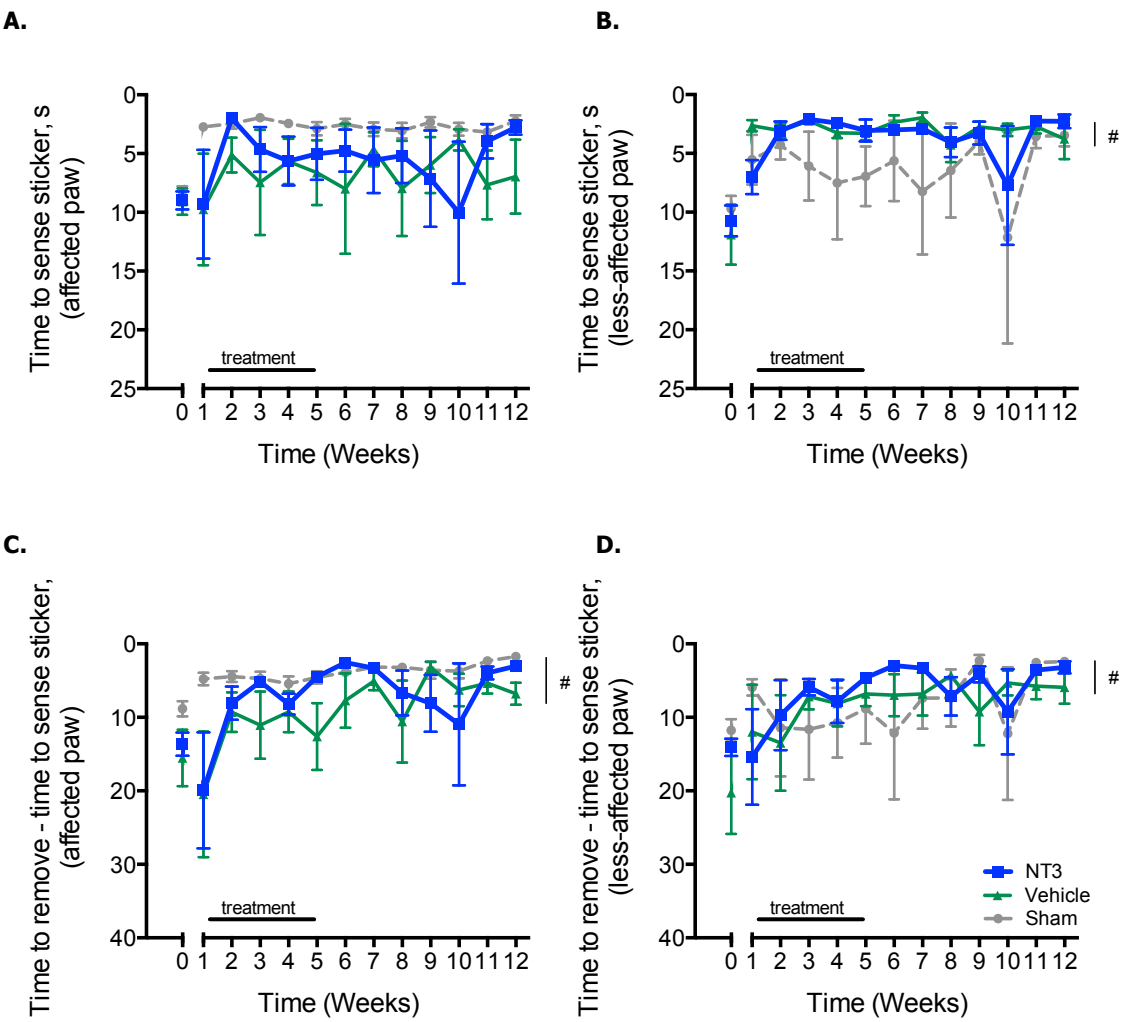


Figure 5-4 : NT3 improves functional sensory outcomes in the bilateral extinction test after distal permanent MCAO in an elderly female stroke model

In the bilateral extinction test the time taken for rats to first contact the sticker label on the affected forelimb was measured (A) and a small difference observed between NT3 treated animals after MCAO compared with vehicle treated animals, although this difference was not significant (p=0.250). This measure was also assessed in the less-affected forepaw (B), in which there was no effect of treatment (p=0.316) although there was an effect of time (p=0.015).

The time taken to subsequently remove the sticker after sensing was not significant, with no effect of treatment group in either the affected forepaw (C) or the less-affected forepaw (D). # denotes difference between stroke groups, p<0.05. Graphs represent mean ± SEM and statistics analysed by linear models with unstructured covariance structures.

5.4.2.3 The single pellet-reaching task revealed a sustained deficit after stroke that was significant, but no effect of NT3 treatment was observed

Recovery of dexterity was also assessed using the single pellet-reaching task. Before stroke surgery, elderly rats were trained on a minimum of 14 sessions to retrieve more than 70% of pellets with their dominant paw and stroke was induced in the hemisphere representing the dominant paw. After stroke, analysis indicated that retrieval of pellets after stroke was slightly greater in the group of rats treated with NT3 than those treated with vehicle (Figure 5-5A), as there was an overall effect of treatment ($F_{2,27.1}$=3.45, p=0.046) and an effect observed between both sham and vehicle (p=0.012) and sham versus NT3 (p=0.049), but there was no significance observed between NT3 and vehicle (p=0.711). The interaction between time and treatment group was not significant although a trend is observed ($F_{22,16.7}$=2.10, p=0.063), suggesting that the sham group has a different behavioural profile to the stroke groups on account of there being no impairment after surgery. There was an effect of time ($F_{11,16.8}$=3.12, p=0.018) over the duration of the study. The less affected paw also showed an effect in pellet retrieval over time ($F_{11,93.0}$=3.15, p=0.001; Figure 5-5B), but no effect of group ($F_{2,5780}$=0.329, p=0.721), and no interaction between group and time ($F_{22,102}$=0.940, p=0.545).

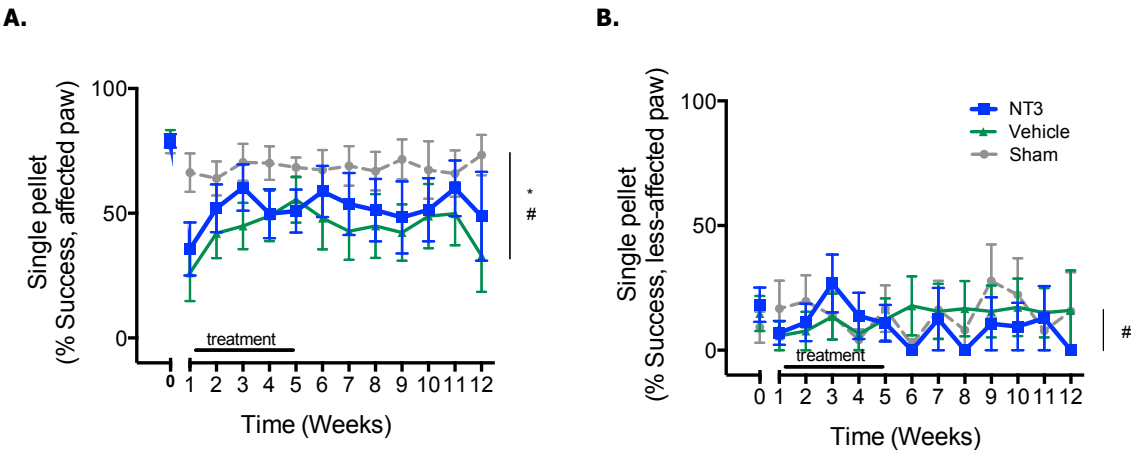


Figure 5-5 : NT3 improves functional sensory outcomes in the single pellet-reaching task after distal permanent MCAO in an elderly female stroke model

