Effects of de-enrichment on post-stroke learning and microglial activation in mice

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Abstract

Stroke is a leading cause of death and lasting disability in adults. The extent of recovery is mediated in part by the patient's environment both before and after stroke. Institutionalisation or loss of work, leisure activities or social contact constitutes a loss of enrichment and impairs recovery, as well as increasing the risk of depression and anxiety. Previous animal studies have not examined the effect of a period of pre-stroke enrichment followed by post-stroke loss of enrichment. Therefore, we aimed to model this situation using three different levels of enrichment after stroke: Continued 'normal' environmental enrichment (EE), de-enrichment (DE), or enhanced enrichment (EEE). Further, we aimed to assess the possible interaction of medial prefrontal cortex (mPFC) lesions and changes in environment and stress on recognition learning and microglial activation, as a marker for inflammation.

Adult male C57BL/6*J* mice were housed for three months in an enriched environment prior to receiving photothrombotic lesions to the mPFC. Immediately after stroke, animals were placed into one of the three environmental conditions: EE, DE, or EEE. Behavioural testing was carried out at one and four weeks post-stroke and included grid walking and cylinder tests to measure motor skills, open field to measure activity levels and anxiety, elevated plus maze and light-dark box to measure anxiety, and novel object and object location recognition to measure learning and memory. Following testing, animals were sacrificed and their brains analysed for stroke volume and secondary degeneration through microglial activation.

DE animals had smaller lesion volumes one week after stroke. Stroke and housing conditions had mixed effects on activity levels and anxiety, and had no effect on object memory. Similar to what we have shown previously, stroke EE and stroke EEE groups showed delayed spatial memory impairment at four weeks. Stroke decreased IBA1-positive microglial staining in several brain regions, except for some dense cores seen in the thalamus and median eminence. EE and EEE reduced staining in stroke animals in several areas, including the thalamus and median eminence, which was associated with spatial memory impairment, and may indicate secondary neuronal degeneration in spatial memory circuits. Interestingly, the stroke DE group showed no impairment in spatial memory seen at 4-weeks post-stroke. In addition, these animals also showed no decrease in IBA1 staining in the thalamus, indicating that IBA1 activation may be

mitigating the secondary neuronal cell loss associated with the spatial memory impairments.

In human patients secondary neuronal loss is common and can lead to delayed cognitive decline. This loss may contribute to post-stroke depression and anxiety. The negative effects of early enrichment may be caused by an increase in stress in the enrichment groups, combined with disruption to the hypothalamic-pituitary-adrenal (HPA) axis caused by mPFC damage. These results may indicate that de-enrichment is protective early after stroke, and that treatment is best delayed for some hours or days to maximise recovery and minimise delayed impairments.

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List of Abreviations

ACC	anterior cingulate cortex
ACAo	anterior cerebral artery occlusion
AChE	acetylcholinesterase
ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
BBB	blood-brain barrier
BCCAo	bilateral common carotid artery occlusion
BDNF	brain derived neurotrophic factor
BPU	Behavioural Phenotyping Unit
BNST	bed nucleus of the stria terminalis
BrdU	Bromodeoxyuridine
CIMT	constraint-induced movement therapy
CMS	chronic mild stress
CORT	corticosterone
COX2	cyclooxygenase 2
CRF	corticotropin releasing factor
CRP	C-reactive protein
DAB	diaminobenzidine
DALY	disability-adjusted life years
DE	de-enrichment
DG	dentate gyrus
DPX	dibutyl phthalate in Xylene
EE	environmental enrichment
EEE	enhanced environmental enrichment

EPM	elevated plus maze
ET-1	endothelin-1
FDA	Food and Drug Administration
GABA	gamma-Aminobutyric acid
GFAP	glial fibrillary acidic protein
GR	glucocorticoid receptor
Hipp.	hippocampus
HPA	hypothalamic-pituitary-adrenal
IBA1	ionized calcium-binding adapter molecule 1
IGF-1	insulin-like growth factor 1
IL-1	interleukin 1
IL-11	interleukin-11
IL-1β	interleukin 1β
iNOS	inducible nitric oxide synthase
IVC	individually ventilated cages
LDB	light-dark box
LH	learned helplessness
LPS	lipopolysaccharide
LV	lateral ventricle
M2	secondary motor cortex
MCAo	middle cerebral artery occlusion
MCI	mild cognitive impairment
MCP1	monocyte chemoattractant protein 1
MCSF	macrophage colony stimulating factor
MDA	malondialdehyde
mPFC	medial pre-frontal cortex
MR	mineralocorticoid receptor

MRI	magnetic resonance imaging
MWM	Morris water maze
NGF	nerve growth factor
NGFI-A	growth factor-induced gene A
NGFI-B	growth factor-induced gene B
nNOS	neuronal nitric oxide synthase
NOR	novel object recognition
OLR	object location recognition
PBS	phosphate-buffered saline
PFC	pre-frontal cortex
PSD	post-stroke depression
PVN	paraventricular nucleus
PVP-40	Polyvinylpyrrolidone
rt-PA	tissue plasminogen activator
RTN	nucleus reticularis thalami
RSC	retrosplenial cortex
SAA	serum amyloid A
SC	primary sensory cortex
SEM	standard error of the mean
sICAM-1	soluble intercellular adhesion molecule
SMART	sensori-motor active rehabilitation training
SNr	substantia nigra pars reticulata
SSRI	selective serotonin reuptake inhibitor
SVZ	subventricular zone
TBI	traumatic brain injury
TBS	Tris-buffered saline
TDCS	transcranial direct current stimulation

Thal.	thalamus
TIA	transient ischemic attack
TNF- α	tumour necrosis factor alpha
v3V	ventral third ventricle
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
VPN	ventroposterior nucleus

Chapter 1. Introduction

1. General Introduction

1.1 Stroke Background

Stroke, or cerebrovascular disease, is the third leading cause of death in New Zealand after heart disease and all forms of cancer, and accounts for around 10% of deaths annually (Ministry of Health, 2011). In 2011, stroke killed approximately 2700 people and caused nearly 38,000 disability-adjusted life years (DALYs), which is a combined measure of life expectancy and disability (Ministry of Health, 2013). Stroke is a neuropathological complication that predominantly affects the aged. However, it should be noted that stroke can affect anyone at any time and approximately 10% of deaths caused by stroke are of people under the age of 65, and this age group accounts for 25% of the disability years. Although death rates have decreased over the last 30 years, an ageing population combined with higher stroke survival rates means that the number of stroke sufferers requiring care increases every year (Stroke Foundation of New Zealand, 2010). In 2013, there were 45,000 stroke survivors in New Zealand and stroke care cost the government approximately $450 million (National Health Committee, 2013).

In New Zealand, there is an increased incidence of strokes in Pacific Island and Maori populations (Feigin et al., 2006). In addition, the age at which stroke occurs is significantly lower in these groups than New Zealand Europeans, which creates a further burden on patients, their families, and the health system (Feigin et al., 2006; Ministry of Health, 2011). Furthermore, access to and funding for rehabilitation facilities is less available to stroke patients under 65, which creates a bias against Pacific and Maori populations (Fink et al., 2006).

1.2 Types of Strokes

There are three main types of stroke: Ischemic, haemorrhagic, and transient ischemic attack (TIA). Of these three types, ischemic stroke accounts for roughly 80% of all strokes (Feigin et al., 2010). This involves a blockage to an artery caused by either plaque build-up at the site where the infarct occurs, or when a piece of plaque becomes detached from a coronary vessel and moves through the circulatory system until it blocks a smaller vessel within the brain. As a consequence to this blockage, tissue “downstream” from the blockage receives insufficient blood flow and may die due to oxygen and glucose deprivation. The second type of stroke is cerebral haemorrhage,

which makes up around 15% of strokes and involves a rupture of a blood vessel and bleeding into the surrounding area. This puts surrounding tissue under pressure, damaging a larger area than ischemic stroke. Mortality rates for this type of stroke range from 30-50%, with much higher rates of impairment and dependence among survivors (Sahni & Weinberger, 2007; Suarez et al., 2006). The third type of stroke is TIA. These are the result of a temporary blood flow blockage and may have similar symptoms to a permanent stroke but resolve in less than 24 hours (Stroke Foundation of New Zealand, 2010). TIAs may result in further strokes and have also been shown to increase the risk of developing dementia (Pendlebury et al., 2011).

Disabilities caused by stroke include problems with motor skills, speech and language, vision, cognitive skills and emotional problems such as depression and anxiety. The type of symptom depends on the location and severity of the stroke, and can include one or more problems as listed above (Dobkin, 2008). Following a stroke, patients' living situations may change. The extent of disability will determine the type of care patients need after stroke. People can remain at home, be sent to an institutionalised aged care facility or be hospitalised. This change in situation, combined with the physical or cognitive disability, results in major disruption to the patient's life. Soon after a stroke, 16% to 20% of patients are institutionalised (Hackett & Anderson, 2006; Lloyd-Jones et al., 2010). After six months, this number drops to around 5% (Hackett & Anderson, 2006). Ability to participate in social and outdoor activities, perform interests and a shorter stay in hospital are all associated with higher scores on a health-related quality of life survey (Almborg, et al., 2010). Even in the medium term, while degree of disability and neurological symptoms remain stable over 6-12 months, up to 66% of patients report a decrease in overall life satisfaction, as well as problems with physical and social functioning and depression (Suenkeler et al., 2002).

There is a clear need to better understand and treat this disease in order to reduce the amount of suffering of those with disability and the cost associated with treating stroke survivors.

2. Consequences of Stroke

2.1 Anxiety

In addition to the motor problems commonly experienced, other neurological problems such as anxiety, depression and cognitive impairment occur frequently after stroke. Approximately half of all stroke patients experience periods of anxiety or depression, or both, following a stroke (Bergersen, et al., 2010). Rates of anxiety stay constant, and may even rise between one and six months post-stroke (Campbell Burton et al., 2012). Struggling with daily tasks was found to make patients almost three times more likely to have a mood disorder (Hackett & Anderson, 2006). Delayed cerebral atrophy increased the risk of anxiety and depression after stroke (Astrom, 1996). Another similar study found that post-stroke anxiety was associated with cortical infarcts, while having anxiety and depression together was associated with subcortical basal ganglia infarcts (Starkstein et al., 1990).

Anxiety is increased in animal models of stroke. When mice received 17 minute transient bilateral common carotid artery occlusion (BCCAo), they spent less time in the middle of an open field test and spent less time in the open arms of an elevated-plus maze (EPM) (Soares et al., 2013). These are commonly used behavioural measures of anxiety in rodents. Occlusion of the right middle cerebral artery (MCAo) for 60 minutes also decreased the amount of time mice spent in the middle region of the open field test, although there was no difference in the time spent in the open sections of the elevated zero maze (O'Keefe et al., 2014). Thirty minute left, but not right, MCAo caused a decrease in the amount of time mice spent in the open arms of the EPM (Kronenburg et al., 2012). Conversely, one study found that after using the vasoconstricter endothelin-1 to impair blood flow in the anterior cerebral artery, rats showed decreased anxiety in the elevated plus maze (Endepols et al., 2014).

2.2 Depression

Low levels of self-efficacy (the ability to deal with challenges – both general and stroke related) and perceived social support, as well as presence of pre-stroke depression all increase the risk of developing post-stroke depression (PSD) (Lewin et al., 2013). PSD is associated with speech and language dysfunction, cognitive impairment, and reduced social activities (De Ryck et al., 2014), as well as higher rates of morbidity and mortality (Williams et al., 2004).

Post-stroke depression has also been modelled in animals. Latency to float and the total time spent floating in the forced swim test, sucrose consumption in the home cage, and escape behaviours in the shuttle avoidance task are often used as measures of helplessness and anhedonia, which are thought to be animal equivalents of depressive symptoms (Bogdanova et al., 2013; Duman 2010). MCAo stroke increases depressive behaviours in these tasks (Kronenburg et al., 2012; Boyko et al., 2013; Sun & Alkon, 2013; Boyko et al., 2013). Depressive symptoms have also been reported in rats following permanent BCCAo (Lee et al., 2014).

2.3 Cognition

Cognitive impairment is another common effect of stroke. A large study in South London found that three months after stroke, 22% of people had cognitive impairment (Douiri et al., 2013). This deficit remained at similar levels over a long period of time – 22% after five years and 21% after 14 years. Another study found rates of 38%, and that impairment was positively associated with stroke severity, age, and cerebral atrophy (Ankolekar et al., 2014). Problems with cognition were related to worse outcomes in several measures of functional recovery including physical disability, dependency, mood and quality of life (Ankolekar et al., 2014). In first-time stroke patients under 50, cognitive impairment following stroke can be as high as 50% (Schaapsmeerders et al., 2013). One study found that although stroke patients had lower cognitive function at baseline compared with healthy controls, they did not decline more than controls (except for verbal memory) unless they had a further stroke (Sachdev et al., 2014). However stroke patients did have a higher risk of developing dementia. Stroke patients who develop dementia are more likely to have another stroke than those who don't (Sibolt et al., 2013). While motor and sensory deficits generally appear very rapidly after stroke, patents often develop cognitive impairments months and even years afterwards (Loeb et al., 1992). Common aspects of cognition affected by stroke are processing speed, working memory, attention, verbal communication, and executive function (Schaapsmeerders et al., 2013; Pinter & Brainin, 2012). Among these specific deficits, executive function and working memory most strongly correlate with post-stroke depression (Hommel et al., 2013).

Stroke causes cognitive deficits in animal models. Temporary anterior cerebral artery occlusion (ACAo) impairs rats' performance in the spontaneous alternation task – a measure of spatial learning and memory (Endepols et al., 2014). BCCAo stroke has been found to impair the spatial memory of mice in the Morris water maze (MWM) at days 7 and 14 following stroke, but performance improved by 28 days (Soares et al., 2013). Only five minutes of BCCAo causes gerbils to have impairments in the win-shift and win-stay task, another spatial memory task (Farrell et al., 2001). Further, vasoconstrictive infarcts to the peri-ventricular white matter caused deficits in the novel object recognition memory task both one and four weeks following stroke (Blasi et al., 2014).

The most common animal model of stroke, the MCAo, also causes learning and memory deficits. Spatial memory is impaired in the MWM following both 60 minute (Truong et al., 2012; Li et al., 2013) and 90 minute occlusion (Ryan et al., 2006). Other spatial memory tasks in which performance is impaired include the Barnes circular maze (Ryan et al., 2006). MWM deficits tend to be absent or mild early (1-3 weeks) after stroke (Li et al., 2013; Bouet et al., 2007), then develop over the following 4-8 weeks (Ryan et al., 2006; Truong et al., 2012; Li et al., 2013).

However, as with the depression and anxiety studies, mixed results have been found with MCAo studies – Bouet and colleagues (2007) found no impairment in the MWM after a 60 minute occlusion. As with white matter strokes mentioned above, recognition memory is impaired by MCAo (Truong et al., 2012). Learning tasks are also impaired by MCAo, including passive avoidance (Bouet et al., 2007; Willing et al., 2002) and various reinforced lever-pressing tasks (Linden et al., 2014; Linden et al., 2015).

The evidence from animal models of stroke and human patients is in agreement – stroke causes increased risk of anxiety, depression, and impairment in a wide range of cognitive tasks.

3. Consequences of Prefrontal Cortex Stroke

3.1 Anxiety

While generally not causing gross motor impairments, stroke to the pre-frontal cortex

(PFC) affects anxiety, depression and cognitive skills, as well as impairing executive function. The relationship between anxiety and PFC stroke has been studied much less than depression, but some studies show anxiety can also be related to PFC lesions. Stroke patients with anxiety are more likely than those without to have infarcts in the right PFC (Tang et al., 2012). Vietnam veterans who suffered injuries to the right orbitofrontal cortex were more likely to have anxiety and depression compared with veterans that suffered injuries in different brain areas (Grafman et al., 1986).

Animal studies into the effects of PFC lesions on anxiety have also shown mixed findings. Electrolytic lesions to the medial prefrontal cortex (mPFC) increased the anxiety of rats (Blanco et al., 2009). However, others have reported no change or a decrease in anxiety after photothrombosis (Zhou, 2013), excitotoxic lesions (Lacroix et al., 1998) electrolytic lesions (Maaswinkel et al., 1996), cytotoxic lesions (Deacon et al., 2003), or injections of a vasoconstrictor (Hewlett et al., 2014). Anxiety can vary within studies, and groups have found conflicting results between different anxiety tests such as the EPM, open field, successive alleys, black-white alley or hyponeophagia (Lacroix et al., 1998; Maaswinkel et al., 1996; Deacon et al., 2003).

Lesions to the mPFC don't seem to have reliable effects on anxiety. Clearly the effect of stroke on anxiety depends on the type, location and extent of the lesion as well as the behavioural tests used.

3.2 Depression

Stroke to the PFC seems to be associated with PSD more than other areas. A large cohort study found that patients with frontal lobe stroke were around twice as likely to develop PSD than those with strokes elsewhere (Shi et al., 2014). PSD is associated with larger lesions in areas of the mPFC including the anterior cingulate cortex (ACC), subgenual cortex, amygdala and subiculum (Terroni et al., 2011). As well as cortical areas, PSD has been linked to subcortical PFC circuits, with lesions to areas including the striatum, globus pallidus, and thalamus resulting in an increased likelihood of developing PSD (Tang et al., 2011). Lesions to inferior frontal regions have been found to be more likely than other frontal regions to be associated with PSD (Singh et al., 2000).

Animal studies of PFC damage find mixed effects on depressive symptoms. Impact injuries (a model of traumatic head injury) to the PFC can cause depressive symptoms in the forced swim and sucrose consumption tests (Moritz et al., 2014), while other studies have found that electrolytic or photothrombotic lesions have no effect on activity levels in the open field (Maaswinkel et al., 1996). Activity levels in the open field are often used as an animal indicator of depression (Krsiak & Janku, 1971; Shao et al., 2015). On the other hand, PFC lesions have been found to cause an increase in activity in the open field (Lacroix et al., 1998; Deacon et al., 2003; Zhou et al., 2015), as do strokes to the ACC (Hewlett et al., 2014).

3.3 Cognition

Strokes targeting the PFC can cause spatial memory deficits, although these tend to be less obvious impairments than those caused by larger artery occlusion strokes. Vasoconstrictive lesions to the ACC of rats resulted in an impairment in spatial memory as observed in the MWM (Hewlett et al., 2014). Similarly, traumatic brain injury (TBI) to the mPFC has been shown to impair performance in the Barnes maze (Moritz et al., 2014). Consistent with these findings, we have recently shown using the photothrombosis model of stroke that damage to the mPFC caused a delayed impairment in spatial memory in the object location recognition (OLR) test, with impairments observed at 4-weeks but not 1-weeks post-stroke (Zhou, 2013). On the other hand, Deacon and colleagues (2003) found no impairments in the spatial Y-maze, spontaneous alternation T-maze, or multi-trial passive avoidance following cytotoxic lesions. Further, studies by Maaswinkel's group (1996) found that not only did animals with pre-limbic electrolytic lesions show no initial deficit when learning the MWM, they actually adjusted faster than shams when the platform position was changed.