*Induction of permanent distal MCAO in elderly rats caused a significant impairment in motor control of the affected (A) and less affected (B) forepaws respectively, in which A and B identifies the ability of pellet retrieval. A sustained deficit was observed in the affected paw of all stroke rats compared with sham, but no improvement is observed with treatment with NT3 compared with vehicle (unstructured symmetry covariance matrix; n=17–19 per group; Table 5-1). Graphs represent mean ± SEM and statistics analysed by linear models with unstructured covariance structures. # denotes difference between stroke groups, and * denotes an effect of time, both p<0.05.*

5.4.3 H-reflex electrophysiology

To measure the monosynaptic Hoffmann (H) reflex (Grey et al., 2008, Toda et al., 2014b) electromyograms were recorded 12 weeks after MCAO stroke from an affected flexor hand muscle (the *abductor digiti quinti*) during stimulation of the ulnar nerve through electrodes placed across the wrist of the rat. The M-wave, the first short-latency EMG wave, is evoked by motor axon excitation, and the H-wave is evoked by excitation of primary sensory afferents synapsing onto motor neurons (Figure 4-11A). The M-wave (Figure 4-11B, middle) and H-wave (Figure 4-11B, right) have been included to indicate that there are no differences between treatment groups in the amplitudes of the individual M- and H-waves between treatment groups and throughout the various intensities of stimuli ($p>0.05$).

In sham rats, the H-wave was depressed by inter-stimulation-intervals less than 1 s. After stroke injury, the H-wave was less depressed at intervals less than 0.5 s, which suggests rats with MCAOs were more hyperreflexic (Figure 4-11B, left). NT3 treatment restored the H-wave depression at these inter-stimulation intervals, indicated by a significant group effect ($F_{2,119}=6.91$, $p=0.014$) and an effect of stimulus ($F_{6,119}=21.27$, $p<0.0001$) but no interaction between group and stimulus ($F_{12,119}=0.826$, $p=0.623$). This is likely to be because we included in our analysis the higher inter-stimulus intervals at which depression is known not to occur as well as intervals at which depression is known to occur (Toda et al., 2014b)—ie, the phenomenon known as frequency-dependent depression. Accordingly we analysed separately just those interstimulus intervals that are known to show depression in naive rodents. At 0.1 s inter-stimulus intervals, *post hoc* t-tests revealed a difference between NT3 treated animals compared with vehicle ($p=0.014$). Differences are also observed at 0.2 s inter-stimulus intervals between NT3 treated animals and vehicle treated stroke animals ($p=0.037$) and vehicle compared with sham ($p=0.024$).

Overall, these data indicate the NT3 may play a role in reversing hyper-excitability, and will be discussed further in Section 5.5.2.

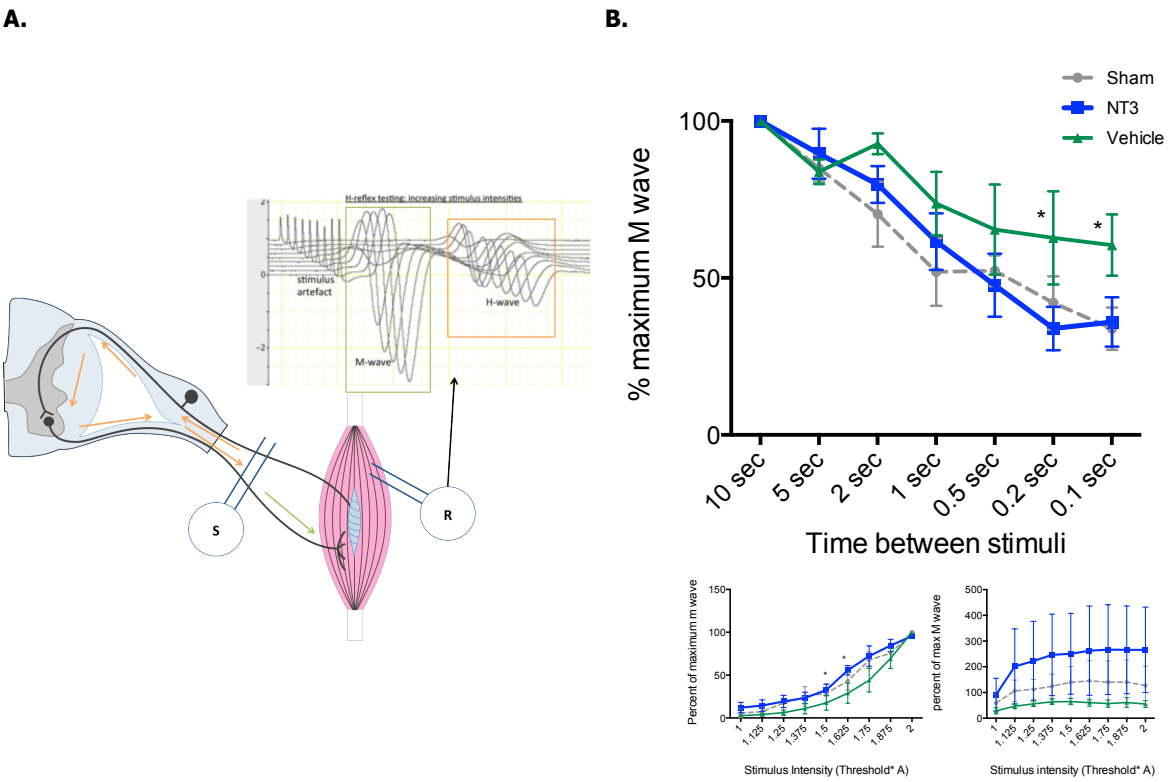


Figure 5-6 : Electrophysiological assessment of the H-reflex indicates rats with strokes develop hyper-reflexia compared to sham, and NT3 reduces this.