3.4 Executive Function

The effects of PFC stroke on executive function have been extensively investigated. The PFC is responsible for executive functions or "higher" cognitive processes such as planning, impulse control, attention, language, sensory integration, and memory formation and retrieval (Wood & Grafman, 2003). Frontal lobe lesions can damage one or several of these functions, depending on where the lesion occurs. Most of the studies below have used groups of patients with PFC lesions caused by stroke as well

as tumour or injury.

Patients with frontal lobe lesions show deficits in planning and strategy application in the Modified Six Elements test, which is a combination of several problem solving tasks (Gouveia et al., 2007). Similar planning and predicting deficits have been found using various problem solving and real-world planning tasks following lesions to the right PFC (Goel et al., 2013; Gomex-Balderrain et al., 2004) and also the left dorsolateral PFC (Colvin et al., 2001). Patients with frontal lobe dementia or focal frontal lobe lesions made more moves, broke rules more often, and took longer to complete the Tower of London task than age- and education-matched controls (Carlin et al. 2000).

Frontal lobe damage can impair the ability to inhibit incorrect responses. Kopp and colleagues (2013) found that patients with lesions to the right inferior frontal gyrus had impaired inhibitory control in the frontal assessment battery (a combination of several cognitive tasks). Damage to the right middle frontal gyrus impaired conceptualisation and mental flexibility.

PFC damage can impair attention and reaction speed in simple reaction time tasks (Dimitrov et al., 2003; Lee et al., 1999). Different areas seem to be responsible for different components of attention: Lesions to superomedial regions slowed reaction time, while lesions to left lateral areas increased errors (Alexander et al., 2005). Similarly, in the Stroop interference task, left dorsolateral frontal lesions caused slower responses and more errors than more posterior lesions or healthy controls, but only bilateral superior medial lesions impaired performance in the incongruent condition (Stuss et al., 2001). PFC lesions can also impair the ability to attend to novel stimuli. Daffner and colleagues (2000) found a reduced P3 wave response to novel stimuli following frontal lobe stroke, which was strongly associated with a reduced viewing time of a novel stimulus. Frontal lesions can also impair memory – patients recalled less items in both free and cued recall, with lesions to the left frontal lobe resulting in poorer performance than right-sided lesions (Dimitrov et al., 1999).

Although executive function is more easily measured in humans, PFC stroke damages executive function in animals as well. Following unilateral frontal cortex lesions, rats had a decision-making deficit when choosing a high-reward high-effort path,

compared with a low-reward low-effort path in a T-maze task (Croxson et al., 2014). This deficit was more marked when the high-reward high-effort arm of the maze was contralateral to the lesion, possibly indicating damage to areas responsible for cost-reward processing (Croxson et al., 2014). Cordova and colleagues (2014) found that rats with vasoconstrictive mPFC lesions had a reduced ability to shift attention between stimuli in different dimensions (odour, material and texture) in a conditioned reward-finding task.

Overall, temporary or permanent MCAo seems to produce more reliable behavioural symptoms of anxiety and depression than smaller, targeted injuries to the PFC, which seem able to increase, decrease, or have no effect on anxiety and depression, and more subtle effects on cognition. This may be due to the variable methods used to induce stroke, the different areas and volumes damaged within the PFC, as well as the much smaller volume damaged compared with MCAo. This may also be due to the much larger number of studies using artery occlusion compared with more recent techniques such as photothrombosis.

4. Photothrombosis as a Model of Stroke

MCAo and other artery occlusion stroke models produce large infarcts that are more similar to fatal or malignant human stroke than the average human infarct (Carmichael, 2005). As a consequence, when animals develop impairments - and in particular cognitive impairments - this does not mimic what usually occurs in humans. Therefore in order to move forward, animal models of stroke that model both the size and location of the stroke and development of the desired impairment (for instance, motor or cognition) need to be established. This will be critical if we are to develop drug treatments that are to translate into the clinic.

Human strokes are commonly smaller (Carmichael, 2005; Lyden et al., 1994; Lindgren et al., 1994), with volumes of 28-80 mm^3. A photothrombotic stroke volume of 1.3-5 mm^3 (Zhou, 2013; Zhao et al., 2015) is more appropriate for comparison than larger MCAo volumes, which can range from 9 ± 2 mm^3 for a 15 minute occlusion up to 69 ± 2 mm^3 for a 60 minute occlusion (McColl et al., 2004).

Following photothrombosis, the infarct volume rapidly evolves to reach its maximum volume by 24 hours post-stroke (Chen et al., 2007; Braun et al., 1996). However, small regions of ongoing apoptosis can still be observed 1 -3 days post-stroke induction, and secondary apoptosis can occur up to four weeks post-stroke (Braun et al., 1996).

5. Current Stroke Treatments

5.1 Drug Treatments

Currently the only drug treatment for ischaemic stroke approved by the Food and Drug Administration (FDA, U.S.A.) is the recombinant tissue plasminogen activator (rt-PA) alteplase. Although this drug can be effective at breaking up clots and restoring blood flow, it is only effective when administered within three to four hours after stroke (de los Rios la Rosa et al., 2012). Often strokes are not recognised soon enough to enable patients to meet this time limit – only 25% arrive at an emergency department within 4.5 hours and of these, only 6.4% are eligible for rt-PA treatment (de los Rios la Rosa et al., 2012). Despite the trial of hundreds of different drugs for early neuroprotection, none have yet made it into the clinic. More recently, trials show newer thrombolytic drugs may be more effective and possibly extend this window (e.g. tenecteplase, Parsons et al., 2012). Promising new treatments are being evaluated for patients with occlusions in larger arteries who do not respond to alteplase such as endovascular clot retrieval (Smith & Schwamm, 2015), but along with alteplase this is only suitable in the hours immediately following stroke. Therefore, there is a need for therapies that enhance recovery in the days and weeks after stroke.

At the moment, the most effective drug treatments for post-stroke disorders seem to be those that target depression and anxiety such as antidepressants. Duloxetine, citalopram and sertraline have been shown to be effective in reducing depression and anxiety following stroke (Karaiskos et al., 2012). A meta-analysis of Fluoxetine treatment post-stroke showed that it reduced the incidence of depression (but not the severity) as well as improving recovery of neurological function and independence (Yi et al., 2010). Antidepressant treatment seems to have beneficial effects beyond anxiety and depression. Three months of Fluoxetine treatment beginning 5-10 days after stroke enhanced performance in the Fugl-Meyer motor scale (Chollet et al., 2011). Fluoxetine or Nortriptyline can both improve recovery on the Rankin disability scale independent of the effects of the drugs on depression (Mikami et al., 2011). A review of selective

serotonin reuptake inhibitor (SSRI) treatment in stroke recovery concluded that treatment reduced dependency and disability scores, as well as neurological deficit, depression and anxiety (Mead et al., 2012).

5.2 Physical Therapy

As motor deficits are one of the most common results of stroke, physical therapy is widely used to aid recovery of motor function. Reviews of physical therapy concluded that intensive, repetitive task-oriented and task-specific training is most effective (Veerbeek et al., 2014; Dobkin 2008). Current best practice is to begin physical therapy as soon as possible – even hours – after stroke (Stroke Foundation of New Zealand, 2010). As well as conventional physical therapy, there is emerging evidence that robotic physical therapy has further benefits, and electrical stimulation of the affected limb, rocking chair therapy, and the Sensori-Motor Active Rehabilitation Training (SMART) Arm system have all shown promise in initial trials (Hayward et al., 2010). Virtual reality training also has shown some positive results compared with conventional therapy (Henderson et al., 2007). Physical therapy alone may help recovery in other areas, such as cognitive skills. One study by Liu-Ambrose & Eng (2015) found that six months of exercise and recreation (two sessions of exercise and one of recreation per week) improved performance in selective attention and conflict resolution in the Stroop test, working memory and functional capacity among stroke patients compared with standard-care controls. Six months of aerobic and resistance training has been shown to improve several areas of cognition (using the Montreal Cognitive Assessment) in stroke patients (Marzolini et al., 2013). Areas improved include attention and concentration, and visuospatial and executive function. There was a reduction in the percentage of patients meeting threshold for mild cognitive impairment (MCI) (Marzolini et al., 2013). One patient showed a large improvement in speech ability after having upper limb mirror therapy (Arya & Pandian, 2014).

When exercises are augmented with cognitive or social aspects, they are more effective than exercise alone. Using an exercycle with a virtual reality cycle tour reduced the risk of progression to MCI by 23% compared with a standard rowing machine (Anderson-Hanley et al., 2012). Active video gaming with motion-sensor controls for one hour per day, four days per week for five weeks caused a larger improvement in motor performance than no intervention (Fritz et al., 2013). Weekly

dancing sessions of 30 to 60 minutes improved performance in several cognitive tests, (Hokkanen et al., 2008; Kattenstroth et al., 2013), as well as posture and reaction times, tactile tests and subjective well being (Kattenstroth et al., 2013). Patients with the lowest scores before therapy benefited the most (Kattenstroth et al., 2013).

5.3 Cognitive Therapy

Many post-stroke therapies target cognitive symptoms of stroke. Similarly to physical therapies, the most successful of these target specific symptoms rather than cognition as a whole. A recent review concluded that there was insufficient evidence of positive results of post-stroke interventions on cognitive functioning (Gillen et al., 2015). However, a review by Xu and colleagues (2013) found positive outcomes for treatment of attention deficits with repetitive drills and exercises targeting the specific type of attention problem, as well as for treatment of visuoperceptual deficits using driving simulators. The review found more limited evidence for treatment of memory and executive function, although training components of executive function such as attention or working memory could help. Another review had similar findings, with some effective treatments for focal injuries causing impairments such as neglect and aphasia, but not for any more "diffuse" impairments (Cumming et al., 2013). Currently, there don't seem to be any effective treatments for executive dysfunction (Chung et al., 2013). In addition to practicing specific cognitive skills to address deficits, there is some evidence that transcranial direct current stimulation (TDCS) in combination with practice can improve performance in auditory and visual tasks more than practice only (Park et al., 2013).

5.4 Enrichment

Several kinds of enrichment activities have been used to improve recovery in human patients. Stroke patients who listened to music daily for two months showed improvements in verbal memory and focused attention compared with those who listened to audio books and controls, and improved mood compared with controls (Sarkamo et al., 2008). A combination of two exercise and one group leisure sessions (e.g. bowling or cooking) per week for six months improved attention, working memory, functional capacity and conflict resolution (Liu-Ambrose & Eng, 2015). Post-stroke apathy was reduced with problem-solving therapy (Mikami et al., 2013).

A study by Janssen and colleagues (2013) used a comprehensive enrichment program. Patients in a rehabilitation hospital were exposed to an environment that included communal enrichments like internet access, newspapers, board games and active video games. Patients also had individual enrichment packages of music, books, puzzles, games, and family members brought hobbies and activities that patients had previously enjoyed. Staff actively encouraged participation in activities and use of the materials. Enrichment increased the amount of patients' activity, both cognitive and social, and decreased likelihood of inactivity and sleep during waking hours compared with control participants housed in standard care. A follow-up study showed that enrichment patients reported more social interaction and motor, sensory and cognitive stimulation, increased feelings of control and less boredom (White et al., 2015).

While there are a number of promising treatments for specific impairments in mood, movement and cognition following stroke, it is clear that there is no holistic therapy that has benefits across all areas. There is a need for a method or methods of treatment that address multiple deficits. One possibility may be housing in environmental enrichment (EE) that includes physical activity, social contact and cognitive activities.

6. Inflammation after Stroke

6.1 General Inflammation

Following stroke, a cascade of pro-inflammatory signals occurs in the brain. In response to cellular damage around the infarct, reactive microglia, macrophages and leukocytes are recruited into the brain and generate inflammatory mediators (Lo et al., 2003). These include inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX2), interleukin 1 (IL-1) and monocyte chemoattractant protein 1 (MCP1), which can have negative effects on lesion volume (Lo et al., 2003). When genes encoding these proteins were knocked out or reduced in mouse studies, the size of ischemia was reduced (Lo et al., 2003). Activity of immediate early genes is upregulated within minutes of injury, followed by chemokine and cytokine release over the next 12-24 hours (Lo et al., 2003, see Figure 1.1).

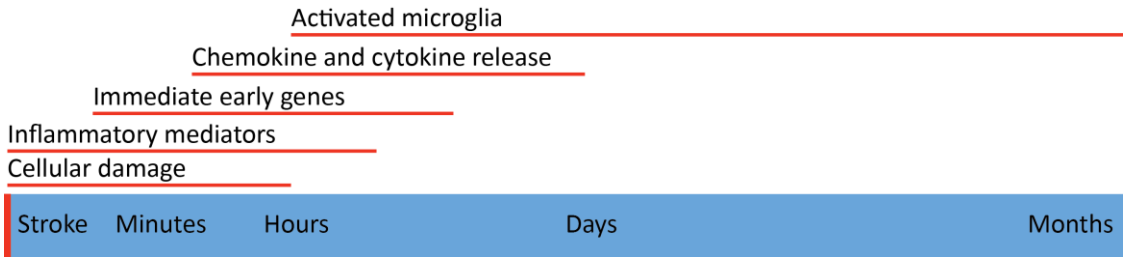


Figure 1.1 Approximate timeline of post-stroke inflammation.

Inflammatory cytokines can have beneficial or detrimental effects in animal stroke studies. For example, when a tumour necrosis factor alpha (TNF-α) binding protein was administered to rats following permanent MCAo, infarct size was reduced (Nawashiro et al., 1997). However, when the TNF-α receptor was completely knocked

out in mice, infarct size increased and functional recovery decreased (Bruce et al., 1996; Scherbel et al., 1999). Similarly, when vascular endothelial growth factor (VEGF) was administered to rats 48 hours after embolic ischemia, angiogenesis and neurological recovery were enhanced, whereas if VEGF was administered only one hour post-stroke, blood-brain barrier (BBB) leakage and infarct size were increased (Zhang et al., 2000).

In human stroke patients, inflammation seems to be associated with negative stroke outcomes. Increased levels of IL-6, C-reactive protein (CRP) and serum amyloid A (SAA) are associated with increased risk of death among hospitalised stroke patients (Rallidis et al., 2006). Stroke patients with higher levels of IL-6 and vascular cell adhesion molecule 1 (VCAM-1) are more likely to suffer further vascular disease, including death (Castillo et al., 2009). Interestingly, chronic inflammation may also contribute to stroke risk. High levels of CRP have been associated with increased risk of first-time stroke in post-menopausal women (Kaplan et al., 2008). No other individual inflammatory markers were significantly associated with stroke, but as the number of elevated markers present increased, so did the risk of stroke.

6.2 The Effect of Stroke on Microglia

In the healthy brain, microglia play an important role in regulating the extracellular environment, clearing debris and maintaining synapses (Beynon & Walker 2012). These cells have a ramified appearance – a structure of branching arms extending from

a central soma. After injury, pro-inflammatory cytokines as well as free radicals induce microglia to change their shape. Ramified, or inactive, cells change to a hyper-ramified appearance where processes thicken, then becoming reactive, where processes shorten and widen further. Even further activation can cause microglia to become amoeboid in shape and become phagocytic (Beynon & Walker 2012). In the healthy brain, microglia can still change shape, particularly in response to chronic stress, which increases the amount of secondary branching.

In the first few days after stroke, inflamed microglia (stained with the radioactive marker PK11195) have been found adjacent to the lesion as seen in magnetic resonance imaging (MRI) in humans, with very little overlap into the lesion itself (Gerhard et al., 2005). From 9 to 28 days post-stroke, the PK11195 positive area increased and began to overlap with the MRI lesion. By 150 days post-stroke, PK11195 binding was present not only around the infarct but in connected areas in the ipsi- and contra-lateral hemispheres. This shows that inflammation continues for some time after stroke and can affect remote regions.

Microglia can have a protective effect following stroke. An interesting study by Neumann and colleagues (2006) investigated the actions of microglia at the cellular level. Using hippocampal slice cultures, and oxygen and glucose deprivation, they showed that applying microglia directly to the slice at 24 or 48 hours post-stroke increased neuronal survival. They then imaged the cells and saw that the microglia migrated into the slice and formed close contacts with neurons. When microglia were treated with anisomycine (a protein-synthesis inhibitor) or minocycline (a monocyte inhibitor) their protective effect was reduced (Neumann et al., 2006). The protective effect of microglia is linked to insulin-like growth factor 1 (IGF-1). When microglia proliferation was stimulated in mice by macrophage colony stimulating factor (MCSF), they increased in number and increased release of IGF-1 (Lalancette-Hebert et al., 2007). When proliferating microglia were ablated after MCAo, there was a decrease in IGF-1 levels, and an increase in inflammatory cytokines, apoptotic cells and lesion size (Lalancette-Hebert et al., 2007). Other beneficial actions of microglia include releasing TNF-α (Lambertsen et al., 2009), phagocytosing neutrophils (Neumann et al., 2006) as well as apoptotic tissue, which may limit secondary damage and aid remodelling following cerebral haemorrhage (Zhao et al., 2007). Microglia in the subventricular zone (SVZ) release IGF-1 following stroke, which promotes

neurogenesis (Thored et al., 2009). Microglial activation can encourage angiogenesis, which is associated with increased blood flow and functional recovery (Baron et al., 2014). Grafted microglia in a damaged spinal cord release growth factors and induce axonal regeneration (Rabchevsky and Streit, 1997).

Microglia can also have negative effects after stroke. High levels of microglial staining (PK11195 in vivo and the activated microglial stain OX42 post-mortem) show that activation is high in areas showing reduced staining for neurons (NeuN), particularly for OX42 (Baron et al., 2014). Fluoxetine treatment following MCAo has been found to suppress microglial activation, inflammatory markers, and neutrophil infiltration, and reduce infarct volume, motor impairment and neurological deficits (Lim et al., 2009). The antibiotic minocycline has also been used to inhibit microglia, which had protective effects in rats (Yrjanheikki et al., 1999), and improved neurological outcome in humans (Fagan et al., 2010). Inhibition using the poly(ADP-ribose) polymerase inhibitor PJ34 reduced cell death in the hippocampus by 84% (Hamby et al., 2007).

The explanation for the dual role of microglia remains to be fully realised. It has been postulated that resident microglia (M1 microglia, see Figure 1.2) are beneficial and help to protect the brain, whereas infiltrating microglia (M2 microglia) and macrophages have negative effects (Ritzel et al., 2015; Evans et al., 2014). Stress may play a crucial role in regulating microglia and influencing differential M1 or M2 phenotype. This is discussed in Discussion section 3.3.3. However, much work is still required to fully define the actions of these cells for all neuropathological conditions including stroke.

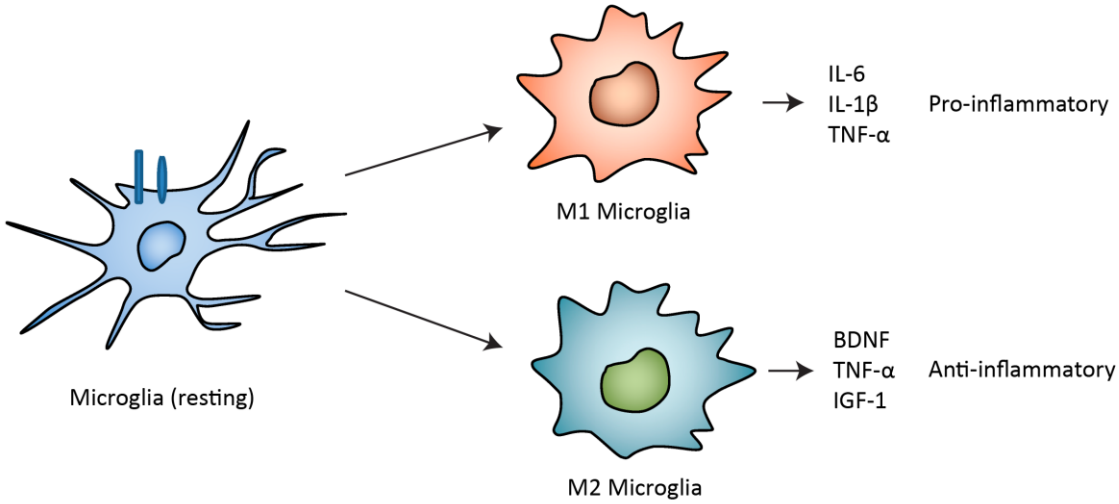


Figure 1.2 Schematic diagram of differential activation of M1 and M2 Microglia. Adapted from Nakagawa and Chiba, 2014.

7. Secondary Neuronal Loss

Stroke can cause neuronal damage in areas not immediately affected by ischemia (also called diaschisis). In one study by Kraemer and colleagues (2004), ten human patients with MCAo stroke underwent MRI. All patients experienced shrinkage of brain tissue in the peri-infarct region, as well as contralateral homolog areas and the striatum and thalamus (Kraemer et al., 2004). Atrophy has also been found in the posterior cingulate cortex six months following stroke, which was associated with increased apathy (Matsuoka et al., 2014). Cerebral atrophy after stroke is associated with depression and anxiety (Astrom, 1996).

Remote damage occurs in the thalamus following stroke. MCAo caused neuronal degeneration in the ventroposterior nucleus (VPN) of the thalamus, even though there was no reduction in blood flow in this area (Rupalla et al., 1998). Three days after stroke, calcium accumulation (an indicator of energy failure) and deafferentation of corticothalamic axons occurs, before neuronal damage (Nagasawa & Kogure, 1990; Iizuka et al., 1990). Seven days post-stroke, neurons shrink, and 14 days post more than half of the neurons in the VPN may be dead (Dihne et al., 2002). Secondary degeneration has also been found in the nucleus reticularis thalami (RTN) after photothrombotic stroke (Block et al., 2005). Remote thalamic damage after MCAo is associated with spatial learning deficit (Kumon et al., 1996).

Secondary neuronal loss occurs at a delay in several other brain areas after stroke, including the hippocampus (Butler et al., 2002) and substantia nigra pars reticulata (SNr) (Block et al., 2005) and may contribute to the delayed development of post-stroke impairments.

8. The Effects of Components of Enrichment on Stroke Recovery

An enriched environment is one that provides increased opportunity for cognitive, social or physical stimulation compared with a standard environment (Nithianantharajah & Hannan 2006). In animal studies, environmental enrichment (EE; or as sometimes referred to, enriched conditions) refers to a type of living condition where animals (usually rats or mice) are kept in large arenas or cages, in groups of 8-12 animals with objects that are changed on a regular basis (daily or weekly, depending on the study). These objects often include running wheels, toys, tunnels, cardboard huts, or other objects with which the animals can interact (Nithianantharajah & Hannan 2006). These conditions are in contrast to standard housing conditions (SC), where animals are housed in smaller cages, in smaller groups (3-6 animals) with no objects.

8.1 Exercise and Stroke Recovery

Even when a running wheel is not included in an enrichment protocol, enrichment is associated with increased activity levels, with the level of physical activity positively correlated with the degree of recovery (Xie et al., 2013). Gerbils with access to a running wheel ran approximately 880 meters per day in the week preceding experimental stroke, and that this improved their post-stroke survival to 90-100%, compared with 21-40% of animals kept in conventional cages (Stummer et al., 1994). Further, post-mortem studies on these gerbils showed decreased cell death in the cortex, striatum, and most areas of the hippocampus.

8.1.1 Pre-Stroke Exercise

Pre-stroke exercise reduces infarct volume (Ding et al., 2006) and cell death (Davis et al., 2007; Liebelt et al., 2010; Stummer et al., 1994) after stroke. Trophic factors such

as brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), IGF-I and VEGF have been found to be increased following exercise (Will et al., 2004; Zhang et al., 2011). Exercise increases expression of genes involved in synaptic plasticity and synaptic trafficking (e.g. synaptotagmin), signal transduction pathways (e.g. CaM-KII) and transcription regulators (e.g. CREB), as well as genes involved in the glutamatergic system (Molteni et al., 2002). On the other hand, genes associated with the gamma-Aminobutyric acid (GABA) system (e.g. $GABA_A$ receptor) were downregulated (Molteni et al., 2002). Both voluntary and forced exercise provide neuroprotection, and at least two or three weeks of exercise training before stroke is enough to give benefits in infarct volume, molecular factors involved in neuroprotection, and functional outcomes.

In human studies, pre-stroke exercise can reduce the risk of stroke as well as reducing the severity of disability if a stroke occurs. A recent meta-analysis showed that pre-stroke exercise increased physical activity and reduced the risk of infarction by 25% and haemorrhage by 33% (Reimers et al., 2009). Interestingly, for both types of stroke, this effect was larger for men than women (Reimers et al., 2009), indicating a sex bias, and highlights the need to examine both sexes in future studies. Another meta-analysis found that moderate physical activity caused an 11% reduction in the risk of stroke outcome (incidence or mortality) over low activity, and high activity caused a 19% reduction (Diep et al., 2010). One study of 673 stroke patients found that those who reported higher amounts of pre-stroke exercise experienced lower levels of impairment (Stroud et al., 2009).

8.1.2 Post-stroke Exercise

Exercise is known to have a multitude of beneficial effects after stroke, such as improved cerebral metabolic capacity (Dornbos et al., 2013; Schubert, 2005), improved angiogenesis (Zhang et al., 2011) and BBB stability (Davis et al., 2007; Ding et al., 2006), which all contribute to improved functional recovery.

Post-stroke exercise has also been found to increase survival rates and body weight, reduce infarct volume, and improve neurological deficit score in aged rats (Zhang et al., 2012). Interestingly, rats that exercised at a lower intensity (both walking speed and duration) showed more improvements relative to the no exercise group than those

that exercised at a moderate intensity (Zhang et al., 2012). Mild exercise five days per week for five weeks following an endothelin-1 (ET-1) injection in the sensorimotor cortex of aged rats increased recovery of sensorimotor performance in the Adhesive Removal Test. The number of proliferating cells staining positive for Bromodeoxyuridine (BrdU) in the ipsilateral cortex also increased (Leasure & Grider, 2010).

Post-stroke exercise in human patients can aid recovery. When chronic stroke patients completed three months of high-intensity treadmill exercise, peak exercise capacity and sustained walking capacity improved, whereas with conventional care they did not (Globas et al., 2012). Maximum walking speed and balance, as well as a mental health subscale of the Medical Outcomes Study improved more in the exercise group. Another study found that increased exercise dose improved recovery of walking speed (Scrivener et al., 2012). Six months of increasing treadmill exercise post-stroke improved cardiovascular fitness and mobility more than stretching and low-intensity walking (Macko et al., 2005). A meta-analysis of various exercise treatments including cardiorespiratory training as well as specific task-related training concluded that the main benefits of exercise are improvements in mobility and a resulting decrease in disability and dependence (Saunders et al., 2013). Another positive effect was improvement in balance. There was not enough evidence to make conclusions about quality of life or mood outcomes.

Exercise before or after stroke has broad positive effects on stroke risk and recovery, and even without a running wheel, due to enrichment causing increased activity, is an important component of enrichment.

8.2 Social Housing

Social housing is another component of enrichment that can have positive effects on behavioural recovery independent of exercise or enrichment. Social housing has similar beneficial effects to enrichment on social and spatial memory, and reduces anxiety in naive animals (Monteiro et al., 2013; Ravenelle et al., 2013). After stroke, social housing can improve motor recovery as much as enrichment (Risedal et al., 2002; Dahlqvist et al., 2003). Social housing increases neurogenesis in the olfactory bulb (Monteiro et al., 2013) and increases NGF gene expression in the hippocampus (Dahlqvist et al., 2003). Oxytocin is increased by social housing, and this has been

shown to reduce infarct size and oxidative stress (Karelina et al., 2011), suppress the hypothalamic-pituitary-adrenal (HPA) axis induced release of corticosterone (CORT) and increase the speed of healing (DeVries et al., 2007).

A person's social environment can influence their risk of having a stroke. Having lower numbers of close contacts and a smaller social network increased a person's relative risk of stroke by 4.1 and 2.7 times respectively (Gafarov et al., 2013). Isolation was also correlated with higher rates of smoking, hypertension, use of cardiovascular medications and depression (Rutledge et al., 2008). High levels of social support decreased the risk of stroke in both high and low alcohol consumption groups of Japanese men (Ikehara et al., 2009). Psychosocial distress as assessed by interviews was found to increase the risk of a stroke incident or stroke mortality (Henderson et al., 2013). Low scores in a social network index were associated with higher levels of the inflammatory markers IL-6, CRP, and soluble intercellular adhesion molecule (sICAM-1) (Loucks et al., 2006). Social factors can also influence a person's ability to identify a stroke. Higher perceived emotional support and frequent social contacts increased knowledge of stroke warning signs (Barger, 2012).

Social factors are important in post-stroke recovery. Pre-stroke social isolation has been associated with increased risk of a post-stroke outcome event (another stroke or death (Boden-Albala et al., 2005)). Having more baseline (17 days post-stroke) social ties and emotional support improved cognitive recovery at 6 months post-stroke (Glymour et al., 2008). Socially inactive patients were less satisfied with life and self-care ability than moderately and highly socially active patients (Boosman et al., 2011). Post-stroke disability and depression are associated with reduced social activity (Carod-Artal et al., 2000). Perceived social support reduces the relative risk of PSD (Lewin et al., 2013), while living alone following stroke increases risk of PSD while also decreasing the likelihood of antidepressant use. (Eriksson et al., 2004).

8.3 Cognitive and Sensorimotor Components

The third key component of EE is the opportunity for animals to interact with a variety of changing objects. This allows constant interaction with new objects and learning of their physical properties and location within the cage. This component is seen as similar to task specific training, and both treatments had similar effects on learning and

formation of long-term memory. They both also had positive molecular effects on the brain, including increased protein synthesis and increased acetylcholinesterase (AChE) activity in the cortex (Rosenzweig & Bennett, 1996). Enrichment objects are almost always used in conjunction with social housing and sometimes exercise, so the effects of objects alone on individually housed animals are difficult to separate. One study by Monteiro and colleagues (2013) found that adding enrichment objects to individually housed mice increased neurogenesis in the dentate gyrus (DG) and the olfactory bulb.

In human stroke therapy, specific training is often used to target specific sensorimotor or cognitive deficits, such as the walking training mentioned above in section 8.1.2, or constraint-induced movement therapy (CIMT) for upper limb impairment (Kwakkel et al., 2015). In addition to the treatments mentioned above in section 5.3, prism glasses can be successfully used to treat hemineglect by shifting the visual field (Shiraishi et al., 2008). Virtual reality training has been used to improve hand dexterity, but this only worked for very specific tasks and was difficult to implement with elderly patients (Merians et al., 2002). Speech therapy improves communication deficits (Bhogal et al., 2003), and specific memory and attention training can improve performance on closely related tasks (Barker-Collo et al., 2009). Goal management training for patients with frontal lobe damage (mainly from stroke) improved performance in a sustained attention task and the tower test (Levine et al., 2011). Working memory training, strategy training, and use of a pager system have all been moderately effective in improving performance in specific related tasks (Poulin et al., 2012).

These therapies are all effective at improving performance in related tasks, but often do not generalise to broader cognitive impairment or daily activities (Cumming et al., 2013). Cross-over can occur – Meinzer and colleagues (2012) found that CIMT improved aphasia as well as motor impairment, and upper limb therapy was found in one case to markedly improve communication deficits (Arya & Pandian, 2014).

8.4 Comparisons Between Enrichment Components

Given the separate effects of exercise, social housing and enrichment objects, several studies have attempted to compare the effects on stroke recovery of these different elements. Enrichment appears superior to social housing when applied before stroke.

Four weeks of pre-stroke enrichment in rats improved performance on several motor tests and the MWM compared with socially housed animals (Xie et al., 2013). Following permanent MCAo, rats enriched post-stroke outperformed individually housed animals with free access to exercise wheels on multiple motor tests over three months of testing post-stroke (Johansson & Ohlsson, 1996). Socially housed animals also outperformed individual animals in some of the tests. There were no group differences in infarct size (Johansson & Ohlsson, 1996). A similar study with an impoverished housing group (individually housed with no objects or running wheel) found similar results – enriched animals outperformed individually housed animals in motor tasks, and the social group outperformed the deprived group (Risedal et al., 2002). Enriched and socially housed animals had higher levels of nerve growth factor-induced genes A and B (NGFI-A and NGFI-B) in the peri-infarct and the CA1 region of the hippocampus than individually housed animals, which was positively correlated to motor performance (Dahlqvist et al 2003).

Although training in specific tasks such as the skilled reaching task can have similar benefits to enrichment (Rosenzweig & Bennett, 1996), a comprehensive review by Will and colleagues (2004) concluded that when compared with each other across a variety of behavioural, cellular and molecular tests, training was found to be more effective than exercise, and enrichment more effective than both. The above results also show that enrichment is superior to social housing alone, and that social housing is more effective than exercise.

9. Effects of Enrichment on Aspects of Stroke Recovery

9.1 Lesion Volume

Enrichment that includes all the factors mentioned in the previous section affects a variety of measures of stroke recovery, including lesion volume, cellular and molecular recovery, and motor and behavioural recovery. The effects of enrichment on lesion size are mixed. Most commonly, studies have found no difference in infarct size after transient MCAo in rats with two weeks (Hirata et al., 2011) or four weeks (Dahlqvist et al., 2004) of post-stroke enrichment. Similar results exist for permanent MCAo in rats with 8 weeks (Matsumori et al., 2006) or 13 weeks (Johansson & Ohlsson, 1996) post-stroke enrichment. There was no difference between infarct sizes when comparing

animals enriched pre- and post-stroke with only post-stroke, or with only individual housing (Ohlsson & Johansson, 1995).

One study did find a reduction in infarct size after post-stroke enrichment in rats that had undergone permanent MCAo (Bucchold et al., 2007). The reduction was correlated with improvement of functional impairments, especially in aged animals. However, a meta-analysis of 19 enrichment studies concluded that despite these mixed effects, enrichment resulted in an 8% increase in infarct size (Janssen et al., 2010). It is possible that placing animals into enriched housing very soon after stroke could increase neuronal activity and exacerbate excitotoxic neuronal loss in the infarct. This does not seem to be the case here, with studies allowing animals to recover for at least 24 hours before differential housing (Bucchold et al., 2007; Johansson & Ohlsson, 1996).

Similarly to cell death in the infarct, there are mixed results for cell death elsewhere. Enrichment has been found to increase cell death in the hippocampus of gerbils after five minutes of transient MCAo (Farrell et al., 2001), while retinal cell death was reduced by enrichment after BCCAo (Kiss et al., 2013).

9.2 Cellular Effects of Enrichment

Enrichment encourages cellular remodelling after stroke. Following stroke, dendritic spine density in the peri-infarct area decreases (Brown et al., 2008). EE and skilled reach training enhances dendritic growth in the peri-infarct area, as well as other brain areas, and is associated with functional recovery (Auriat et al., 2010). Similar results have been found with axon growth – EE encourages axon growth from peri-infarct areas into lesioned areas, and this is also associated with behavioural recovery (Papadopoulos et al., 2009). Two weeks of pre-stroke enrichment increased synapse density in the peri-infarct area (Hirata et al., 2011). Post-stroke EE increased synapse density in the parietal cortex and hippocampus, which was associated with improved performance in the MWM (Xu et al., 2009). Eight weeks of enrichment in naive rats caused an increase in neural activity in the barrel cortex in response to whisker movement (Alwis & Rajan 2013).

Enrichment increases neurogenesis. Eight weeks of EE after MCAo caused an increase in neurogenesis and neuronal differentiation in both sham and stroke rats in the dentate gyrus (Matsumori et al., 2006). This effect occurred even in enriched animals housed individually (Monteiro et al., 2013). EE increased neurogenesis in the subventricular zone of rats after MCAo and also improved survival and migration of transplanted stem cells (Hicks et al., 2007). This effect is not only present in rodents – Salvanes and colleagues (2013) found that enrichment increased neurogenesis in the forebrain of juvenile Atlantic salmon.

Astrocytes show mixed responses to enrichment. EE in intact animals can increase the number and mean size of astrocytes in the occipital cortex (Sirevaag & Greenough, 1991) and the dentate gyrus (Diniz et al., 2010; Williamson et al., 2012). After permanent MCAo in rats, four weeks of EE caused an increase in proliferating astrocytes in the peri-infarct cortex and an increase in proliferating oligodendrocytes in intact cortex (Komitova et al., 2006). EE causes astrocyte growth and remodelling around synapses, and seems to be involved in cortical remodelling in the peri-infarct area (Nilsson & Pekny, 2007).

On the other hand, decreased glial fibrillary acidic protein (GFAP) labelling has been found in the ipsilateral cortex of rats after two weeks of post-stroke enrichment (Auriat et al., 2010). 30 days after stroke, enriched animals had fewer proliferating astrocytes and smaller glial scar volumes, which were correlated with functional recovery in aged and young rats (Bucchold et al., 2007). EE reduced the number and size of hypertrophied astrocytes in the hippocampi of aged rats (Soffie et al., 1999). The role of astrocytes in stroke recovery is complex, and they seem to have both positive (e.g. supporting cortical remapping) and negative effects (e.g. gliosis and scar formation).
Enrichment has positive effects on neurogenesis and cellular remodelling, and seems to encourage cellular recovery, despite mixed effects on astrocytes. Despite all of this, timing and intensity of enrichment appears to be a major factor, which still needs to be addressed further, especially with respect to strokes within different brain regions.

9.3 Molecular Effects of Enrichment

9.3.1 Inflammation

Enrichment reduces the release of inflammatory cytokines. In naive animals, EE from 30 days to 5 months of age in mice reduced pro-oxidative factors and inflammatory mediators and increased anti-oxidative factors (Herring et al., 2010). EE reduces the expression of TNF-α and IL-1β, and subordinate animals in social housing have reduced production of cytokines and T cells (Singhal et al., 2014). Seven weeks of EE in rats reduced the inflammatory response to lipopolysaccharide (LPS) injection in the hippocampus (Williamson et al., 2012). Four months of EE in mice reduced inflammation caused by influenza infection, and improved performance in the MWM. (Jurgens & Johnson, 2012).

Microglia can be variously affected by enrichment, depending on the area of the brain. EE can cause increased microglia expression in the DG (Williamson et al., 2012), decreased newborn microglia in the amygdala (Ehninger et al., 2011), and no difference in the cortex or striatum (Auriat et al., 2010). One study found that while EE did not cause an increase in microglia, wheel running did in some cortical layers (Ehninger & Kempermann, 2003). Exercise is thought to reduce inflammation in a variety of ways including regulation of microglia and hippocampal T cells, and reduced secretion of adipokines.