Stimulation of the ulnar nerve (stimulating electrode, S) and recording of the M- and H- waves from a distal flexor muscle (recording electrode, R; A). The effect of decreasing the inter-stimulus intervals are assessed (B, top). Sham animals show no depression of the H-wave at inter-stimulus intervals of 1 s or more. Stroke animals exhibit hyper-excitability at shorter latencies between stimuli. An effect of vehicle vs *NT3 is shown (*), p <0.001. M- and H-wave (B, bottom) have been included to show no difference between groups and a consistent relationship between stimulus intensity and contraction in all treatment groups. Graphs show mean ± SEM and statistics are repeated measures two-way ANOVA (n=6–7, as some animals were used for RNASeq; Table 5-1).*

5.4.4 RNASeq

To gain insight into the mechanism of peripheral NT3-mediated changes in the spinal cord after subcutaneous delivery of NT3 after stroke, and because our lab has previously provided evidence that intramuscular NT3 not only affects spinal networks *via* proprioceptive afferents but also that NT3 is trafficked to DRG after intramuscular overexpression (Duricki et al., 2016; Kathe et al., unpublished), an exploratory analysis was done to investigate transcriptional changes in the stroke-affected cervical DRGs contralateral to the stroke injury at 5 and 11 weeks after stroke injury.

Tables containing significantly dysregulated genes for each comparison can be found in Appendix 1 (DVD). The PANTHER tool was used to identify pathways for these genes, which are displayed in Figure 5-7, which include the FGF and apoptotic signalling pathways, among others, which will be discussed further in section 5.5.4. These genes were then compared within groups across time-points (Appendix 2) and within time-points across groups (Appendix 3) for genes common to both conditions, respectively. Genes that are differentially expressed between weeks within conditions are presented in Table 5-2 and Table 5-3.

For example, levels of Emr1 were downregulated in vehicle treated stroke animals compared with sham at 5 weeks, but this was reversed by week 11. This gene corresponds to F4/80, a protein expressed on the surface of macrophages (Baud et al., 1995, McKnight et al., 1997), for which the role of this protein is still not fully understood, but has been suggested to play a role in macrophage accumulation in obesity (Weisberg et al., 2003). If this is the case, with the knowledge that macrophage-related inflammation occurs in stroke and may contribute to some of the deficits seen (Kochanek and Hallenbeck, 1992, del Zoppo et al., 2000), it could be suggested that presence of this glycoprotein might be associated with the amount of inflammation observed after stroke. The treatment with NT3 then might modify the abundance of macrophages in the blood to similar levels to those seen in sham, although it is important to note that it is difficult to determine at which point in the pathway NT3 might be producing its effects.

5 week Veh vs sham			11 week Veh vs sham		
logFC	PValue	gene_symbol	**logFC**	PValue	gene_symbol
-0.905	0.0411	Aoah	**1.326**	0.0024	Aoah
-0.778	0.0022	C3	**0.924**	0.0259	C3
-0.643	0.0012	Cd4	**0.624**	0.0323	Cd4
-0.758	0.0001	Cd74	**1.404**	0.0054	Cd74
-0.888	0.0016	Emr1	**0.782**	0.0204	Emr1
-0.721	0.0005	RT1-Ba	**1.138**	0.0087	RT1-Ba
-0.676	0.0037	RT1-Bb	**0.894**	0.0258	RT1-Bb
-0.774	0.0001	RT1-Da	**1.571**	0.0033	RT1-Da
-0.876	6.55E-05	Thbs1	**0.447**	0.0249	Thbs1

Table 5-2 : Differentially expressed gene in vehicle treated animals vs sham animals at 5 weeks and at 11 weeks

Log fold change (Log FC) is used to compare sham animals to vehicle at 5 and 11 weeks in a subset of genes where p<0.05 at both time points. A positive value indicates that the gene is upregulated in vehicle compared to sham. The dysregulated genes in this table are dysregulated after stroke, and the directionality of change is reversed after 11 weeks, which could indicate genes involved in spontaneous recovery after stroke injury.

5 week veh vs NT3			11 week veh vs NT3		
LogFC	PValue	gene_symbol	**logFC**	PValue	gene_symbol
-0.767	2.09E-05	Aif1	**0.605**	0.0191	Aif1
-0.766	2.33E-06	Cd74	**1.6054**	0.0012	Cd74
-0.611	0.0176	Emr1	**0.674**	0.0392	Emr1
-0.477	0.0458	Evi2a	**0.612**	0.0467	Evi2a
-0.786	0.0002	Fcer1g	**0.591**	0.0266	Fcer1g
-0.756	0.0017	Fcgr1a	**0.628**	0.0212	Fcgr1a
-0.579	0.0421	Gad1	**1.220**	0.0130	Gad1
-0.472	0.0255	Gbp2	**0.471**	0.0289	Gbp2
-0.561	0.0005	Lyz2	**0.562**	0.0044	Lyz2
-0.776	1.46E-05	RT1-Ba	**1.249**	0.0038	RT1-Ba
-0.860	1.05E-05	RT1-Bb	**0.938**	0.0206	RT1-Bb
-0.901	6.88E-08	RT1-Da	**1.744**	0.0013	RT1-Da
-0.807	0.0093	Tnfrsf14	**1.374**	0.0035	Tnfrsf14

Table 5-3 : Differentially expressed genes in vehicle treated animals vs NT3 treated animals at 5 weeks and 11 weeks

Log fold change (Log FC) is used to compare NT3 treatment to vehicle at 5 and 11 weeks in a subset of genes where p<0.05. A positive value indicates that the gene is upregulated in vehicle compared to NT3. The dysregulated genes in this table are dysregulated after NT3 treatment, and the directionality of change is reversed after 11 weeks, which could indicate genes involved in NT3-related recovery after stroke injury.

Another consideration was for genes that were uniquely dysregulated in the vehicle versus NT3 treatment compared with vehicle versus sham condition at each time point, or uniquely expressed at 11 weeks versus 5 weeks within each condition. Genes uniquely expressed in NT3 treated animals compared with vehicle at 11 weeks and show differences from gene expression at 5 weeks are shown in Table 5-4.

A priori, we were interested in genes that were normalised with neurotrophin-3 treatment at 11 weeks compared to 5 weeks, or genes that were uniquely dysregulated at 11 weeks after NT3 treatment. 61 genes were significantly dysregulated (Tables 5-2 and 5-3). One gene that was differentially regulated is *Plxnb3* (plexin B3, Table 5-4). Semaphorins are axon guidance molecules, which have been shown to be important for specificity of afferent connectivity of sensorimotor axons within the spinal cord (Pecho-Vrieseling et al., 2009). *Sema5b* is anchored in the membrane and its binding partner Plexin B3 is expressed in neurons of the CNS. *PlxnB3* was up-regulated in afferent neurons after corticospinal tract injury. Gria1, glutamate receptor 1 (AMPA subunit 1), is another gene that is uniquely dysregulated at 11 weeks, showing downregulation in NT3 treated animals versus vehicle treated animals at 11 weeks after stroke.

11 week veh vs NT3		
logFC	PValue	gene_symbol
0.975	0.0097	Aoah
-0.305	0.0394	Atf3
-0.755	0.0294	Bag6
-0.736	0.0380	Ccl11
0.626	0.0395	Ccl6
0.873	0.0175	Cd22
1.817	0.0089	Clec2g
-0.545	0.0492	Cmtm8
0.606	0.0397	Cxcl9
-0.439	0.0360	Cytl1
1.097	0.0088	Doc2b
0.566	0.0092	Efcab2
0.567	0.0481	Enc1
-0.231	0.0470	Entpd2
0.806	0.0494	Fcrla
-0.805	0.0152	Fgf5
0.788	0.0415	Gria1
0.781	0.0293	Gucy1a2
0.903	0.0227	Hmox2-ps1
0.990	0.0196	Hpca
1.335	0.0178	Igsf1
0.960	0.0035	Il1b
1.858	0.0050	Lpp
0.688	0.0068	Lrguk
1.320	0.0288	Lrrc7
1.248	0.0040	Ly6h
4.207	5.86E-05	Mobp
3.788	0.0002	Nrgn
1.549	0.0060	Nts

-0.537	0.0391	Plac9
-0.242	0.0257	Plxnb3
1.205	0.0112	Prkcg
-0.537	0.0419	Prr22
0.708	0.0376	Prtg
0.875	0.0363	Psd2
-0.726	0.0486	Rarg
1.184	0.0411	Robo3
0.285	0.0277	Rps27
0.928	0.0075	RT1-Db1
1.225	0.0310	S1pr5
-0.718	0.0301	Sbk1
0.743	0.0476	Slamf8
2.462	0.0011	Slc12a5
-0.595	0.0229	Tal1
-0.313	0.0486	Tcf7l1
-0.593	0.0355	Top2a
1.069	0.0153	Trank1
1.390	0.0092	Wscd1

Table 5-4 : Uniquely expressed genes in vehicle treated animals vs NT3 treated animals 11 weeks compared with at 5 weeks

Log fold change (Log FC) was used to compare NT3 treatment to vehicle at 5 and 11 weeks. Genes are included if uniquely significant at 11 weeks (compared to 5 weeks, $p<0.05$). A positive value indicates that the gene is upregulated in vehicle compared to NT3. The dysregulated genes in this table are dysregulated after NT3 treatment at 11 weeks, but this dysregulation is not observed at 5 weeks, which could indicate genes involved in NT3-related recovery after stroke injury. Genes are presented alphabetically.

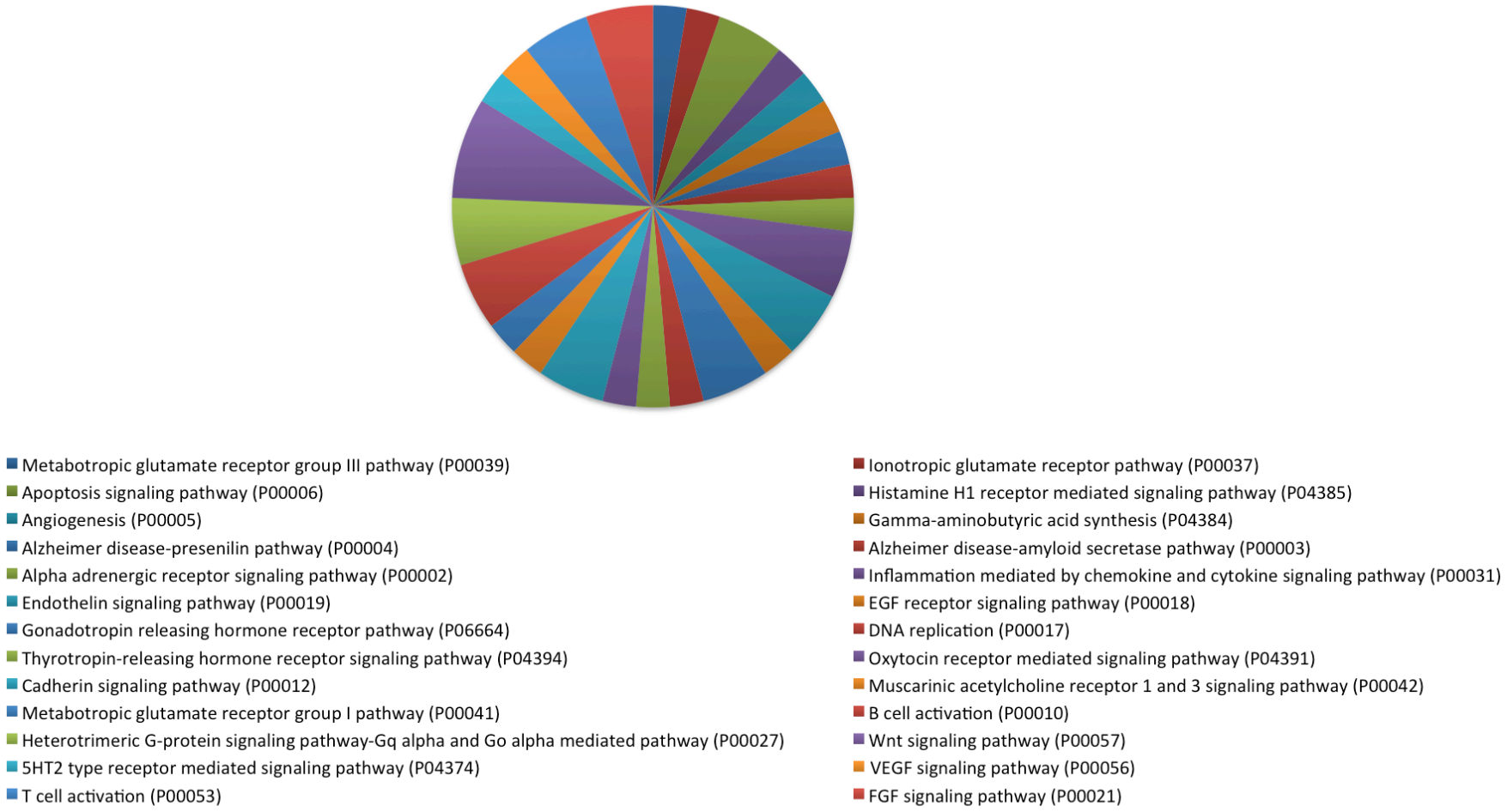


Figure 5-7 : PANTHER analysis of pathways dysregulated at 11 weeks with NT3 treatment

Please note that the legend reads from left to right, with Metabotropic glutamate receptor group III pathway at the top and working clockwise

5.5 Discussion

5.5.1 Study overview

This chapter confirms that permanent, distal MCAO with bilateral common carotid artery occlusion is a feasible model of cortical stroke in elderly rats and that human recombinant NT3 can be infused subcutaneously as a clinically relevant administration route. Although little benefit was observed after NT3 treatment in behavioural tasks, electrophysiology revealed significant enhanced plasticity in the NT3 treated group, indicating a reversal in hyper-reflexia of NT3 treated animals compared with vehicle controls. RNASeq data indicated differential gene expression that could be explored in the future to further elucidate the mechanisms by which NT3 brings about benefit after stroke. Delayed subcutaneous infusion of recombinant human NT3 is a viable method of drug delivery in which treatment initiated in a clinically-relevant time frame induced spinal neuroplasticity.