9.3.2 Neurotrophins

Neurotrophins are essential to the maintenance of healthy neurons and networks, and are involved in plasticity and in injury response (Allen & Dawbarn, 2006). After striatal stroke in mice, endothelial cells in the striatum produced BDNF, and migrating neuroblasts expressed the p75NTR BDNF receptor (Grade et al., 2013). Treatment with BDNF can improve recovery from stroke in rats (Schabitz et al., 2004). BDNF levels are increased by enrichment in the hippocampus in both naive (Williamson et al., 2012) and stroke animals (MacLellan et al., 2011; Gobbo & O'Mara, 2004) and in the motor cortex, which is associated with improved recovery (MacLellan et al., 2011). On the other hand, pre-stroke enrichment was found to have no effect on BDNF levels in the peri-infarct cortex, even though enriched animals showed more neurological recovery. Enrichment also increases VEGF, which had antidepressant effects (Huang et al., 2012), and NGFI-A and NGFI-B, which were higher in the peri-infarct and the

CA1 region of the hippocampus and were correlated with improved functional recovery from stroke (Dahlqvist et al., 2003).

9.4 Behavioural effects of Enrichment

9.4.1 Motor deficits

Enrichment improves motor recovery after stroke. Various studies have found that post-stroke enrichment improves performance on the rotating rod test (Ravenelle et al., 2013), inclined plane, limb placement and beam traversal tests (Johansson & Ohlsson, 1996). Enrichment combined with daily reach training improves upper limb deficits after stroke (Biernaskie et al., 2004) and functional recovery in the staircase and cylinder tasks (MacLellan et al., 2011). Aged rats show faster and more complete recovery from MCAo when housed in EE (Bucchold et al., 2007). Enrichment is also effective in improving motor recovery after cortical impact injury (Moritz et al., 2014). A meta-analysis of the effect of post-stroke EE on sensorimotor function found consistent positive effects on several motor tasks such as the rotating pole, limb placement, horizontal beam and ladder tests, with enriched animals scoring 0.9 standard deviations higher than controls (Janssen et al., 2010).

9.4.2 Learning and Memory

Enrichment improves spatial memory performance of naive animals in the MWM (Frick et al., 2003; Diniz et al., 2010) and the radial arm water maze (Sampedro-Piquero et al., 2013). EE is protective of MWM performance after chronic cerebral hypoperfusion (Zhu et al., 2011), as well as MCAo (Dahlqvist et al., 2004; Xu et al., 2009). Janssen and colleagues' review (2010) concluded that EE animals showed a 25.1% increase in performance in the MWM compared with standard housing controls. Benefits in spatial memory are also seen in the T maze (Farrell et al., 2001) and the radial arm water maze (Sampedro-Piquero et al., 2013). Both young and aged mice housed in EE from birth performed better than standard housed animals in an episodic-like memory test that had aspects of object and spatial recognition (Diniz et al., 2010). One month of enrichment also improved performance in the novel object test in healthy adult mice (Doulames et al., 2013). Pre- and post-stroke enrichment improved performance in odour discrimination and object exploration (Gobbo & O'Mara, 2004). Social memory persistence was improved by object enrichment among individually

housed mice (Monteiro et al., 2013). These studies show that enrichment is beneficial in a wide range of learning and memory tasks in different stroke models as well as healthy animals.

9.4.3 Depression and Anxiety

There is some evidence that EE can improve symptoms of depression. Five weeks of EE reduced learned helplessness (LH) behaviour in young animals bred for high LH (Richter et al., 2013). Further, in mice exposed to seven days of EE there was increased sucrose preference and mobility on the tail suspension test compared with standard housed animals (Huang et al., 2012). This effect remained even when mice were subjected to chronic stress, which worsened symptoms in standard housed animals (Huang et al., 2012). After stroke, EE restored saccharine preference to pre-stroke levels (Branchi et al., 2013). Enrichment also moderated the effects of fluoxetine treatment – fluoxetine improved depressive symptoms in EE animals, but worsened symptoms in an induced-stress environment (Branchi et al., 2013).

EE has been shown to have mixed effects on anxiety behaviours. EE reduced anxious behaviour in both high and low anxiety rat strains (Ravenelle et al., 2013). Two months of enrichment for three hours per day reduced anxiety in the elevated zero maze, which was associated with a decrease in COX activity in brain regions associated with anxiety such as the infralimbic cortex, paraventricular nucleus of the hypothalamus (PVN), basolateral amygdala, and the ventral hippocampus (Sampedro-Piquero et al., 2013). On the other hand, Moritz and colleagues (2014) found that EE had no effect on depressive or anxious symptoms after cortical impact injury. Overall, enrichment has beneficial effects on depressive symptoms, but mixed effects on anxiety.

Despite mixed results for some measures such as astrocytes, microglia, and anxiety, enrichment has positive effects on a large variety of cellular, molecular and behavioural measures of stroke recovery, and should be considered a reliable treatment for stroke in animal models. The timing (before and/or after stroke) as well as the length of enrichment changes the extent of this recovery, and is an important factor in evaluating the effects of enrichment.

10. Timing of Enrichment

10.1 Environmental Preconditioning

Pre-stroke exercise has been extensively studied and provides many benefits (see section 8.1.1). Enrichment is more commonly used after stroke, but several studies have seen benefits from environmental preconditioning. One month of enrichment before stroke reduced the inflammatory mediators iNOS, neuronal nitric oxide synthase (nNOS), and altered the phospho-ERK1/2 and malondialdehyde (MDA) signalling pathways and improved motor performance (Yu et al., 2013), and spatial memory (Xie et al., 2013).

Continuous enrichment starting before and continuing after stroke can be equally as effective as post-stroke EE in improving motor recovery (Ohlsson and Johansson, 1995). However, EE before stroke may be more important, as pre-stroke EE reduced motor deficits as much as continuous pre- and post-stroke EE, and both were better than post-stroke EE only (Held et al., 1985).

10.2 Post-stroke Enrichment

In general, earlier enrichment seems to be more beneficial. Enrichment beginning at five days post-stroke caused large improvements in several motor tasks and increases in dendritic branching (Biernaskie et al., 2004). Beginning at 14 days had only modest effects, and at 35 days there were no differences. However, immediate intervention can have negative effects. Rats with access to a running wheel from days 0-6 after fluid percussion injury did not show an increase in exercise-induced BDNF levels, and animals showed significant impairment in the MWM, while those with access to the running wheel on days 14-20 showed elevated BDNF levels and improved performance in the MWM (Griesbach et al., 2004). Studies giving animals a 24 or 48 hour recovery period after stroke before re-entering enrichment have reported greater benefits of EE in motor and cognitive tasks (Ohlsson & Johansson, 1995; Gobbo & O'Mara, 2004). Allred and colleagues (2014) reported that the intervention needs to be timed to the progression of brain changes that occur after injury. In the first one to two weeks, there is an increase in synaptic remodelling, migration of new cells, dendritic growth and other repair processes (Allred et al., 2014), and this is a good time for interventions. After this window, it may be more difficult for cells to create new connections.

11. Environmental Impoverishment

Although standard housing may be impoverished in terms of objects, because it mostly involves social housing, it is not completely empty of stimulation. Impoverishment usually refers to individual housing with no objects and no access to a running wheel. In many studies of social housing or enrichment, animals are also housed in isolation. Some of the disadvantages of individual housing compared with social housing have been discussed above (section 8.2 and 8.4), for both animal experiments and human patients after stroke.

Social isolation can have dramatic negative effects on mammalian development. For example, macaques isolated from birth had severely impaired socialisation ability (Harlow, 1965). Post-weaning isolation of male rats increased anxious behaviour and startle reflex (Weiss et al., 2004). In addition, isolation caused an increase in both basal adrenocorticotropic hormone (ACTH) and stress-triggered ACTH release in males (Weiss et al., 2004). Experimental isolation is more often in adulthood, or after stroke for periods of a few weeks, but this can still have negative effects on health and recovery. Environmental impoverishment for 30 days can reduce cortical thickness (Diamond et al., 1972). As little as seven days of impoverishment can reduce neurogenesis in the olfactory bulb, and decrease performance in a food-finding task (Monteiro et al., 2013). Mice in impoverished housing showed a decline in spatial memory in the Y-maze, and failed to improve in the novel object recognition (NOR) task compared with socially housed and enriched animals (Doulames et al., 2013).

Impoverishment has negative effects on stroke recovery. Individual housing before and after MCAo increased infarct size and edema at 24 and 72 hours after stroke. This lead to a lowered survival rate of 40% seven days after stroke, compared with 100% of socially housed mice (Karelina et al., 2009). Two weeks of impoverishment after MCAo impaired performance in an inclined plane test and neurological severity score test compared with enrichment, and lowered synapse density in the peri-infarct area (Hirata et al., 2011). In one study that induced retinal hypoperfusion, two weeks of post-stroke social isolation increased lesion size compared with standard housing and enrichment (Kiss et al., 2013). This effect was more pronounced in female mice, suggesting that they are more susceptible to isolation. A study by O'Keefe and colleagues (2014) found that mice isolated immediately after stroke showed increased

neural atrophy and glial scarring and decreased BDNF expression compared with mice housed in pairs. Isolated mice also showed increased depressive behaviours in the forced swim test 13 days post-stroke, although this effect was gone by 33 days post-stroke

Changing environments can also have negative effects. Mice switched between enrichment and impoverishment had smaller cerebellums and lower brain weights than mice kept constantly in either environment (Klippel, 1978). When mice were switched from enrichment to de-enrichment they increased voluntary cocaine consumption, losing the protective effect of enrichment on cocaine consumption seen in continuously enriched animals (Nader et al., 2012). This was associated with increased levels of corticotropin releasing factor (CRF) mRNA in the bed nucleus of the stria terminalis (BNST), an area involved in anxiety response (see introduction section 12.4). Administration of the CRF antagonist antalamin blocked the increase in cocaine consumption, indicating that the stress of de-enrichment was causing the increased drug sensitivity (Nader et al., 2012).

Just as social housing confers many benefits on recovery, impoverishment increases stress responses, and impairs several cellular, molecular and behavioural measures of recovery. Despite the different combinations of enrichment and/or impoverishment before and after stroke, to our knowledge no one has examined a long period of pre-stroke enrichment followed by de-enrichment immediately after stroke that would more closely match the situation seen by human stroke patients.

12. Stress and Stroke

12.1 Modelling Stress in an Animal Model of Stroke

Several studies have used chronic mild stress (CMS) in combination with MCAo to induce post-stroke depression. This usually involves combinations of different mild stressors such as periods of food and water deprivation, overnight illumination, and forced swims (Wang et al., 2009). This combination causes depressive behaviours in the sucrose consumption test, the open field, and the forced swim test (Shao et al., 2015; Wang et al., 2009; Yan et al., 2013). Repeated restraint is also widely used to model stress (Neigh et al., 2009; Blanco et al., 2009; Faraji, Ejaredar et al., 2011;

Faraji, Metz & Sutherland, 2011). These methods do not mimic what happens in humans, where it is the combination of neurological effects of stroke with post-stroke disability and a change in living environment that causes stress (Stuller et al., 2012). Given the effects of impoverished housing on stress and the HPA axis mentioned in the section above, we think that this is a more realistic model of post-stroke stress.

12.2 General Effects of Stress

Chronic stress increases microglial activation in naïve animals. Rats exposed to stress showed increased microglial and neural activation in the PFC, as well as impairments in spatial working memory (Hinwood et al., 2012). Minocycline treatment suppressed microglial activation and reduced neuronal activation and the memory impairments (Hinwood et al., 2012). Chronic stress increases the amount of microglial secondary branching towards hyper-ramification and activation (Beynon & Walker, 2012; Walker et al., 2013). In hand with increased microglial activation, stress increases inflammatory cytokine release (Walker et al., 2013). This can have negative effects on neurogenesis, plasticity and memory (Yirmiya & Goshen, 2011) and may contribute to depression (Anisman & Merali, 2002). Rats given chronic mild stress and CORT injections showed dendritic atrophy and loss of synapses in CA3, CA1 and granule cells of the hippocampus, which was associated with spatial memory impairments (Sousa et al., 2000). These changes reversed after ending stress and CORT treatment (Sousa et al., 2000). Elevated glucocorticoid levels can reduce BDNF levels, which could hinder cellular recovery and plasticity in the peri-infarct area (McKlveen et al., 2015).

12.3 Effects of Stress on Stroke Recovery

Stress is common in human stroke patients (Walker et al., 2014), with around 66% of patients reporting high psychological distress soon after stroke (Hilari et al., 2010). Stress, activation of the HPA axis and increased concentrations of ACTH, and CORT or cortisol affect the immune response to stroke and subsequent recovery.

12.3.1 Pre-stroke stress

Chronic stress is a risk factor for developing stroke, and pre-stroke stress impairs recovery. Among middle aged men, presence of severe psychological stress increased

the risk of stroke by 2.1 times (Harmsen et al., 1990). A review by Stuller and colleagues (2012) concluded that the level of glucocorticoid release is important. Low levels of glucocorticoids can suppress cytokine release, but high levels, especially early after stroke, increase the release of inflammatory cytokines, which are associated with increased neuronal death and worse functional outcome (Stuller et al., 2012). Chronic social stress (being placed in the home cage of and fighting with a large, aggressive male mouse) or CORT injections before stroke increased infarct size and impaired passive avoidance retention in mice (Sugo et al., 2002). These differences were reversed after treatment with a glucocorticoid receptor antagonist (Sugo et al., 2002). Daily restraint stress for 16 days before hippocampal stroke impaired spatial cognition, but rats injected with CORT daily for the same period were not impaired (Faraji, Ejaredar et al., 2011). This shows stress has its effects not only through CORT expression. Three weeks of daily restraint stress before cerebral hypoperfusion induced by cardiac arrest in mice increased neuronal damage, microglial activation and anxious behaviour (Neigh et al., 2009). Minocycline treatment (which reduces microglial activation) reduced these effects (Neigh et al., 2009).

12.3.2 Post-stroke stress

Post-stroke stress also has negative effects on recovery. In stroke patients, there is a large surge of ACTH and cortisol soon after stroke, and cortisol remains high for some days (Fassbender et al., 1994). High levels of ACTH were associated with larger infarct volume, acute post-stroke confusion and worse functional outcome (Fassbender et al., 1994). MCAo followed by chronic mild stress increased levels of CRF, IL-1β and TNF-α in the hippocampus of rats (Wang et al., 2013). Glucocorticoid receptors in microglia and astrocytes increased from days 3 to 7 post-stroke to over 200% of baseline levels (Hwang et al., 2006). Microglial activation is important after stroke as increased activation can have negative effects, but decreased activation has detrimental effects as well. After motor cortex photothrombotic stroke, ionized calcium-binding adapter molecule 1 (IBA1) staining was increased, and there was some neuronal death in the thalamus (Jones et al., 2015). Four weeks of stress post-stroke decreased microglial levels, which was associated with further neuronal loss in the thalamus.

Stress generally impairs behavioural recovery, including motor function (Stuller et al., 2012). Chronic mild stress following MCAo is used as a model of post-stroke depression – animals show lower locomotor activity, less frequent rearing, and a lower

preference for a sucrose solution (Wang et al., 2009). Acute stress after mPFC lesions increases anxious behaviour in the elevated T-maze (Blanco et al., 2009). On the other hand, mild restraint stress after hippocampal stroke has been found not to affect lesion size, and to actually reduce spatial deficits in the MWM (Faraji, Metz & Sutherland, 2011). The authors speculate that a low level of stress may be beneficial to recovery, or that spatial memory may be improved by stress while other functions are not.

Post-stroke stress contributes to poorer functional outcomes. At the cellular level this is represented through neuronal loss, increased CORT and changes in microglial activation at sites distant to the infarct. Minimising stress both before and after stroke seems to be key to improving recovery. However, like most aspects of post-stroke recovery, establishing a time for starting treatment that may induce stress needs to be further investigated.

12.4 Stress and the mPFC

Medial prefrontal cortex stroke is associated with a variety of mood and cognitive problems in humans and animals (see section 3). One of the reasons for this is the role of the mPFC in regulating the HPA axis. The mPFC has mineralocorticoid (MR) and glucocorticoid (GR) receptors. These sense systemic CORT and CRH levels and are involved in a feedback loop to regulate the HPA axis (McKlveen et al., 2015). For example, when crystalline CORT was directly applied to the mPFC, blood ACTH and CORT after stress was lowered (Diorio et al., 1993). The mPFC can have inhibitory or excitatory effects on the HPA, via a pathway from the anterior BNST to the PVN (Radley & Sawchenko, 2011). For example, stress-induced salivary cortisol levels were inversely correlated with rate of glucose metabolism in the rostral mPFC, indicating that this area has an inhibitory effect on cortisol release (Kern et al., 2008).

Excessive stress can have adverse effects on the mPFC. Six hours per day of restraint stress for three weeks in rats decreased dendritic spine volume and surface area, reduced the number of large spines and increased the number of small spines (Radley, Rocher et al., 2008). Stress impairs many executive functions controlled by the mPFC such as set-shifting, decision making and planning, and increases habitual behaviour (McKlveen et al., 2015). In both humans and animals, these behavioural changes are accompanied by atrophy and lower activation in the mPFC (McKlveen et al., 2015). In

lesion studies, this could cause a cycle of damage where mPFC lesion reduces HPA inhibition, which increases stress response and further damages cells in the mPFC and related stress-sensitive areas such as the hippocampus.

Damage to the mPFC can disrupt normal stress regulation. Lesions to the mPFC (including cingulate gyrus, pre-limbic and infra-limbic areas) caused an increased adrenal response to immobilisation stress (Ondicova et al., 2012). Cingulate gyrus lesions increased blood levels of ACTH and CORT after acute restraint stress compared with shams (Diorio et al., 1993). mPFC lesions increased c-fos mRNA (an indicator of neuronal activation) in the PVN and increased ACTH blood concentration (Figueiredo et al., 2003). Ablation of noradrenergic innervation of the dorsal mPFC from the locus ceruleus reduced restraint-induced Fos and CRH mRNA in the PVN, and reduced acute secretion of ACTH and CORT (Radley et al., 2008).

mPFC damage has stress-related behavioural effects as well as HPA changes. Chronic mild stress combined with social subordination and left ACC stroke increased anxious behaviours in rats (Hewlett et al., 2014). ACC stroke alone did not change basal CORT levels, but did increase suppression of the HPA in the dexamethasone test (a test of negative feedback in the HPA axis) which indicates increased stress sensitivity, and caused adrenal hypertrophy (Hewlett et al., 2014). mPFC patients reported higher feelings of stress after a social stress test than healthy controls or patients with brain damage in other areas (Buchanan et al., 2010). Greater volume of mPFC damage was associated with increased cortisol response in women, but lower cortisol response and higher heart rate in men. It seems that mPFC regulation of stress response is different in men and women (Buchanan et al., 2010).

The mPFC has an important role in regulating stress. Damage to this area disrupts stress regulation, which can further exacerbate stress caused by environmental factors after stroke. Dysregulation of the stress response has negative effects on stroke recovery, including cellular recovery in the mPFC itself.