5.5.2 H-reflex electrophysiology shows that hyper-reflexia after stroke is ameliorated through treatment with NT3

Over-expression of NT3 after ischaemic stroke causes functional spinal circuit re-organisation, reduces sensorimotor abnormalities, normalises hyper-reflexia, and enhances mobility. We have now shown that hyper-reflexia is normalised in two studies in this thesis, and we can say with some confidence that NT3 is mediating this modulation of spinal reflexes. However, despite the normalisation of hyper-reflexia in stroke animals with NT3 treatment, additional behavioural functional recovery did not occur. It might not be enough to modulate a single reflex; perhaps the benefit observed in this study is not sufficient to confer improvement in sensorimotor behavioural tasks.

Non-human primate studies have characterised cortical map changes after focal ischaemic injury, which has greatly enhanced the knowledge of the effects of rehabilitation, axonal projections, and infarct size in stroke research (Nudo, 2007, Cramer, 2008). A study by Nudo and Milliken (1996) used intracortical microstimulation to map forelimb movement representations in the primary motor cortex, before and a few months after a focal ischaemic lesion. They found that the infarcts caused a marked but transient deficit in use of the contralateral forelimb to the lesion, with a corresponding increase in the use of the ipsilateral forelimb for retrieval tasks.

5.5.3 Conclusions from behavioural tasks

Following a human stroke, the most common impairments are hemiparesis of the contralateral upper limb, deficits in sensory function (Cramer et al., 1997, Langhorne et al., 2009), and interruption of the CST. This permanent distal model of three-vessel MCAO incorporates these three key processes, resulting in a sustained deficit in some of our motor and sensory tests when used in elderly rats. In some tasks, deficits were sustained as shown in experiment 3 of chapter 4 (Figure 4-8–Figure 4-10); however, on the bilateral tactile-extinction task (Figure 5-4), spontaneous recovery towards baseline is seen. This could be because of varying sizes of lesions between experiments, or inter-experimenter variability. Even with 60-minute occlusions, there was not a consistently sustained deficit on these tasks, resulting in inconsistent recovery in treatment groups; significant improvement with behavioural tasks was only seen in experiment 2 of chapter 4 (Figure 4-4–Figure 4-6). NT3 also showed improvement in a smaller, more focal model of stroke in both young and elderly rats (Duricki et al., For submission). This could suggest that NT3 is particularly efficient at modulating and improving behavioural outcomes when the lesions are small enough to permit cortical reorganisation, although the most convincing evidence for NT3 recovery was with the large lesions produced in Chapter 4. Another possibility is that medium size subcutaneous doses of NT3 can promote changes in the H-reflex but that larger subcutaneous doses (eg, 47 µg or more, as used in the previous Chapter) are required to promote sensorimotor recovery.

Motor deficits may severely impair use of the preferred limb in unilateral models, which can result in the animal switching to the use of its non-preferred (and healthy) ipsilateral forelimb as a compensatory action. In the staircase test, this is prevented by an experimental design that forces use of both hands. However, in the single pellet task, a bracelet can be used to prevent the use of the ipsilateral paw during the task and reinforces the use of the contralateral paw, enabling the measure of lesion outcome or therapy (Whishaw et al., 1986). This restraining process can also be used in humans in order to force use of an affected limb (Tillerson et al., 2001, Taub and Uswatte, 2003), and could potentially be used in future to maximise the potential of a statistically beneficial effect of treatment after stroke.

5.5.4 RNASeq

NT3 has previously been shown to be overexpressed in the muscle and retrogradely transported into the ipsilateral cervical DRG following intramuscular AAV-NT3 delivery after stroke in rats (Duricki et al., 2016b), and even after administration of recombinant NT3 protein after spinal cord injury in rats (Kathe et al., under review).

The results from the RNASeq analysis are complex and there will be many false positives and secondary associations, as a lowering of one element of a pathway will doubtless have a knock on effect on other factors. The results warrant a systematic literature review to find associations between the genes identified as differentially regulated and potential roles in neuronal injury, axon growth, and plasticity. Unfortunately, this review was beyond the scope of this thesis, so I have identified a few pathways and genes of interest, which I will discuss here.

One significant finding of interest was the use of PANTHER to identify potential biological processes to identify pathways most affected by NT3. The presence of the apoptotic signalling pathway among these is surprising as it would be unexpected for DRG neurons to die after (remote) stroke as these neurons do not normally undergo apoptosis even after proximal axotomy in the adult (Kenney and Kocsis, 1998). An alternative possibility is that other cells in the DRG undergo apoptosis—for example, inflammatory cells—after recruitment.

In other RNASeq analyses in our lab, one of the semaphorin family (semaphorin 4C) was found to be down-regulated in cervical DRG after spinal cord injury in adult rats (Kathe et al, unpublished). Plexin B3, a semaphorin receptor for semaphorin 4a and 5b was found to be upregulated in NT3 treated rats at 11 weeks in this study after stroke. Some semaphorins have been shown to have a role in axon growth and guidance, particularly after injury (Koncina et al., 2013), and interacts with vascular endothelium growth factor (VEGF) and the MAP kinase pathway, among others. One study has shown the importance of Sema3A in axon injury, but the role of the plexin b3 receptor still needs to be elucidated (Ueno and Yamashita, 2008).

Functional analysis of RNASeq data is shaped by the available literature used to compile the PANTHER database, which might be incomplete because of missing gene ontology annotations for a large proportion of genes. It is therefore possible that the functional information provided here will become more refined as gene ontology and pathway annotations evolve. Additionally, DRG may have low sensitivity in RNASeq experiments because of low (66%) expression of Trk receptors in DRG neurons (Bradbury et al.,

1999b), in particular the high affininty NT3 receptor, TrkC. Additionally, a small proportion of DRG neurons are muscle afferents (McMahon et al., 1994), resulting in a diluted effect of neurons that are potentially undergoing plasticity mechanisms following NT3 treatment. This could be counteracted in future by doing laser microdissection on DRG neurons to specifically target the proprioceptive neurons. This would increase the sensitivity to detect changes from subsets of neurons in future RNASeq analyses. The gene changes observed here in this study could underlie the changes in the hyper-reflexia observed in the electrophysiological analysis, but larger changes in these genes might be necessary to promote behavioural recovery, either through higher doses of NT3, or through the targeting of the identified genes. From an initial comparison with other datasets, it seems that these findings are novel and have not been seen elsewhere. However, the resource GEO2R permits the comparison with other datasets, and would be useful to thoroughly analyse these data alongside other datasets (Lee et al., 2012b)

5.5.5 Study conclusions

This study has shown that delayed subcutaneous treatment of disabled forelimbs with recombinant human neurotrophin-3 (NT3) results in amelioration of hyper-reflexia observed with electrophysiological recording directly from affected muscles during stimulation compared with vehicle treatment in elderly female rats following three-vessel MCAO.