12.5 Enrichment and Stress

Enrichment appears not to change levels of stress hormones, but reduces an animal's response to these hormones. In a large study of EE, social housing, and individual housing, there were no differences in levels of plasma CORT concentration, glucocorticoid receptor expression in the hippocampus or CRH mRNA in the paraventricular nucleus (Dahlqvist et al., 2003). Social housing did not change glucocorticoid concentrations, but did reduce the expression of inflammatory cytokines (Karelina et al., 2009). Enrichment in rats increased interleukin-11 (IL-11) and caused microglia to move towards M2a type, which act to reduce inflammation (Pusic et al., 2014). EE decreased the response to amphetamine treatment and stress-induced CORT, which was associated with reduced anxiety (Ravenelle et al., 2013). Enrichment after chronic mild stress combined with fluoxetine increased neuronal proliferation in the hippocampus (Tanti et al., 2013).

Enrichment seems to have beneficial effects on behavioural changes caused by stress. EE protects against stress-induced memory impairments (Wright & Conrad, 2008) and depressive symptoms (Huang et al., 2012). Fluoxetine treatment worsened depressive symptoms after chronic stress, but when stress was followed by EE, fluoxetine reduced depressive symptoms (Branchi et al., 2013)**.** Social housing can cause stress among submissive animals after confrontation, but animals housed socially after these conflicts showed behavioural improvements and lower arousal than those housed individually (Singhal et al., 2014). Conversely, one month of EE in mice increased behavioural responses to acute (von Frey fiber) and chronic injury (formalin injection). This may indicate a sensitisation to pain or inflammation (Shum et al., 2007). Generally, stress seems to have negative effects, but can be neutral or positive in small amounts. Enrichment is usually protective against the negative effects of stress. This may be because enrichment itself is a mild stressor which inoculates against more serious stress events in the future (Crofton et al., 2015)

13. Summary

A patient's environment, both before and after a stroke, has a large impact on their resilience and recovery. Disability caused by stroke can result in a loss of enrichment in the patient's environment, as they are housebound or institutionalised. This can be

associated with symptoms of depression and anxiety, and poorer outcomes (Campbell Burton et al., 2012). Impoverishment has a wide range of negative effects on stroke recovery including increased infarct sizes, impaired behavioural scores, increased stress, and worsened cellular and molecular recovery. An enriched environment following stroke, including cognitive and social activities, can improve activity levels in human patients (Janssen et al., 2013). EE has a wide range of positive effects on stroke recovery including improved cellular recovery, reduced inflammation, reduced stress, and improved behavioural scores.

Our study aimed to more closely approximate the situation that many human stroke patients encounter, that is, an enriched environment pre-stroke followed by a change in post-stroke environment that is impoverished or enriched to varying degrees. We assessed the effect of changes in post-stroke environment on lesion volume and measured microglial activation as a measure of inflammation in various brain regions. We also assessed functional recovery in a variety of behavioural tasks, as well as behavioural models of mouse anxiety and depression. We expected that DE would increase infarct volume, increase remote inflammation and worsen behavioural recovery due to increased stress and loss of the benefits of enrichment.

Chapter 2. Methods

1. Animals and Housing

Male C57BL/6*J* mice aged 2-3 months and weighing 28-32 g were obtained from the Hercus Taieri Resource Unit, University of Otago. All procedures described in the study were approved by the University of Otago Animal Ethics Committee. Animals were housed in groups of ten in mildly enriched conditions on a 12:12-hour day-night cycle at 22 ± 2 °C for three months prior to stroke. Animals had *ad libitum* access to standard chow and water. During the three-month period where we maintained the mice in mild enrichment conditions, we used open-topped cages (430 x 280 mm and 100 mm high) with wire lattice lids. Cages were provided with shredded paper along with toys and objects that were changed weekly. These objects included plastic cups, trucks, plastic and rubber balls, plastic and wooden ice cream sticks, polystyrene balls and stars, and plastic and cardboard tubes and shelters (Figure 2.1). The cages themselves were changed weekly, with new bedding material.

Figure 2.1. Example of cage with enrichment objects. Other objects were used such as cardboard, wooden and polystyrene objects, and other toys such as balls and cups.

Because of the large number of animals (n=15-20 per treatment group), the procedures of stroke, differential housing and behavioural testing were split into two rounds. The first half of the animals received stroke after three months of enrichment, and the second half received stroke after approximately five months of enrichment. There were some minor behavioural differences between rounds one and two (see Appendix A), but these were generally due to small sample sizes in individual rounds (see Discussion section 4.1).

Immediately following sham or stroke surgery (see below), animals were split into three separate housing groups, and after 3-4 days of post-surgical monitoring transferred to the Behavioural Phenotyping Unit (BPU), University of Otago. The groups of ten animals from pre-stroke housing were randomly split into two groups of five animals and each group of five went into a different housing condition. Some groups continued in the mildly enriched housing with weekly object changes (EE), some groups started enhanced environmental enrichment (EEE) with object changes three to four times per week, while the third group was housed in a de-enriched environment (DE) where animals were housed individually with no toys or objects.

Upon moving all mice to the BPU, all groups were transferred from open-top cages to individually ventilated cages (310 x 140 mm and 120 high; Tecniplast, Italy). In the EE and EEE groups these smaller cages necessitated a change from groups of five animals to two groups – one of three animals and one of two animals. It should be noted that moving mice from open-top caging into individually ventilated cages (IVC) acts as a mild stressor to the animals. Moving the animals from large groups into smaller groups can have similar effects. These changes in conditions mean that all mice are subjected to mild stress, which mimics what happens to human patients. In the BPU, all animals were maintained on a reversed 12:12-hour day-night cycle. These housing conditions continued for five weeks throughout behavioural testing until the animals were sacrificed. Figure 2.1 shows the design and timeline of animal housing.

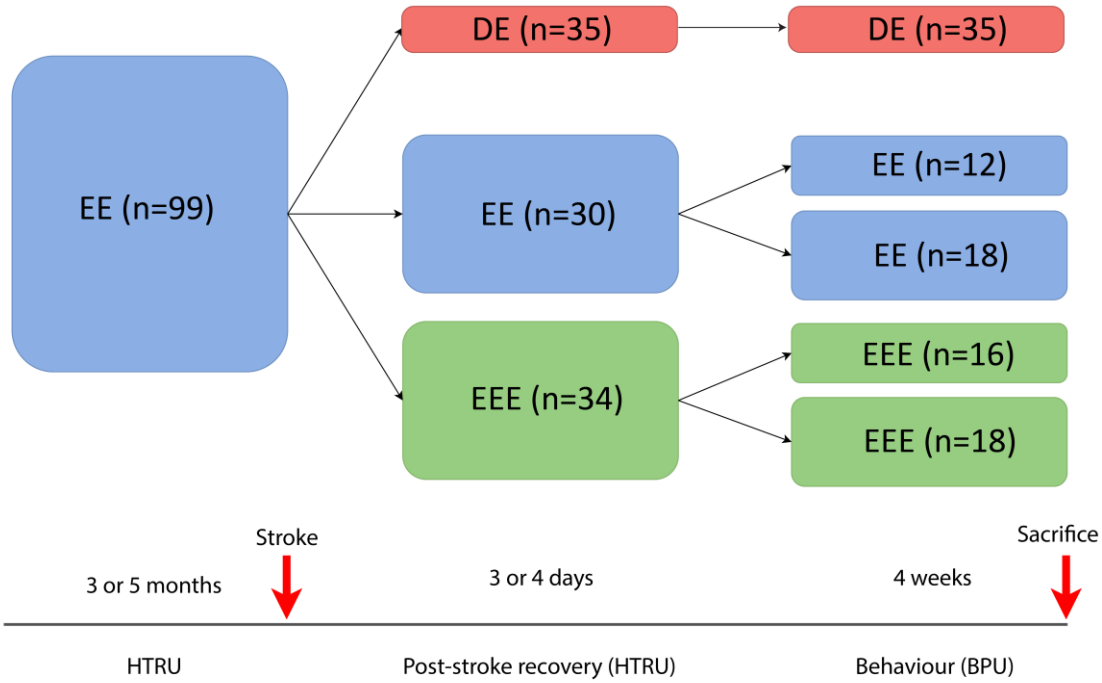


Figure 2.2. Schematic diagram of experimental design and timeline. n-numbers refer to the number of mice in each group. The size of the coloured blocks represents the number of animals in each cage, pre-stroke being 10, post-stroke recovery 5 for EE and EEE and 1 for DE, and during behaviour testing 2 or 3 for EE and EEE and 1 for DE. EE = environmental enrichment; EEE = enhanced environmental enrichment; DE = de-enriched environment; HTRU = Hercus Taieri Resource Unit; BPU = Behavioural Phenotyping Unit.

2. Photothrombotic Stroke

Focal ischemia in the medial prefrontal cortex (mPFC) was induced using the photothrombosis model (Clarkson et al., 2010). In this procedure, animals were initially anaesthetised with 4 % isoflurane in an oxygen/air mix in a closed plexiglass container (VetEquip; U.S.A.) before being transferred to a stereotaxic frame (Model: 9000RR-B-U, KOPF; California, U.S.A.). At this time, the anaesthetic (delivered via a nose-cone) was reduced and maintained between 1.5 - 2.5 % isoflurane during the course of the surgical procedures. Body temperature was monitored and maintained at 36.9 ± 0.2 °C using a rectal thermometer attached to a homeothermic blanket (Harvard Apparatus; Massachusetts, Unites States of America).

Once steady-state anaesthesia had been reached in the animals, the top of the head was shaved with an electric razor (Remington). The animal's eyes were protected from the light and from drying out using Vaseline (Unilever). The shaved area was then sterilised using a cotton bud with hibitane (30 % chlorhexadine in 70 % ethanol). A midline incision 10 – 15 mm long was made in the scalp to expose the skull. Using a sterile cotton bud, the periosteum was cleared from the top of the skull, and the skull dried to minimise light scatter. Bregma was located and marked on the skull with a marker. A 3300 KW cold light source attached to a 20 x objective with a 2 mm aperture (Olympus KL1500 LCD; New Zealand) was attached to the stereotaxic frame and centred 1.2 mm anterior to bregma on the midline and lowered until it just touched the skull. Rose Bengal dye (200 µl of a 10 mg/ml solution in saline) was injected intraperitoneally (i.p.) into the mouse. After waiting five minutes for uptake into the blood, the brain was illuminated for a period of 22 minutes. This illumination time was chosen based on prior studies conducted by Ms Lisa Zhou (Honors and now PhD candidate in the laboratory of Dr Clarkson), which she demonstrated resulted in reliable infarction to the mPFC (Zhou et al., 2015).

Following illumination, the light was switched off and the scalp was closed with surgical glue and the animals were placed in an individual cage on a heating pad to recover from anaesthesia, before being randomly assigned to one of the housing conditions. Animals were kept in the surgery room for 2-4 days for post-surgical monitoring.

3. Behavioural Testing

All behavioural testing was carried out during the animals' night phase (when mice are more active (Aoki et al., 2014)) in the BPU, University of Otago. Animals were transferred to the testing room just prior to testing, which was carried out under white fluorescent lights. The lighting was under moderate lux conditions to allow cameras to operate effectively and to allow anxiety-related behaviour under light exposure, while not being to bright to avoid excessive anxiety and disturbance of animals' daily rhythms. All arenas were cleaned with 10 % ethanol between each trial. During each trial, the experimenter stood behind a curtain to avoid influencing the animals' behaviour. The grid-walking and cylinder tests were carried out one week prior to stroke and one week post-stroke to ensure there was no effect of stroke on simple

motor behaviour that would confound further tests of activity such as the open field test. The open field, novel object recognition, object location recognition, elevated plus maze and light-dark box tests were carried out at one and four weeks post-stroke to determine whether any deterioration or recovery occurred in activity levels, anxiety, or memory over the weeks following stroke.

3.1 Grid-walking

The grid-walking test measures forelimb and hindlimb motor performance (Clarkson et al., 2010). It was carried out one week prior to surgery to obtain baseline measurements, as well as one and four weeks following stroke to test whether or not the strokes had any effect on motor performance. Animals were placed on a wire grid measuring 200 x 320 mm with 12 mm grid squares and dark opaque plastic walls suspended 500 mm above the ground for five minutes. A mirror was placed underneath the grid to enable a video camera to record the underside of the grid. Videos were analysed by hand for the total number of steps taken and the number of foot faults by an observer blind to the experimental conditions. A foot fault was defined as a step where a limb slipped through the grid or where the wrist was placed on the grid wire instead of the paw. The number of foot faults was expressed as a percentage of total steps taken.

3.2 Cylinder Test

The cylinder test measures the use of each forelimb and is a test of motor asymmetry (Clarkson et al., 2010) and indicates that a stroke has affected one side of the brain more than the other. Similar to the grid-walking test, the cylinder test was carried out one week prior to surgery to obtain baseline measurements, and one week following stroke to measure the effect of stroke on motor performance. Immediately following the grid-walking task, mice were placed in an upright clear plexiglass cylinder (100 mm diameter x 150 mm high) for five minutes. A mirror was placed behind the cylinder to allow the video camera to see all movements of the animal. Paper towels were placed underneath the cylinder to allow cleaning between animals. As mice rear up to a standing position to explore the cylinder, they use their right, left or both forelimbs to press on the cylinder for support. Videos were analysed by hand by an observer blind to the experimental conditions for the amount of time each forelimb

was used during rearing. This was expressed as the percentage of time spent using the left relative to the right forelimb, and is a measure of forelimb asymmetry.

3.3 Open Field

The open field test involves observing how an animal behaves in a large box with no objects, possibly revealing anxiety related behaviours and abnormal activity levels (Shao et al., 2015). Animals were placed in a 400 x 400 mm white opaque Plexiglas arena with sides 200 mm tall for ten minutes. Activity was recorded with overhead cameras and analysed using TopScan software (CleverSys Inc; Virginia, U.S.A.). Several different variables were measured. These included distance travelled (both total as well as assessment of individual one-minute sections), and the number of activity bursts that occur at various speed thresholds, which can be a measure of hyper- or hypo-activity. Several different threshold speeds were considered, and 200 mm/s was chosen as the speed showing the least variance and the largest differences between groups (See Appendix D Figure A18). Another measure was the amount of time spent in the middle of the arena vs. around the outside, which is often used as a measure of anxiety – more anxious animals spend less time in the open middle section. In TopScan, the arena was divided up into a 4 x 4 grid of even-sized squares. The middle zone was made up of the four middle squares and the outside zone was made up of the 12 squares around the border (see Figure 2.3.).

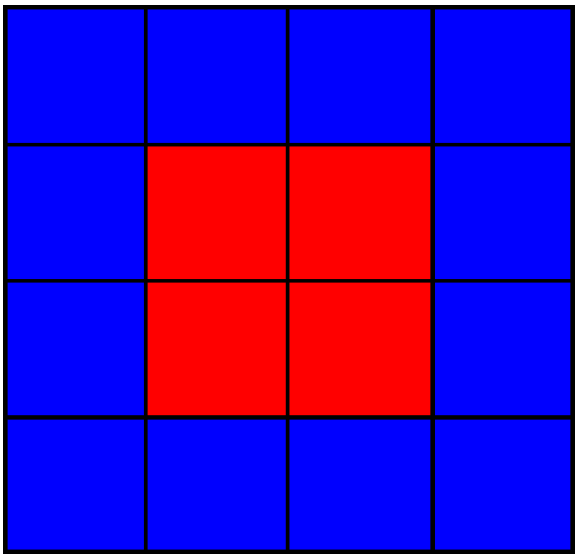


Figure 2.3. Open field grid arrangement for TopScan analysis. The middle zone (red) measured 200 x 200 mm.

3.4 Elevated Plus Maze

The elevated plus maze is a measure of anxiety related behaviour – animals that spend less time out on the open arms are thought to be more anxious (Ravenelle et al., 2013). The maze consisted of a plus-shaped maze made of white opaque plastic. Two of the arms were open with a small vertical lip, and two of the arms were enclosed with walls measuring 150 mm high. The arms were all the same length and measured 650 mm from the middle. The whole maze was elevated 600 mm above the ground on a metal base (see Figure 2.4.). Animals were placed in the maze with their heads in the middle of the intersection of all four arms, facing the open arm away from the investigator, and could immediately choose which arm to move into. Trials lasted for three minutes and activity was recorded with overhead cameras and later analysed by hand. Variables measured included the percentage of time spent in the open arms and the number of entries into the open arms. An entry into an open arm was defined as when more than 50% of the animal's body entered the arm.

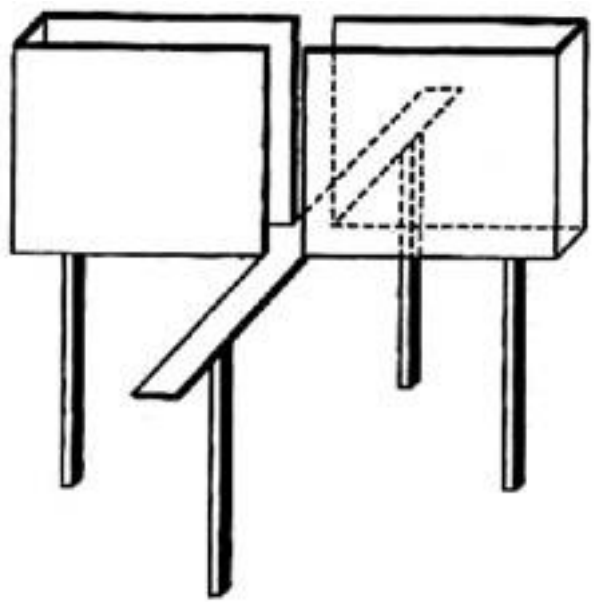


Figure 2.4. Diagram of elevated plus maze arena (Steinman & Trainor, 2010).

3.5 Light-Dark Box

Like the elevated plus maze, the light-dark box (LDB) is used as a test of anxiety – animals that spend less time in the light half are thought to be more anxious (Campos et al., 2013). The light-dark box consisted of a rectangular arena 460 x 270 mm and 270 mm high, split into two by a wall with a small opening in the centre at the bottom

for animals to move through. Slightly less than half of the arena, including the dividing wall and lid, was made of black opaque plastic, measuring 190 mm long x 270 x 270. Slightly more than half (270 mm cube) including the lid was made of clear plastic, and was illuminated by the room lights. The mouse was initially placed in the light half of the arena, and its activity recorded for five minutes by an overhead camera and later analysed using TopScan. Variables measured were the percentage of time spent in the light half of the arena, and the number of times the animal moved between the light and dark sections. Due to the absence of grid-walking and cylinder testing in the second round, the light-dark box test was included for the second round of strokes and behavioural testing only, so approximately half of all animals were tested in this arena.

3.6 Novel Object Recognition

The novel object recognition test measures time spent with a new and old object, and is a measure of visual recognition memory (Grayson et al., 2015). One day after the open field test, animals were put back into the same arena for ten minutes, this time with two identical objects. Objects were placed in two corners of the arena, 80 x 80 mm away from the two back corners (see Figure 2.5. A). This constituted the habituation phase, and provided time for the animals to investigate and become familiar with each of the objects. Mice were then placed back in their home cages for approximately one hour (the retention period). During the retention period objects were removed, cleaned and then one of the objects from the habituation phase was substituted with a new object and along with the other original object introduced back into the arena in the same locations (see Figure 2.5. B). Animals were then re-introduced into the arena for a 3 minute test period. During week one behavioural testing, the familiar objects used were two identical yellow soft drink cans and the novel object was a red plastic rectangular prism with a base of approximately the same size as the soft drink cans (see Figure 2.6.). During week four testing, the familiar objects were ceramic bear salt-shakers of a similar size to the week one objects, and the novel objects were ceramic figures of different colour and shape but a similar size to the familiar objects.