Importantly, although there was little improvement in behavioural tasks, this therapy resulted in functional improvement of hyper-reflexia even when treatment was initiated 24 hours after stroke and measured 11 weeks after injury. This is important as the gold standard therapy for stroke (tPA) is most effective when given less than 4.5 hours after stroke, yet less than 50% of European stroke victims are diagnosed within 6 hours (Hacke et al., 2008, Gargano et al., 2011). RNASeq analysis reveals some genes that could be involved in mechanisms of recovery following NT3 treatment, including glutamate receptor 1 and plexin B3 receptors, which could be explored further in the future.

Chapter 6 Discussion

6.1 Thesis overview and key findings

Stroke is the leading cause of adult disability in the Europe and the USA (Lloyd-Jones et al., 2010) and yet clinically there is no drug therapy that is effective after the initial treatment window in which tPA is effective, although rehabilitation has been shown to have some benefit. This thesis aimed to identify whether delayed NT3 therapy could be used to improve functional outcome following a stroke injury. This thesis provides some evidence that treatment with NT3 improves sensory and motor recovery following a focal ischaemic stroke, but NT3 is also not detrimental to outcome after stroke, and electrophysiological data suggest that there is some improvement of hyper-reflexia. This treatment seems to be particularly effective when used in an elderly model of stroke in Chapter 4 compared with the use of AAV-NT3 in young adult rats in chapter 2.

In the first study (chapter 2), a comparison was made between middle cerebral artery occlusion of stroke with or without the simultaneous occlusion of the common carotid arteries in young adult rats. 4 weeks later the affected biceps and triceps were injected with an AAV encoding either human NT3 or GFP. A more sustained deficit was observed in the sensory and motor behavioural tasks in the three-vessel occlusion model—although spontaneous recovery was seen at the end of the study—but no deficit was seen with the distal MCAO model. Importantly, no difference was observed on any task between the AAV-NT3 group relative to the AAV-GFP group. This result was not unexpected, as researchers have shown that activation of the immune response is required to produce axonal sprouting after spinal cord injury when substantially delaying NT3 treatment (Chen et al., 2008). This experiment was the setting up of the surgical model for fine-tuning in later studies. Although functional recovery because of treatment was not likely in this experiment, it was decided that the administration of the viral vectors worth trying as the resources were available.

The third chapter explores functional MRI images during stimulation of the affected paw after MCAO stroke with carotid artery occlusion (used in chapter 2) to map novel longitudinal changes in brain activity after stroke. This confirmed that the somatosensory cortex was active in all rats before stroke but not active in the ipsilesional side at 1 week following stroke. At weeks 4 and 12, recovery was observed through recruitment of the

secondary somatosensory cortex and the bilateral striatum. The study also used resting state MRI techniques to determine the connectivity of the primary somatosensory cortex and lobules of the cerebellum associated with motor coordination; changes observed after stroke and during the recovery period. The results from this study showed the increased connectivity of the contralateral secondary somatosensory cortex, and bilateral regions of the striatum over the first 4 weeks after stroke, which could be participating in the spontaneous recovery observed in behavioural tasks observed in chapter 2.

In the fourth chapter, the decision was made to try the three-vessel occlusion stroke model used in the first results chapter in aged rats. First, a pilot was used to determine the length of time needed for vessel occlusion of the contralesional common carotid artery in conjunction with permanent occlusion of the ipsilateral common carotid and middle cerebral arteries. A second experiment used elderly female rats to assess the effect of delayed subcutaneous administration of NT3 from 24 hours for 4 weeks. In this experiment, an effect of NT3 was observed in behavioural tasks such as the horizontal ladder task, and in an analysis of CST sprouting in the spinal cord. An aged cohort of male and female rats were also used to assess the effect of recombinant NT3 protein delivered subcutaneously at 24 hours for 4 weeks. Structural MRI taken at 24 hours and at 8 weeks showed no differences between elderly rats at either time-point, indicating that NT3 is not neuroprotective when delivered subcutaneously 24 hours after stroke. The findings from this final experiment showed no effect of treatment in behavioural tasks, but a moderate effect was observed in electrophysiological assessment of the H-reflex, indicating that NT3 ameliorates hyper-reflexia after stroke. This could be partly explained by the use of a heterogeneous experimental group; despite the use of large numbers of animals to try to account for this in the sample size, the use of both male and female rats in the third experiment of chapter 4 resulted in little significant observations in the behavioural tasks, despite improvements in the NT3-treated stroke animals in the second experiment using only elderly female rats. Although NT3 is unlikely to work only in female rats, it is worth considering that elderly male rats were not motivated to complete the behavioural tasks, and their use in future should be limited to histology, electrophysiology, or "involuntary" behavioural tasks such as those withdrawal responses to Von Frey stimuli that measure pain reflexes.

Therefore, the final study of this thesis looked at potential mechanisms of action of NT3 through the analysis of mRNA through RNASeq analysis. Behavioural assessment showed no functional effect of NT3, but electrophysiological effect was observed in the

NT3 treated group, reducing hyper-reflexia after stroke. RNAseq revealed altered levels of genes such as *Gria1* and *Plxnb3* genes, coding for glutamate 1 and plexin b3 receptors respectively, which are dysregulated at 11 weeks but not at 5 weeks after stroke.

6.2 Three-vessel MCAO as a model for stroke

Clinically, approximately 80% of all strokes are of ischaemic origin with the most commonly seen deficits including hemiparesis of the contralateral upper limb (Cramer et al., 1997, Langhorne et al., 2009) and impairments in sensory processing. The middle cerebral artery (MCA) is the largest of the vessels that draws supply from the circle of Willis and stems from the internal carotid artery (Mohr et al., 2004). The MCA is the cerebral vessel most commonly affected in ischaemic stroke, with strokes in this territory accounting for 65% of all ischaemic strokes (Sacco et al., 1984, Hossmann and Heiss, 2009). In this thesis we used MCAO to produce a unilateral ischaemic stroke lesion, which resulted in sustained and significant sensory and motor disabilities in elderly rats.

In the human stroke condition, the majority of surviving stroke victims that survive have infarcts that occupy between 4–14% of the ipsilateral hemisphere, although malignant stroke involves more than 39% of the ipsilateral hemisphere (Carmichael, 2005). In this thesis this clinical outcome was achieved using a model of stroke that incorporated a similar proportion of the affected hemisphere. The lesions produced by the occlusion of the middle cerebral artery were of a reliable severity and the experiments in this thesis have shown that this model is reproducible in magnitude of deficit between animals, and is a suitable method for assessing neurorestorative therapies. However, this model is not without its disdvantages. There was an element of spontaneous recovery observed throughout the studies presented in this thesis; as such it might be better used in short-term (< 4 week) studies.