A) B)

Figure 2.5. Novel object recognition arena arrangements. On the left are the two cans in the habituation period. On the right are the familiar object and the novel object in the test period. Photos courtesy of Lisa Zhou.

Figure 2.6. The objects used in the novel object and object location recognition tasks. The soft drink can and the ceramic bear were the familiar objects used in both tasks. The red plastic rectangle and the ceramic girl were the novel objects (Photo courtesy of Lisa Zhou).

Activity was recorded with overhead cameras and videos were analysed using TopScan. Measured variables included time spent sniffing (investigating) each object and the number of times an object was sniffed. To analyse sniffing, a zone was created in TopScan around the objects measuring 20 mm in width. When the animal was sniffing in this area it was deemed to be investigating the object. The accuracy of this method was checked by analysing some videos by hand (see Appendix E Tables A1 and A2).

3.7 Object Location Recognition

The object location test measures time spent with familiar objects in a familiar or new location, and is a test of spatial memory (Luine, 2015). One day after the novel object test, animals were again placed back into the same arena for ten minutes with the two original familiar objects from the novel object test. Objects were in the same position as for the novel object test (see Figure 2.7. A). This was the habituation phase for the object location recognition test. Animals were again placed back in their home cages for approximately one hour. One of the familiar objects was moved to a new location (the bottom right-hand corner 80 x 80 mm out from the corner) and the animals were then reintroduced to the arena for three minutes (see Figure 2.7. B). The object location recognition test was carried out at one and four weeks post-stroke. The objects used were the same as the familiar objects in the novel object recognition task – soft drink cans during week one testing and ceramic bear salt shakers during week four testing.

This test period was later analysed using TopScan in the same way as in the novel object recognition test. Activity was recorded with overhead cameras. Similarly to the novel object recognition test, the measured variables were the time spent sniffing each object and the number of times each object was sniffed.

A) B)

Figure 2.7. Object location recognition arena arrangement. On the left are the two familiar objects during the habituation period. On the right, one of the objects has been moved to the unfamiliar location. Photos courtesy of Lisa Zhou.

4. Tissue Processing

4.1 Cardiac Perfusion

Animals were sacrificed following the completion of either week one or week four of behavioural testing. Mice were given an anaesthetic overdose of pentobarbital (100 µl i.p. of a 75 mg/ml solution). While still breathing, the animals were placed in a supine position with their forelimbs and one hindlimb taped to an absorbent mat in a fume hood. To ensure the mouse was fully unconscious, a foot-pinch was used to test the pain reflex.

An incision was made with scissors in the medial abdomen immediately posterior to the sternum. Lateral incisions were made along the bottom of the ribcage on both sides. Further incisions were made through the diaphragm and the sternum to expose the chest cavity. Incisions were also made laterally and connective tissue cleared away in order to provide access to the heart. A further small incision was made in the right atrium to allow the blood and perfusate to drain following circulation through the body. A 25-gauge needle attached to a 1 ml syringe and a pressurised perfusion system (Perfusion One, Leica Biosystems, Germany) was inserted into the left ventricle, and approximately 10 ml of a 0.125 M phosphate-buffered saline (PBS) was injected at a

constant pressure, followed by approximately 30 ml of an ice-cold 4 % paraformaldehyde fixative solution. The mouse was then decapitated with scissors and skin and muscle cleared to expose the skull. The skull was then carefully removed using scissors and forceps, the optic nerves severed and the whole brain removed. The brain was then stored in 4 % paraformaldehyde for approximately one hour before being transferred into a 30 % sucrose solution in PBS to protect the brain prior to freezing and sectioning. Brains were left in sucrose solution for at least two days or until brains had sunk indicating they were ready to be cut.

4.2 Brain Tissue Sectioning

Brains were sectioned with a freezing microtome at -20 °C (Leica Jung, RM2025). Before mounting, the cerebellum was removed giving a flat surface at the posterior part of the brain to mount onto the freezing stage. The stage of the microtome was covered with a layer of 30 % sucrose and two brains at a time were placed onto the stage with the anterior surface facing the blade. The stage was then turned on to cool down and further sucrose solution was added around the base of the brains to secure them to the stage, and a polystyrene cover was placed over the brains to insulate them for approximately five minutes until they were completely frozen. The blade was set at 45° and a small amount of cryoprotectant solution was applied to the blade to protect the sections during the cutting process. Coronal (40 μm thick) sections were cut and carefully collected from the blade using a paintbrush and placed into wells containing cryoprotectant (1 % Polyvinylpyrrolidone (PVP-40), 30 % sucrose, 0.9 % NaCl, 30% Ethylene glycol in 0.1M phosphate buffer) in a 24-well plate (Falcon, U.S.A.). Twelve wells were used for each brain (two brains per 24-well plate), and sections were placed sequentially into these wells, so that each well contained every 12^{th} section. The sections were then stored at -4 °C until mounting or staining.

4.3 Cresyl Violet Staining

Tissue was taken from two non-consecutive wells to ensure an even distribution of sections throughout the brain, for instance wells 1 & 7 for each brain. This ensures an accurate measure of lesion size. Sections were transferred into a shallow glass dish containing Tris-buffered saline (TBS; 0.05 M Tris and 0.15 M saline in dH_2O) then using a paintbrush gently mounted on microscope slides subbed with a gelatine

solution (0.2 % gelatine and 0.025% Chromium Potassium Sulfate ($KCr(SO_4)_2$) in dH_2O) and allowed to dry overnight. The following day, slides were taken through ascending ethanol concentrations (50, 70, 95 and 100 % ethanol). This process partially dissolves the fatty cell membranes and allows the Cresyl violet to enter the cell bodies to stain the Nissl substance. Slides were then taken back through descending ethanol concentrations, dH_20, then immersed in a Cresyl violet solution (0.1 % Cresyl violet dissolved in 250 ml of a 0.07 % acetic acid solution) for ten minutes. Slides were rinsed quickly in 0.07 % acetic acid solution to clear away excess stain then taken back through ascending ethanol solutions to dehydrate the sections before two Xylene baths and coverslipping with dibutyl phthalate in Xylene (DPX).

Photos were taken of each section to quantify lesion size. A montaging microscope (Olympus BX61, Japan) connected to a computer running the Volocity imaging program (PerkinElmer, USA) was used to take digital images of each section and exported TIFF files. These files were then opened in the ImageJ processing software (National Institutes of Health, USA) and the drawing tool used to define the edges of the infarct and calculate the area.

4.4 IBA1 Chromagen Staining

IBA1 is protein expressed by microglia, and as its expression is increased in response to microglial activation, is often used as a marker of inflammation (Walker et al., 2013). Tissue was taken from one well per brain, placed into net wells using a small paint brush and washed in TBS (3 x 10 min). Where possible, tissue was left in the net wells and transferred to another well plate containing a different solution, in order to reduce damage caused by handling with the brush. Following this the sections were quenched in glycine (0.76 % in 0.1 M phosphate buffer) for 10 min. Glycine blocks formaldehydes left from fixation and prevents their reaction with peroxidases used later in the procedure. Sections were washed in TBS (3 x 10 min) then placed into an endogenous peroxidase blocking solution (8 ml methanol, 11.4 ml TBS and 600 µl 30 % H2O2). This solution prevents non-specific peroxidases from reacting with the diaminobenzidine (DAB) used later in the procedure and increasing the background staining. Sections were again washed in TBS (3 x 10 min) then placed into incubation in the primary antibody solution (rabbit anti-IBA1, Wako Pure Chemical Industries

Ltd., Japan; diluted 1:10,000 in a TBS/0.3 % triton/0.25 % bovine serum albumin (BSA) solution with 2 % normal goat serum) for approximately 40 hours at 4 °C on an orbital shaker. The primary antibody concentration of 1:10,000 was determined following a titration series that we carried out that ranged from 1:1,000 to 1:10,000.

Following the primary antibody incubation, sections were washed for 10 min in 4x TBS and then 2 x 10 min in 1x TBS before incubation in the secondary antibody solution (biotinylated goat anti-rabbit IgG, Vector Laboratories, U.S.A.; diluted 1:400 in a TBS/0.1 % triton/0.2 % BSA solution) for approximately 70 min. Sections were then washed in TBS (3 x 10 min) and then incubated in A/B solution (1 % A and 1 % B in TBS/triton/BSA, prepared 30-40 min in advance to allow the avidin to bind with the biotinylated enzyme, Vectastain ABC kit, Vector Laboratories, U.S.A.) for approximately 70 min. The avidin proteins bind to the biotin on the secondary antibody and so the IBA1 molecules are connected with the biotinylated enzyme complex. After another 3 x 10 min wash in TBS, sections were incubated in a DAB solution (1 x 10 mg tablet dissolved in 20 ml 0.1 M phosphate buffer (PB), syringe filtered then 6.8 µl of 30 % H202 added) for 7-10 minutes. DAB is oxidised by the biotinylated enzyme and forms a brown stain wherever IBA1 is present. During the reaction process sections were wet-mounted on glass-slides and DAB staining checked using a light microscope. Once a desired level of DAB staining had been reached, sections were washed in dH_20 (3 x 10 mins), transferred to TBS and mounted on 22 x 60 mm gelatine subbed microscope slides and left to dry overnight. Sections were then dehydrated in ascending concentrations of ethanol for 1 minute each (50, 75, 95, 100, 100 %) followed by 2 x 1-minute incubations in xylene before being coverslipped with DPX.

4.5 IBA1 Analysis

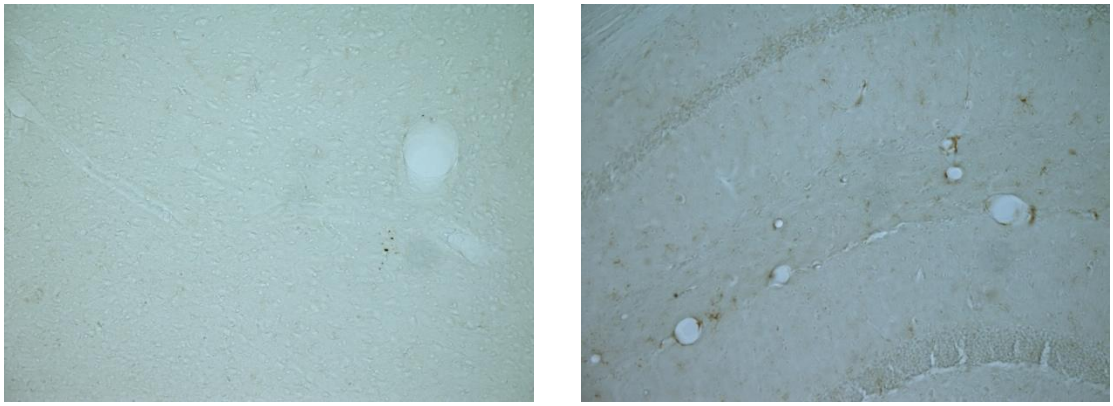
IBA1 staining was first analysed in a semi-quantitative way. Sections were examined using a montaging microscope, and staining intensity and location recorded on maps of the brain taken from the Mouse Brain Atlas (Franklin & Paxinos, 2007). The intensity of staining in various areas of interest was rated on a scale of 0-3: 0 being no staining; 1 for occasional stained cells, very low density; 2 for moderate even staining of low to medium density; and 3 for dense heavy staining. A representative example of each

staining intensity can be seen in Figure 2.8. Each animal received a score for a particular brain region and these scores were used to calculate group averages. The regions of interest included the secondary motor cortex (M2), sensory cortex (SC), lateral ventricles (LVs), the arcuate nucleus and median emminence of the ventral third ventricle (v3V), hippocampus (Hipp.), substantia nigra pars reticulata (SNr), retrosplenial cortex (RSC), and thalamus (Thal.). The M2 region was analysed as it was the primary location within the mPFC damaged by the stroke. Other regions were chosen because of their connections to the mPFC (SC, Hipp., Thal., RSC (van Eden et al., 1992)), involvement in spatial memory (RSC, Hipp., Thal. (Mendez-Couz et al., 2015)), neurogenesis (LV (Pan et al., 2013)), the HPA axis (v3V (Smythe et al., 1996)) or because of observed staining patterns (v3V, SNr, Thal.). Many of these areas spanned several sections and had a variety of staining intensities, so an overall score was given for the maximum amount of staining observed in an area, even if there were other parts of that area that had less staining.

The second method used to analyse IBA1 staining was a cell count. Photographs were taken using a montaging microscope. The number of IBA1 positive cells was counted by hand using the cell counter plugin in the Fiji version of ImageJ image processing software. Cells were counted when there was a clear cell body and at least one process extending from the nucleus, or in the case of more amoeboid cells when there was a large cell body and the area of the cell was distinct from any neighbouring or overlapping cells. Cell counts were taken from selected sections only – one per animal per brain region. This was because of the large number of animals. Sections used were those that were intact, from as close as possible to certain measurements. These measurements are shown in part A of Figures 3.16, 3.18, 3.19 and 3.20 of the Results section, as well as in Appendix B.

The third method used to analyse IBA1 staining was thresholding. Again using Fiji ImageJ, an image was converted into 8-bit grayscale, then the threshold adjusted to the correct level. As images had differing levels of background staining, this level was different for each image, and was determined by the investigator as the highest level before artefacts and non-cellular stains were included. The software reports the number of pixels in the image that are darker than the threshold.

A) B)



C) D)

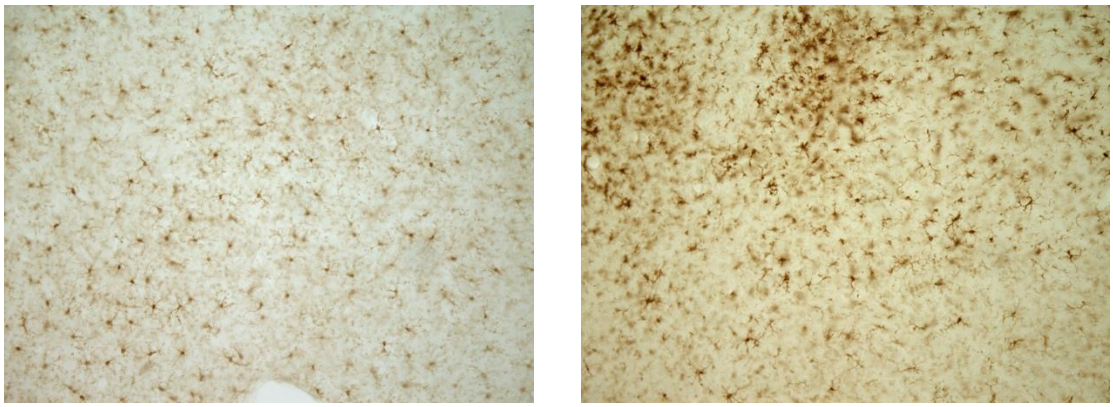


Figure 2.8. Photomicrograph examples of each IBA1 staining intensity observed. A) 0 – no staining. B) 1 – low density, occasional cells. C) 2 – moderate density. B) 3 – high density.

Due to the time-consuming nature of the cell-counting and thresholding methods, and the large number of animals used and brain regions studied, these methods were restricted to the v3V region in all animals. We chose this site for the most thorough analysis due to the involvement of this area in the HPA axis (Smythe et al., 1996; Spinedi et al., 1991; Owens et al., 1991) and the observation of dense IBA1-positive staining. The other regions mentioned above were analysed only in the qualitative manner, except for 2-5 animals randomly chosen from each treatment group, which also received cell counting and thresholding in all brain areas. This was in order to assess the accuracy of the qualitative method.

5. Statistical Analysis

Experimental groups were in a two (stroke and sham) by three (de-enrichment, enrichment, enhanced enrichment) design, so two-way analysis of variance (ANOVA) was used to compare the main effects of stroke and housing, and the interaction of these variables. Multiple pair-wise comparisons were used to compare individual groups (e.g. sham de-enriched vs. stroke de-enriched). The same statistical tests were used to compare results between weeks one and four post-stroke, and also between rounds one and two of testing. All data are reported as mean ± standard error of the mean (SEM), and the two-tailed minimum level of significance is $p<0.05$.

Sample-sizes (n) were not equal due to the timing of tests and the withdrawal of some animals. In round one, all sham groups had n=5, and all stroke groups had n=10. In round two, all sham groups had n=10, and stroke groups n=10. Unfortunately, five animals from the stroke EE group and one from the stroke EEE group had to be removed from the study. The stroke EE animals were fighting excessively and were placed into individual housing to prevent further injury. This confounded the housing conditions. The animal from the stroke EEE group died due to stroke. This meant that in round two these groups had n=5 and n=9, respectively.

Tests carried out only in round one (grid-walking and cylinder) and only in round two (light-dark box) had lower n-values. It was decided after we had completed the first round of testing that inclusion of the LDB would add value, which meant only mice that were tested during the second round took part in this test. With the inclusion of the LDB testing for the second cohort of animals, we also decided not to test these mice on the grid-walking and cylinder tasks, so as not to over-test the animals. Our group has previously published that an n=6-8 is sufficient to observe significant impairment on this task (Clarkson et al., 2010, 2011). For histology, some tissue was sent elsewhere for analysis. The values for each test are shown in Table 2.1.

Table 2.1. Sample sizes (n) for each test

Test		sham-DE	sham-EE	sham-EEE	stroke-DE	stroke-EE	stroke-EEE
Behaviour	Grid-walk	5	5	5	10	10	10
	Cylinder	5	5	5	10	10	10
	OF	15	15	15	20	15	19
	EPM	15	15	15	20	15	19
	NOR	15	15	15	20	15	19
	OLR	15	15	15	20	15	19
	LDB	10	10	10	10	5	9
Histology	Cresyl violet	N/A	N/A	N/A	9	11	7
	IBA1	10	10	10	15	11	13

Chapter 3. Results

1. Behavioural Tests

For most behavioural tests, results have been combined between the two rounds of testing. The exceptions are the grid-walking and upright cylinder tests as they were only performed in the first round, and the light-dark box, as this was only performed in the second round. As explained below, and shown in Appendix A, there were some differences between round one and round two of behavioural testing. The possible reasons for these differences are explored in the discussion section 4.1. Despite these differences, results from both rounds have been combined for analysis.

1.1 Grid-walking Test

Performance in the grid-walking test is a measure of motor coordination. Although all animals experienced the same housing conditions prior to stroke, data for the pre-stroke grid-walking and cylinder tests has been separated into the post-stroke groups in order to make within-group comparisons across the testing period.

One week before stroke, sham DE animals (6.33 ± 0.25) made fewer foot faults than both sham EEE (8.29 ± 0.72, $p<0.05$,) and stroke DE (8.41 ± 0.64. $p<0.01$) animals. There was an interaction effect (two-way ANOVA, $p<0.05$). Since all animals received the same housing treatment pre-stroke, and experimenters were at this stage blind to the assignment of animals into groups, we must conclude that this difference is an anomaly and possibly due to remarkably low variance within the sham DE group.

One week after stroke, stroke animals made more foot faults than shams (two-way ANOVA, $p<0.001$, Figure 3.1 B), and there was a main effect of housing, ($p<0.05$), with DE animals making more foot faults. Among shams, DE mice (9.84 ± 0.22) made more foot faults than either EE (7.53 ± 0.84, $p<0.05$) or EEE mice (8.06 ± 0.68, $p<0.05$). Stroke EE (10.04 ± 0.43) and stroke EEE (10.27 ± 0.28) showed increased foot faults compared with their sham counterparts ($p<0.05$ and $p<0.01$ respectively). These data indicate that mPFC stroke has caused a slight impairment in motor skills. It also seems that DE among shams has worsened motor performance.

Four weeks after stroke, there was an interaction effect (two-way ANOVA, $p<0.01$, Figure 3.1 C), due to EEE increasing foot faults among shams, while decreasing faults among stroke groups. There was a main effect of stroke where sham DE mice (6.25 ± 0.29) made less foot faults than stroke DE mice (8.27 ± 0.28, $p<0.001$). Among stroke animals, the EEE group (6.38 ± 0.36) made less foot faults than either DE ($p<0.001$) or EE (8.78 ± 0.65, $p<0.01$) groups. These data indicate that motor skills have recovered more in the stroke EEE group than the stroke DE or stroke EE groups.

Overall, foot faults increased from pre-stroke to one week post-stroke (two-way ANOVA, $p<0.001$, Figure 3.1 B). Multiple comparisons showed that this was also true for the sham DE mice ($p<0.001$) and stroke EEE mice ($p<0.01$). Stroke DE and stroke EE groups showed a similar trend.

The number of foot faults then decreased overall from one to four weeks post-stroke (two-way ANOVA, $p < 0.001$). Multiple comparisons showed that this was true for the sham DE mice ($p<0.001$) as well as the stroke DE ($p<0.001$) and stroke EEE mice ($p<0.001$). The stroke EE mice showed a similar trend, although not significant. There were no differences between the number of faults pre-stroke and at four weeks post-stroke, indicating that any motor effects of stroke had disappeared after four weeks. Overall, these data show that our stroke has caused a small temporary impairment in motor skills at one week post-stroke, which has then recovered by four weeks post-stroke. Among shams, the de-enriched animals showed the same pattern.

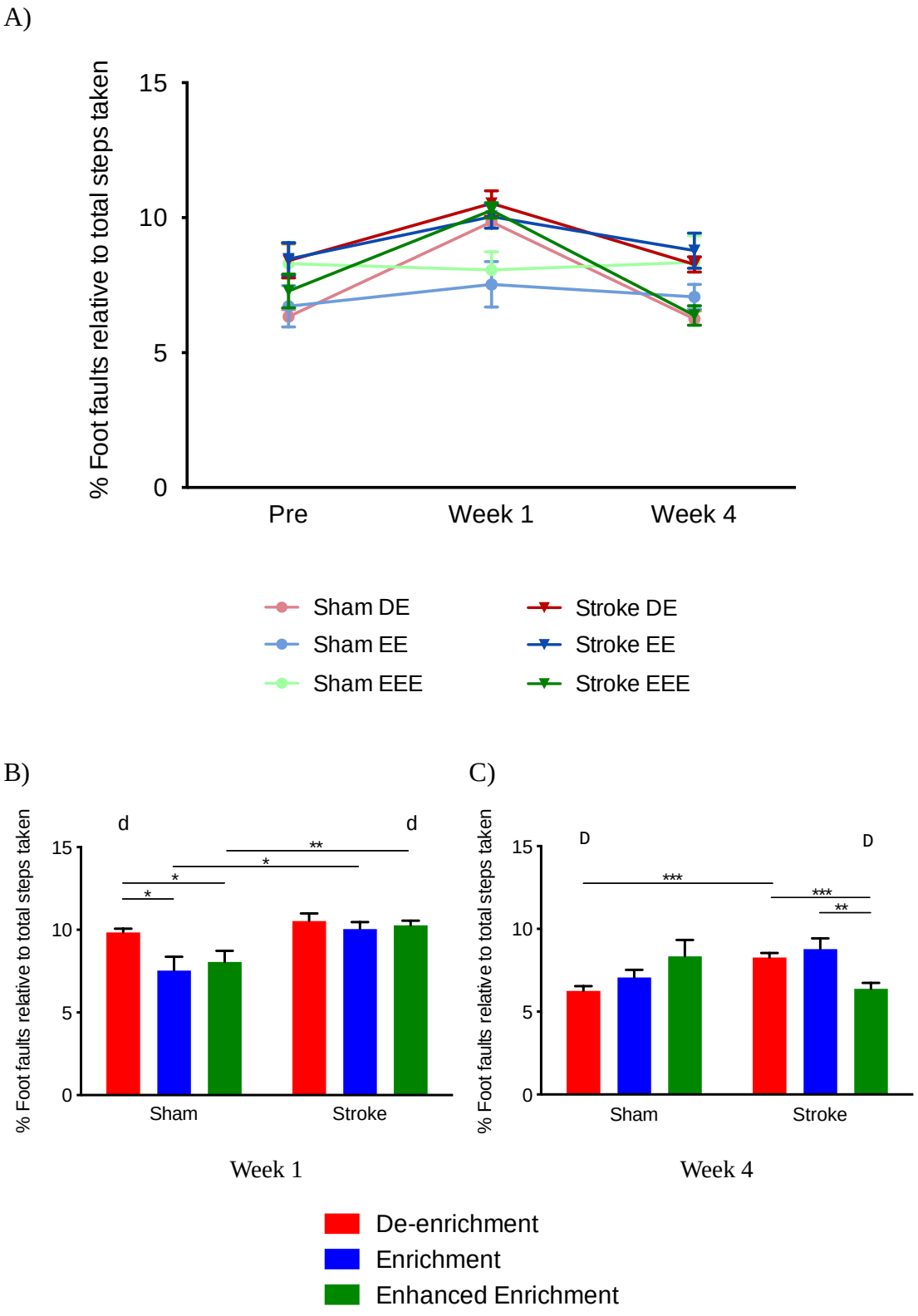


Figure 3.1. A) The percentage of foot faults made relative to total steps taken in the grid-walking task. The time periods refer to one week pre-stroke and weeks one and four post-stroke. B) and C) A closer look at weeks 1 and 4 post-stroke. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$. δ = significantly different from pre-stroke, Δ = significantly different from week 1.

1.2 Cylinder Test

Similarly to the grid-walking test, the cylinder test data has been separated into the post-stroke groups in order to make within-group comparisons.

Pre-stroke, sham DE mice (9.52 ± 1.41 %) favoured their left forepaw more than stroke DE mice (1.02 ± 2.79 %, $p<0.05$, Figure 3.2 A). Among shams, DE mice favoured their left forepaw more than EEE mice (0.87 ± 3.59, $p<0.05$). Similarly to the grid-walking test, there should be no difference between groups as they all received the same treatment pre-stroke. Again, the variance of the sham DE group is much lower than the other groups, which may account for this result.

One week post-stroke, there were no differences found (Figure 3.2 B). Four weeks after stroke, there were still no differences (Figure 3.2 C). There were no differences found between time points. These results indicate that stroke, as well as housing condition, had no effect on the preference of either forepaw.

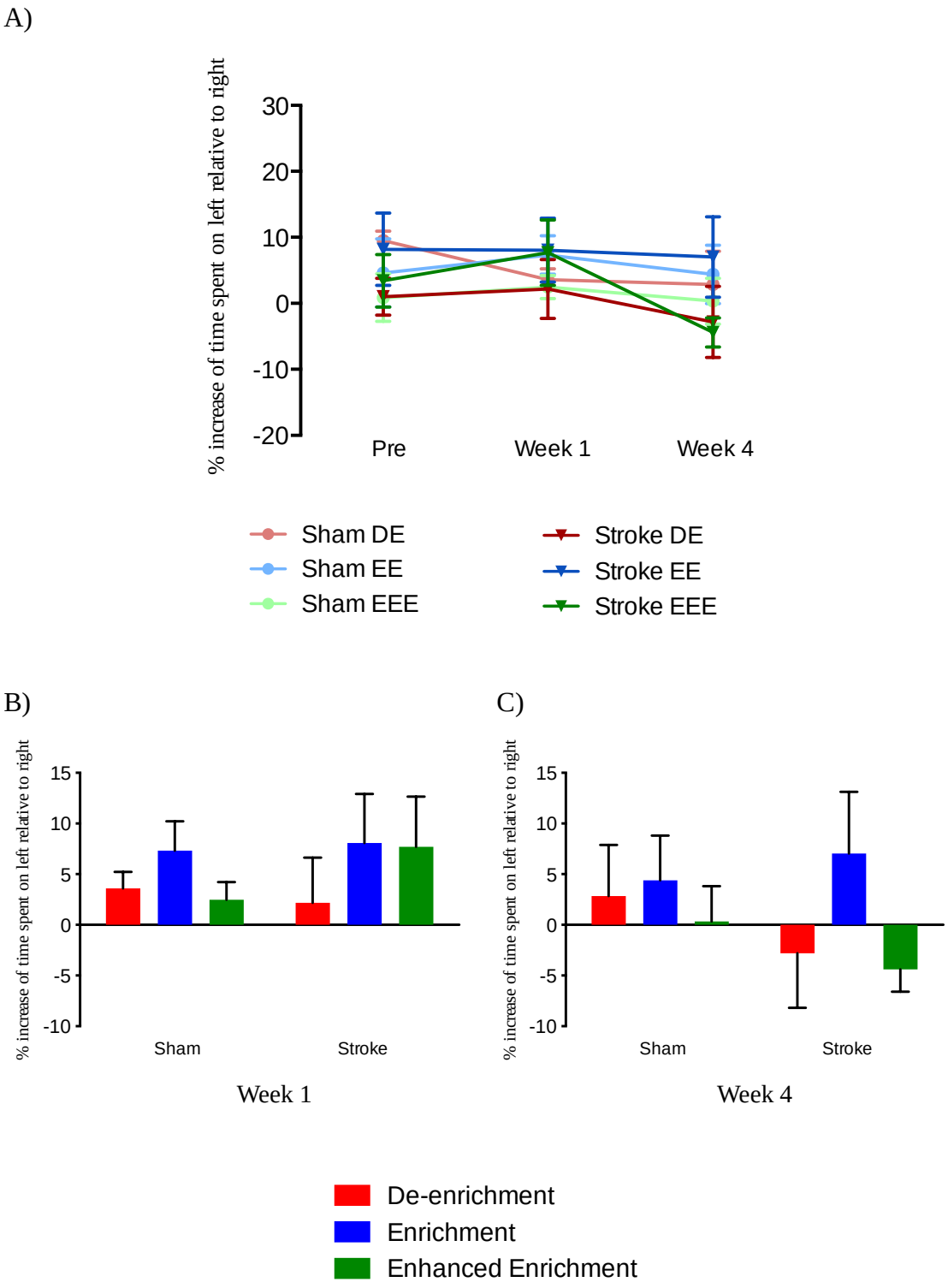


Figure 3.2. A) The percentage increase of time spent using the left forepaw compared with the right in the cylinder test. The time periods refer to one week pre-stroke and weeks one and four post-stroke. B) and C) A closer look at weeks 1 and 4 post-stroke.

1.3 Open Field

Abnormal activity levels in the open field can be indicators of dysfunction. Low activity levels are seen as an animal correlate of depression (e.g. Shao et al., 2015). We used three measures of activity in the open field: Total distance travelled in the ten-minute trial, distance travelled in each of ten one-minute blocks, and the number of high-speed bursts.

1.3.1 Total Distance Travelled

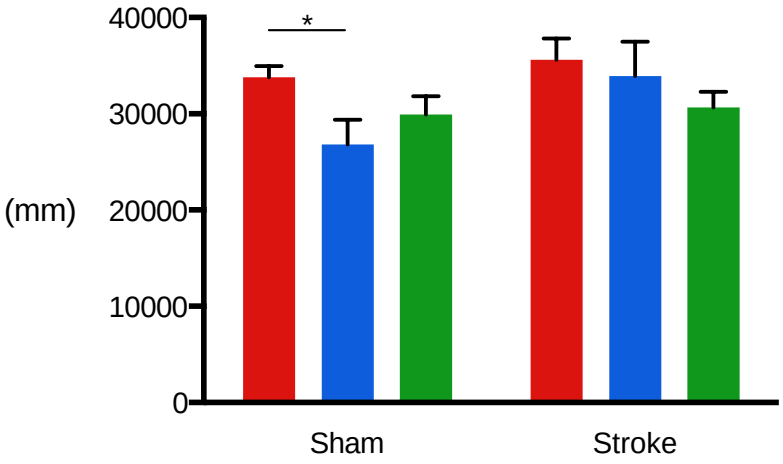
One week post-stroke, sham DE mice (33788 ± 1168 mm) travelled further than sham EE mice (26831 ± 2555 mm, $p<0.05$, Figure 3.3 A). Overall, there were no significant main effects of stroke or housing, and no significant interaction effect (two-way ANOVA).

Four weeks post-stroke, there was an interaction effect (two-way ANOVA, $p<0.001$) where EE animals travelled further than EEE animals among the stroke groups, but less far among sham groups. There were no main effects. Stroke EEE animals (23942 ± 1346 mm) travelled further than sham EEE animals (14843 ± 1572 mm, $p<0.001$, Figure 3.3 B). Among the sham mice, the EEE animals travelled less than both the DE (20106 ± 1803 mm, $p<0.05$) and EE animals (22673 ± 2665 mm, $p<0.05$). Among the stroke mice, the EEE animals travelled further than the EE animals (17854 ± 1603 mm, $p<0.01$).

Overall, animals travelled less in week four than week one (two-way ANOVA, $p<0.001$, Figure 3.3 B). All individual groups travelled significantly less in week four, except the sham EE animals (sham DE $p<0.001$, sham EEE $p<0.001$, stroke DE $p<0.001$, stroke EE $p<0.01$, stroke EEE $p<0.01$).

The results for round one and two comparisons are shown in Appendix A. Overall, there was no difference between rounds. In week one the stroke EE group travelled further in round two than round one ($p<0.05$); in week four, there were no differences (see Appendix A Figure A1 A).

A) Week 1



B) Week 4

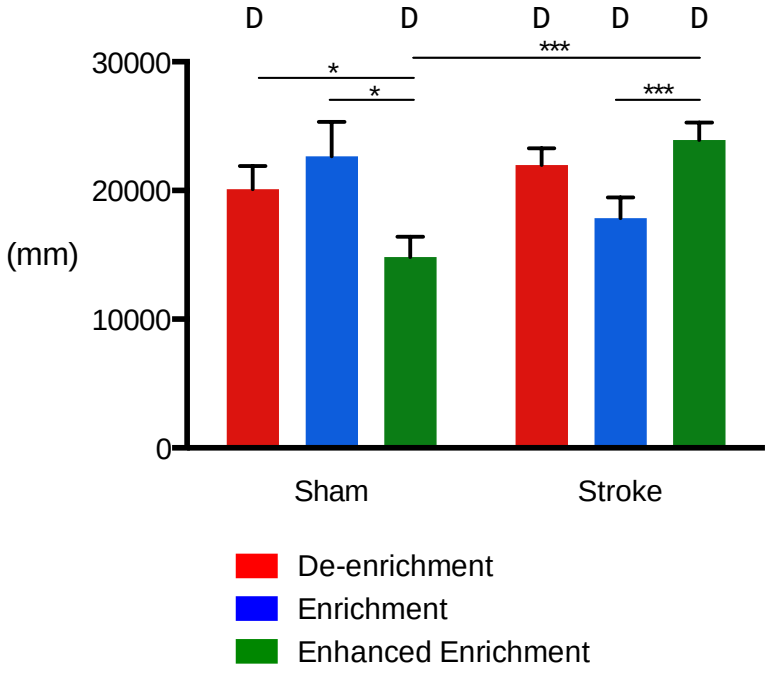


Figure 3.3. Total distance travelled in the open field test. A) Week one post-stroke. B) Week four post-stroke. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, Δ = significantly different from week one.

1.3.2 Distance Travelled – 60-second Blocks

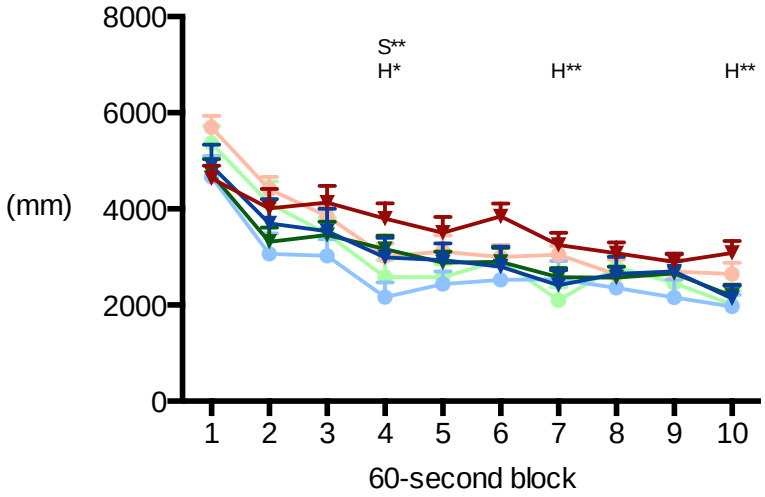
To compare changes in activity across the ten-minute trial, data were split into ten 60-second blocks. Animals tended to travel more at the beginning of the test, and then less as time went on. Two-way ANOVAs were carried out for each block. In week one, there was a main effect of stroke only in block four – stroke animals travelled further than shams ($p<0.01$, Figure 3.4 A). There were main effects of housing in blocks four ($p<0.05$), seven ($p<0.01$) and ten ($p<0.01$). Multiple pair-wise comparisons were also carried out for each block. DE sham animals travelled significantly more than EE shams in blocks one ($p<0.05$), two ($p<0.05$) and four ($p<0.05$), which is consistent with the difference seen in the total distance travelled.

In week four, interaction effects were found in blocks 1 (two-way ANOVA, $p<0.001$, Figure 3.4 B), 2 ($p<0.01$), 4 ($p<0.001$) and 9 ($p<0.001$). These were consistent with the interaction effect found in the total distance analysis – among shams, EE mice travelled further than EEE mice, whereas in stroke animals, this effect was reversed. Stroke animals travelled further than shams only in block five ($p<0.05$). There were multiple significant results which agree with those found in the total distance travelled.

Overall, animals travelled less in week four than week one in each of the 60-second blocks (two-way ANOVA, $p<0.001$). Multiple comparisons for each time block are shown in Figure 3.5. For most of the groups, mice travelled further in week one in almost all blocks. However, animals in the sham EE group only travelled more in week one in two out of ten blocks. This is consistent with the results for total distance travelled where the sham EE group travelled the same distance in weeks one and four.

Round one and two comparisons are shown in Appendix A. Figure A2. In week one, the stroke EE animals travelled more in round two in nine out of ten blocks (Appendix A Figure A2 D), but other groups showed only occasional differences. In week four, there were only occasional differences between rounds (Appendix A Figure A3).