The permanent, distal coagulation MCAO model of ischaemic stroke was used throughout this thesis. A comparison was made in chapter 2 between the occlusion of a single vessel and the occlusion of three vessels through additional permanent occlusion of the common carotid artery ipsilateral to the lesioned cortex and transient (1 hour) occlusion of the common carotid artery on the contralateral side. The three-vessel occlusion model was shown to produce larger and less variable lesion volumes, and was selected as the preferred model for the remainder of the thesis with a few modifications

to animal care and procedures made throughout the chapters to reduce mortality. This resulted in all stroke rats showing similar functional deficits in contralateral skilled locomotion, sensory neglect, fine motor control, and postural support.

One undesirable feature of this model is the mortality rates that result; this was particularly true in the elderly model studies, where the occlusion times of the transient occlusion of the common carotid artery had to be reduced due to the number of animals that were not surviving the surgery (chapter 4). Other groups have found a 95% survival rate in this model, even in elderly rats (Wendy Kartje, personal communication; occlusion times ranging from 30 to 60 minutes), which suggests either an effect of operator between laboratories, an effect of occlusion times, or another source of variation (Fox et al., 1993, Kahveci et al., 1999). It is known that blood vessel architecture varies between species, but what is often not accounted for in studies such as those presented in this thesis is the effect of variation within the same species; blood vessel architecture in the brain can vary significantly between litters of animals, resulting in more variable effects of producing stroke lesions.

Sex differences are important to consider in stroke models (Fisher and STAIR, 1999); through these studies, we have used mostly female animals. It is important to use both males and females to identify sex differences. There is evidence (from chapter 4) that male animals have larger lesion volumes than their female counterparts, and it has been shown in other studies that ovarectomised female rats have infarct volumes similar to male rats (Alkayed et al., 1998); differences in hormones such as progesterone and oestrogen are thought to account for this, and some studies have shown that application of exogenous oestrogen reduces infarct volume in male rats (Toung et al., 1998). It is possible that using post-menopausal animals in the elderly studies resulted in less variable lesions, which could explain the improved deficit in function after stroke in elderly female animals.

Clinically, ischaemic stroke is caused by a thrombus or embolism and so technically the use of this model (which involves coagulation and occlusion) does not result in the obstruction of the vessel as is observed in the human condition, but instead causes vascular damage and irreversible occlusion. Therefore, it could be argued that this was not the most representative model for the investigation of neuroprotective therapies (Carmichael, 2005), although it is similarly worth noting that no single model is able to model all aspects of the disease. However, this concern is largely irrelevant in this thesis because the therapy being assessed is administered 24 hours after stroke, by which

time, neurons in the ischaemic region are irreversibly damaged. The therapy presented in this thesis does not target cell death mechanisms, rendering the method of occlusion less important since cell death and any reperfusion would have already occurred.

Furthermore, delays in diagnosis in the clinical setting (Evenson et al., 2009) make many neuroprotective treatments not suitable for all patients as current methods do not permit the hospitalisation and treatment within a timeframe that enables the "saving" of the ischaemic core or penumbral regions. Therefore, the focus of this thesis was to investigate the possibility of neuroregeneration through enhancing plasticity of the CNS following a delayed, clinically relevant treatment. The delay of treatment by one week was too delayed to elicit an effect (chapter 2); but 24-hour treatment showed a amelioration of hyper-reflexia as measured with electrophysiological recording of the H-reflex.

This model of ischaemic stroke used throughout this thesis successfully produced sustained significant deficits in elderly female rats. The stroke model was a valuable tool for assessing a regenerative therapy following stroke in aged rats.

6.3 Moving towards the use of NT3 clinical trials for stroke

Five Phase 1 and Phase 2 clinical trials have already shown that subcutaneous recombinant NT3 is safe and well tolerated in more than 200 humans to date for Charcot-Marie-Tooth neuropathy and severe constipation (Parkman et al., 2003, Sahenk, 2007, Sahenk et al., 2014). Clinical approval for use in people with stroke should, theoretically, be viable. The use of NT3 generated from mammalian cells means that the pro form of the NT3 protein can be produced, along with the incorporation of post-translational structures and folding (Demain and Vaishnav, 2009), meaning that there is no need for renaturing and allowed the initial production of large proteins such as tPA (Swartz, 1996); the NT3 used in chapter 4 was the mature form of NT3 (14 kDa) generated by proteolytical processing of the 30 kDa proNT3 in mammalian cells. Advancements in recombinant technology, *E Coli* is now frequently used for the production of tPA, and indeed, the NT3 used in the final results chapter of this thesis was made in *E Coli*. Consequently, the NT3 has a leading methionine, which the mammalian form did not, indicating minor differences between the different forms of NT3. It would be interesting to elucidate whether these differences have any functional consequences.

An NT3 clinical trial would be useful once preclinical studies have shown reproducible resuls, to identify an optimal dose and duration of treatment for NT3 following stroke injury. Methods should be identified to assess deficits and recovery in patients treated with NT3, following the identification of suitable patients. NT3 potentially binds to receptors on sensory neurons and enhances transmission of information about muscle movement and tactile sensation, causing improvements in proprioception, assessments using H-reflex testing (Schieppati, 1987), and standard clinical tests (Brott et al., 1989), including Fugl-Meyer (Gladstone, 2002), and Action Research Arm test for hemiparetic function (Lang et al., 2006).

6.4 Future therapies for stroke: what next?

The development of new therapies for stroke has proven to be a difficult task, with very few resulting in clinical approval. Currently, drug treatment for the acute ischaemic stroke phase is still limited to tPA which was implemented in 1987 (Papadopoulos et al., 1987). The main push in clinical trials has been neuroprotective compounds, which significantly reduce infarct volume in animal studies and showed promising phase 1 and phase 2 trials, however none have made it to the clinic. This lack of efficacy is thought to be related to numerous factors including underpowered trials, incorrect inclusion of patients who are not likely to respond, and lack of linkage between the animal model and the clinical situation (Fisher, 2003). However, in recent years, clinical trials have also shown benefits to the use of mechanical thrombectomy using new devices (Smith, 2006, Nogueira et al., 2009).

One major problem with stroke therapies in the clinic is that stroke is a heterogenous disease. The severity of the stroke often vary between individuals since the locations and size of the infarct are not uniform because of the vessel architecture and the cause of the stroke, be it thrombotic or embolic. Therefore, modifying therapy to the individual and stratifying patients into lesion types and sizes may be a key strategy to maximise recovery.

One therapy that has emerged and gained popularity recently is the use of functional imaging and brain stimulation to visualise and modulate cortical excitability and plasticity in the cortex after stroke (Liepert et al., 2000, Ward and Frackowiak, 2006). Functional MRI or transcranial magnetic stimulation (TMS) are used frequently in stroke clinical trials. fMRI can be used to visualise patterns of activity following stroke and longitudinal

fMRI can be used to see how these patterns change in response to either spontaneous plasticity or in response to rehabilitation or a pharmacological therapy (Johansen-Berg et al., 2002a, Ward and Frackowiak, 2006). fMRI can also be coupled with TMS to deliver a pulse of current flowing through a coil generates a magnetic field, producing an electric current flowing in parallel to the MR coil (Hallett, 2007), which can alter the activity of underlying neurons. This procedure alters neurotrophic substance levels and enhances neurotransmitter release (Strafella et al., 2001, Liebetanz et al., 2002, Siebner and Rothwell, 2003). Repetitive TMS can be used at a low frequency to inhibit overactive areas or at a high frequency to excite certain neuronal areas (Kobayashi et al., 2004, Mansur et al., 2005). A more recent imaging technique, which is currently being clinically assessed, is the Glasgow Oxygen Level Dependent (GOLD) MRI technique, which uses perfluorocarbon as a carrier for oxygen for simultaneous detection and treatment of the penumbral area following stroke and has been successful to date in animal models (Deuchar et al., 2013). Using these techniques allows patients and treatments to be monitored and modulated more carefully. It allows understanding of the contribution of the contralesional and ipsilesional hemisphere in response to motor tasks and allows for the manipulation of these to result in an enhanced motor output and produce functional benefits following stroke.

6.5 Further studies

6.5.1 Future *in vivo* studies:

Based on the experiments in this thesis, and those in previous studies in our group, we have shown a significant effect of NT3 in two models of stroke; one producing a small, focal stroke, and the second, presented in this thesis, producing large, strokes. Both models have assessed the effect of NT3 treatment in young and elderly rats, and found that plastic changes in in spinal circuitry led to a significant effect of treatment, which has been shown by both electrophysiological and anterograde tracing. It is still unknown by exactly which mechanism this occurs, although the results from the RNASeq data in Chapter 5 provide some suggestions that can be explored further in the future. Additionally, stroke comorbidity models could be used (such as spontaneously hypertensive rats), which would be more similar to the human conditions than those used to date. Additionally, combination therapy of NT3 with tPA would be useful before progressing to Phase 1 or 2 clinical trials to determine combined effectiveness.

6.5.2 Understanding mechanisms of recovery:

To understand the mechanisms by which NT3 is producing recovery after stroke, more research needs to elucidate how NT3 is modifying descending and ascending transmission between spinal and supraspinal cord or segmental spinal transmission between afferents and motoneurons.

6.5.2.1 Tracing experiments:

Over the course of this thesis, whilst tissue was taken after cortical injection of AAV-mCherry with the intention of doing histological analysis of CST projections after three-vessel middle cerebral artery occlusion (chapter 4), it was not possible to complete it in the time available. Previous studies have shown that NT3 treatment results in midline sprouting of the CST axons on the contralateral side to the lesion. It would be useful to co-localise the CST axons with antibodies to elucidate the available receptor group of the sprouting axons. It has been suggested that axons containing glutamatergic (Woolf and Salter, 2000) or serotonergic (Bregman, 1987) receptors might form the subgroup of axons that undergo plasticity after injury.

6.5.2.2 Brain imaging/recordings using EEG-fMRI

During my PhD, I was fortunate to receive a scholarship from the Japanese Society for the Promotion of Science to study at Tohoku University, Sendai, Japan. There, I was able to learn a technique allowing the recording of full scalp EEG with simultaneous BOLD-fMRI in rats. This lab was the first to develop this technique and have patented a cap that can be used in rats (Sumiyoshi et al., 2011). This cap has 31 electrodes, which reduces noise and permits simultaneous collection of EEG and fMRI data. The BOLD response occurs over approximately 10 seconds so to understand more about the processes occurring, it would be useful to use a technique that has a high temporal resolution in combination with fMRI, especially to understand more about default connectivity in the brain and pathological changes following stroke and spontaneous, as well as pharmacological, recovery.

Moreover, simultaneous rsfMRI and single-channel electroencephalogram recordings have revealed that synchronised low-frequency BOLD signals within functional networks are associated with distinct electrophysiological signatures (Laufs et al., 2003, Lu et al.,

2007, He and Liu, 2008, Schmuel and Leopold, 2008), which support the potential use of multi-channel EEG-fMRI to decipher network activity. This technique could be used to monitor somatosensory recovery after stroke and NT3 treatment; combined with information about spontaneous network connectivity after stroke (measured with both rsMRI and forepaw stimulation fMRI) that was shown in Chapter 3, we could identify networks that are uniquely modulated with NT3 after stroke to bring about novel functional recovery.

6.5.2.3 NT3 tracking: Where is NT3 transported to after delivery?

One unresolved problem within this thesis is the understanding of how NT3 affects spinal and supraspinal plasticity after peripheral administration. To test where NT3 is transported to after delivery, we could deliver an epitope-tagged NT3 to the muscle, in a similar vector and route of administration as used in Chapter 2. Two MSc students in our lab, Bruce Fanshawe and Adam Tyson, have constructed a vector with an AAV backbone to encode prepro-NT3 with GFP, which could be used in future to determine where NT3 is deposited following *in vivo* delivery after ischaemic stroke.

6.5.2.4 Mechanism of action: genes involved with improved functional outcome after stroke treatment with NT3 may provide insight

The manipulation of genes thought to be involved in the mechanisms of injury or neuroprotection could be important for treatment, or might even just allow further discovery into the pathways involved. Results from RNASeq (Chapter 5) identified numerous genes that could be involved in injury after stroke, and the recovery associated with both spontaneous recovery, and functional recovery following NT3 treatment. Genes such as Plxnb3 and Emr1 could have interesting consequences on the trajectory of recovery. In order to identify the most promising genes from this study, the datasets obtained could be compared with datasets from GEO2R, such as the GSE38074 (Lee et al., 2012a). This dataset is of particular interest because it has identified gene changes that occur in proprioceptive neurons ($TrkC^+$) in the DRG after either overexpression of NT3 in the muscle (*mlcNT3*) or NT3 knockout (neurons were kept viable by knocking out *Bax*). This could be exciting as it would allow the identification of genes that are differentially regulated in $TrkC^+$ DRG neurons from wildtype versus *mlcNT3* mice of the GSE38074 dataset. Additionally, genes could be identified that are

differentially regulated in $TrkC^{+}$ neurons from $Bax^{-/-}$ mice versus $NT3^{-/-}$ $Bax^{-/-}$ mice. The resulting lists reveal genes that are regulated by NT3 in proprioceptive neurons.

Additionally, a systematic review of the literature around these genes would be a worthwhile endeavour before using these to inform the following experiments, although pursuing the most promising targets and performing knock-out experiments in mice would be informative into the role that these genes play in the recovery induced by NT3 after stroke.

6.6 Thesis Conclusions

Within this thesis I have shown that subcutaneous delivery of NT3 improves function in elderly rats when treatment is initiated 24 hours after stroke. This was particularly shown through the use of electrophysiological stimulation of the H-reflex, which indicated that NT3 ameliorated the hyper-reflexia observed after stroke compared with vehicle treated animals, and behavioural data supported this finding (at least in Experiment 2 of chapter 4). This thesis provides further evidence for the beneficial use of NT3 following CNS insult and provides novel evidence for the use of this therapy following an ischaemic stroke.

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