A) Week 1



B) Week 4

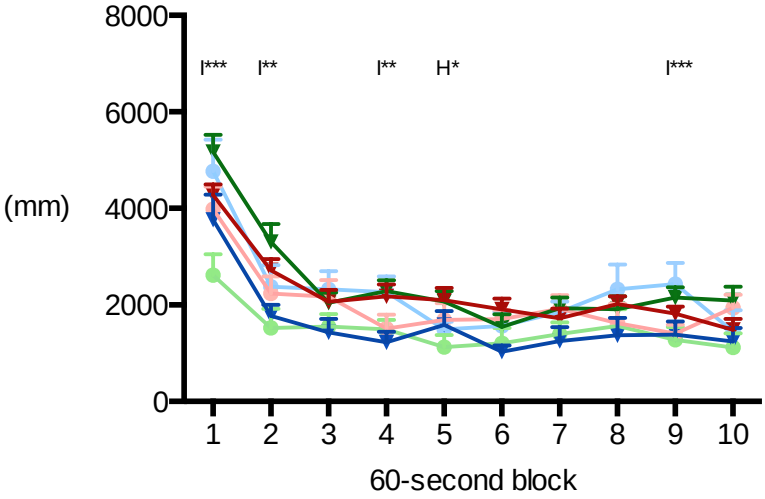


Figure 3.4. Distance travelled in the open field divided into 60-second blocks. The data from week one post-stroke are shown in A), and week four in B). The letters I, S and H above each block indicates the significance levels of the interaction and main effects of stroke and housing respectively, for a 2-way ANOVA carried out separately for each time block. Significance levels are: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

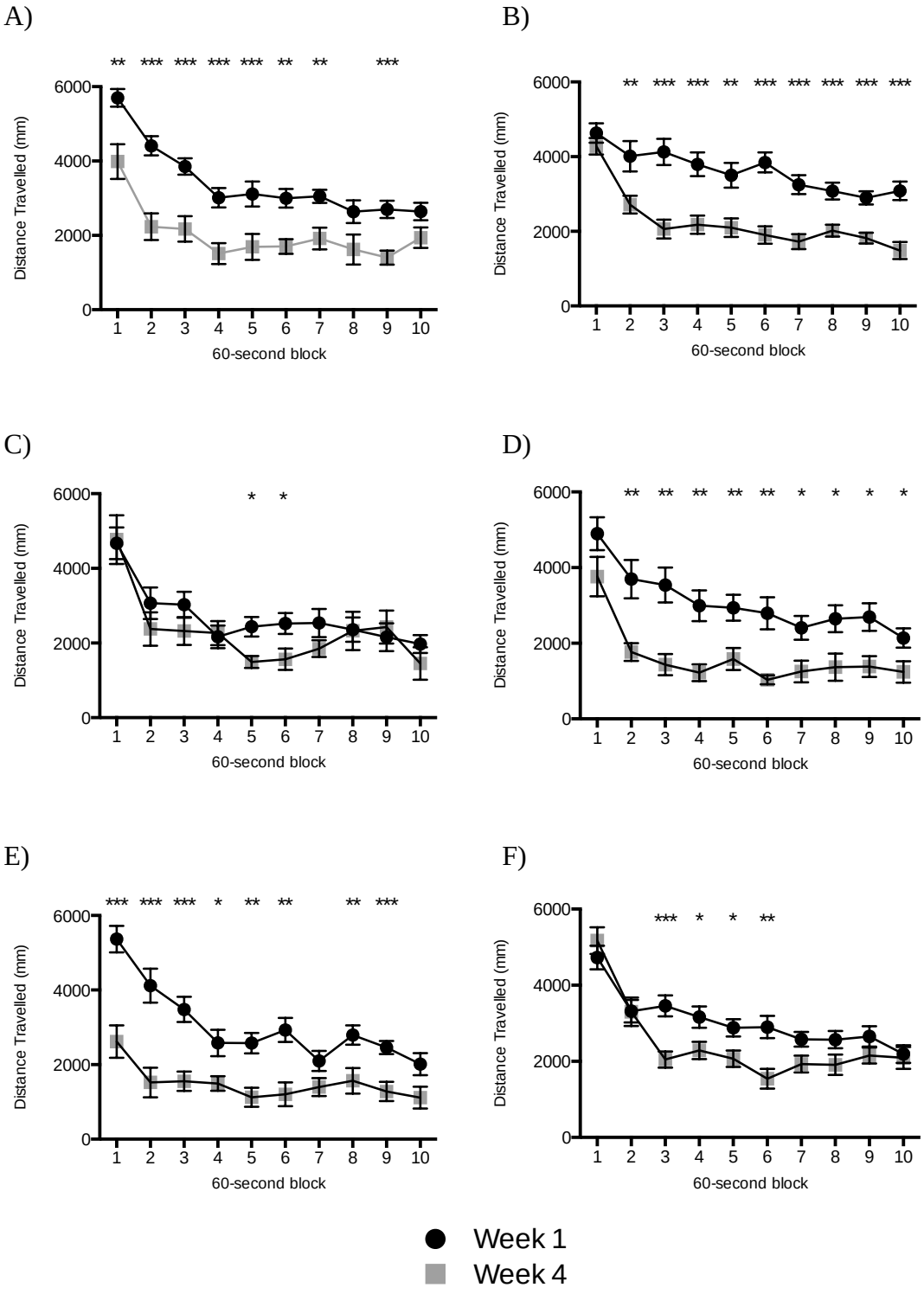


Figure 3.5. Distance travelled by each group in 60-second blocks. Each graph shows the data from weeks one and four for one experimental group. A) sham DE; B) stroke DE; C) sham EE; D) stroke EE; E) sham EEE; D) stroke EEE. Stars above each 60-second block indicate significance level of the t-test comparing week one and four for that block. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

1.3.3. High-Speed Bursts

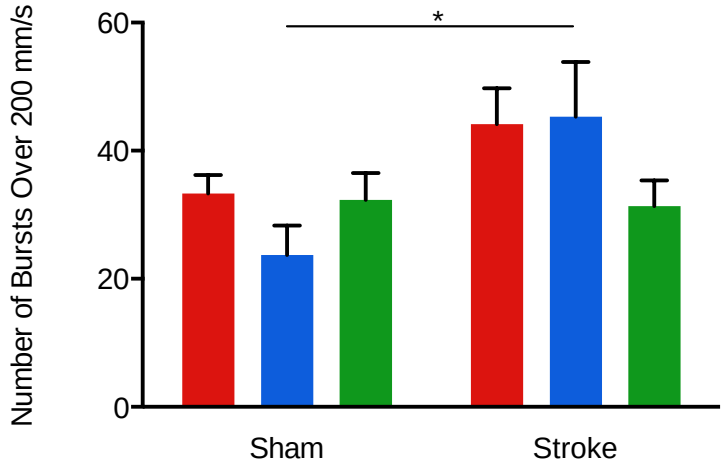
The number of high-speed bursts in the open field can be another indicator of abnormal activity levels. In week one, stroke animals made more high-speed bursts ($p<0.05$, two-way ANOVA, Figure 3.6 A). Stroke EE mice (45.3 ± 8.6) made more high-speed bursts than sham EE mice (23.7 ± 4.6, $p<0.05$).

In week four, there was a significant interaction effect where EE decreased bursts in sham animals, but increased bursts in stroke animals (two-way ANOVA, $p<0.05$, Figure 3.6 B). Stroke EEE animals (23.6 ± 2.6) made more bursts than sham EEE animals (10.9 ± 2.2, $p<0.01$,). Within the stroke groups, EEE animals made more bursts than DE animals (16.2 ± 1.6, $p<0.05$).

Overall, mice made less high-speed bursts in week four than in week one (two-way ANOVA, $p<0.001$, Figure 3.1.3 B). Multiple comparisons showed that four out of the six groups made significantly less bursts in week four: Sham DE ($p<0.001$), sham EEE ($p<0.001$), stroke DE ($p<0.001$), and stroke EE ($p<0.05$).

There were some differences between round one and round two. In week one, animals made more bursts in round two (two-way ANOVA, $p<0.05$, Appendix A Figure A4. A). In week four, there were no significant differences.

A) Week 1



B) Week 4

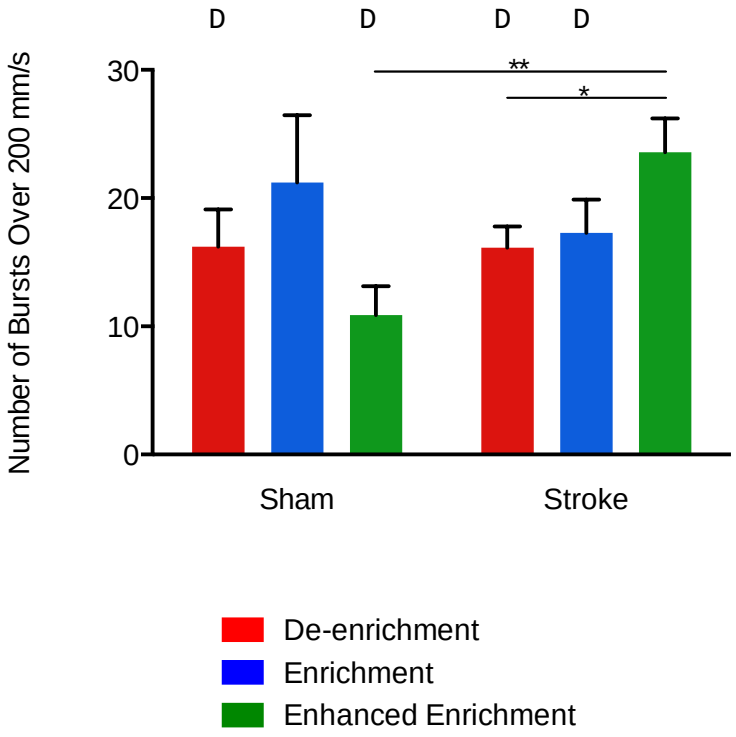


Figure 3.6. The mean number of high-speed bursts over 200 mm/s performed in the open field. * = p<0.05, ** = p<0.01, *** = p<0.001. Δ = significantly different from week one.

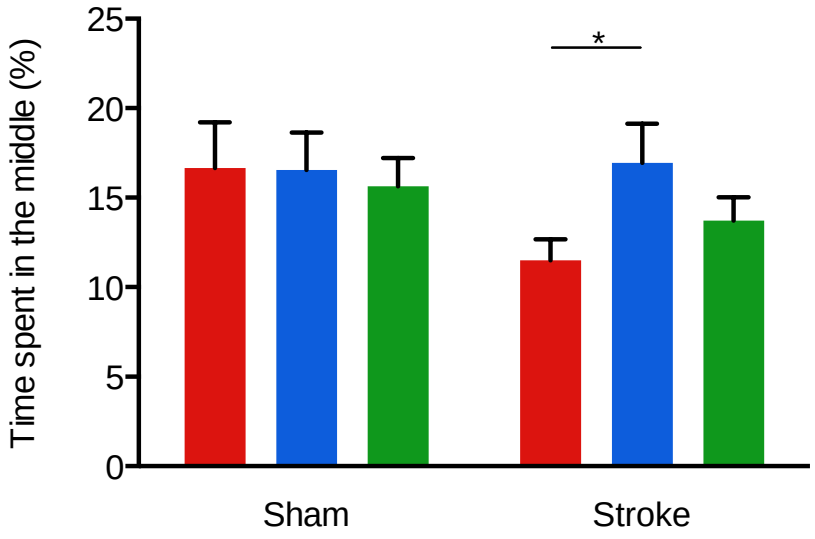
1.3.4 Time Spent in Middle of Arena

The percentage of time animals spent in the middle of the open field is a measure of anxiety. In week one, stroke EE animals (16.9 ± 2.2 %) spent more time in the middle than stroke DE animals (11.5 ± 1.2 % $p<0.05$, Figure 3.7 A). There were no significant ANOVA results. In week four, there were no significant differences.

Animals spent less time in the middle of the arena in week four than week one (two-way ANOVA, $p<0.001$, Figure 3.7 B). Multiple comparisons revealed that only sham EEE animals spent less time in the middle in week four (8.8 ± 1.7 %) than week one (15.6 ± 1.6 %, $p<0.01$, Figure 3.7. B).

In week one, animals spent less time in the middle in round two (two-way ANOVA, $p<0.001$, Appendix A Figure A5), however only the stroke DE group showed this effect ($p<0.05$). In week four, there was no main effect of round. The sham EE group spent less time in the middle in round two ($p<0.05$, Appendix A Figure A5 B).

A) Week 1



B) Week 4

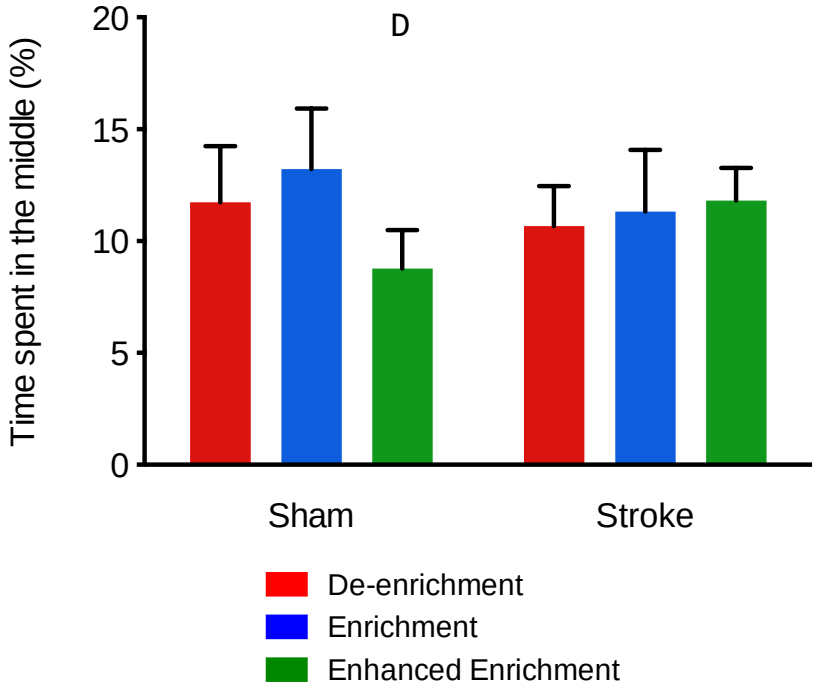


Figure 3.7. The mean percentage of time spent in the middle of the arena in A) week one and B) week four of the open field test. * = $p<0.05$. Δ = significantly different from week one.

1.4. Elevated Plus Maze

Two measures were used in the elevated plus maze – the percentage of time spent in the open arms, and the number of times an animal entered the open arms. More anxious animals spend less time in the open arms and make fewer entries to the open arms.

1.4.1. Time Spent in the Open Arms

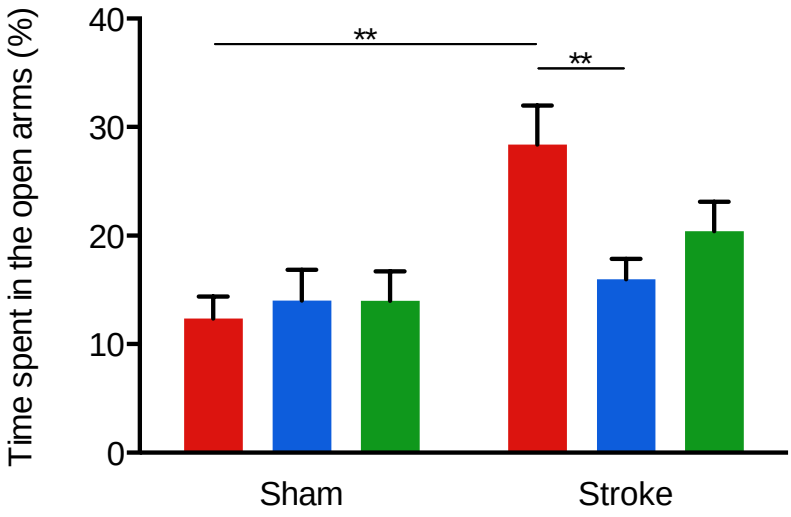
There was an interaction effect where DE had no effect in shams but increased time in the open arms in stroke animals (two-way ANOVA, $p<0.05$, Figure 3.8 A). There was a main effect where stroke animals spent more time in the open arms than shams, ($p<0.001$). Stroke DE animals (28.39 ± 3.58 %) spent more time in the open arms than sham DE animals (12.36 ± 2.04 %, $p<0.01$). Among stroke groups, DE animals spent more time in the open arms than EE animals (15.98 ± 1.89 %, $p<0.01$).

In week four, stroke DE mice (13.31 ± 1.51 %) spent less time in the open arms than sham DE mice (5.92 ± 1.93 %, $p<0.01$, Figure 3.8 B). There were no significant effects in the ANOVA.

Animals spent less time in the open arms in week four than week one (two-way ANOVA, $p<0.001$, Figure 3.8 B). Four individual groups showed this same change: sham DE ($p<0.05$), stroke DE ($p<0.001$), stroke EE ($p<0.05$) and stroke EEE ($p<0.01$, Figure 3.8. B).

Overall in week one, there was no difference in time spent in the open arms between round one and round two, but the sham EE group did spend more time in the open arms in round two than round one ($p<0.01$, Appendix A Figure A6 A). In week four, animals spent less time in the open arms in round two (two-way ANOVA, $p<0.001$, Appendix A Figure A6 B). This effect seems to be due to large differences between rounds one and two in both EEE groups – sham and stroke ($p<0.001$ and $p<0.05$, respectively).

A) Week 1



B) Week 4

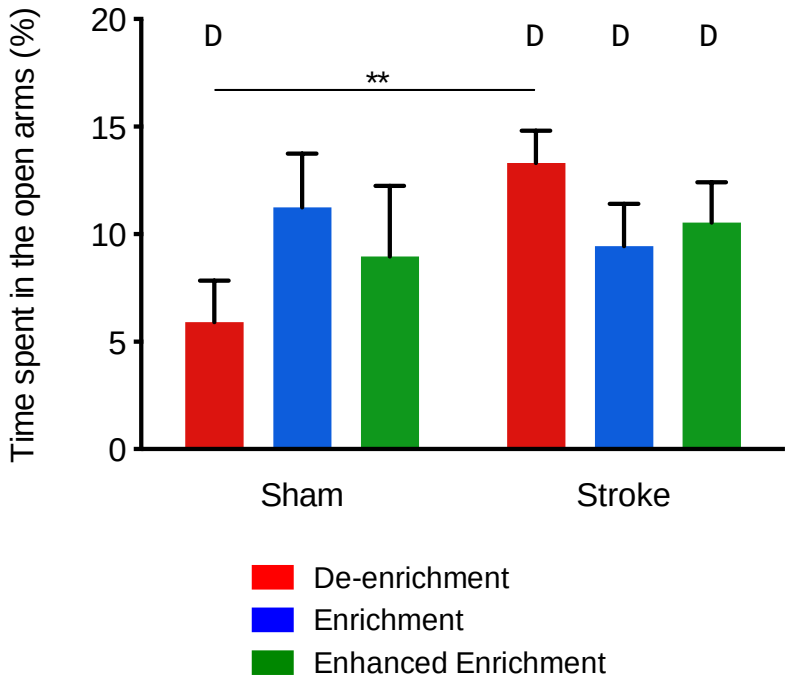


Figure 3.8. The percentage of time spent in the open arms of the elevated plus maze. ** = $p<0.01$. Δ = significantly different from week one.

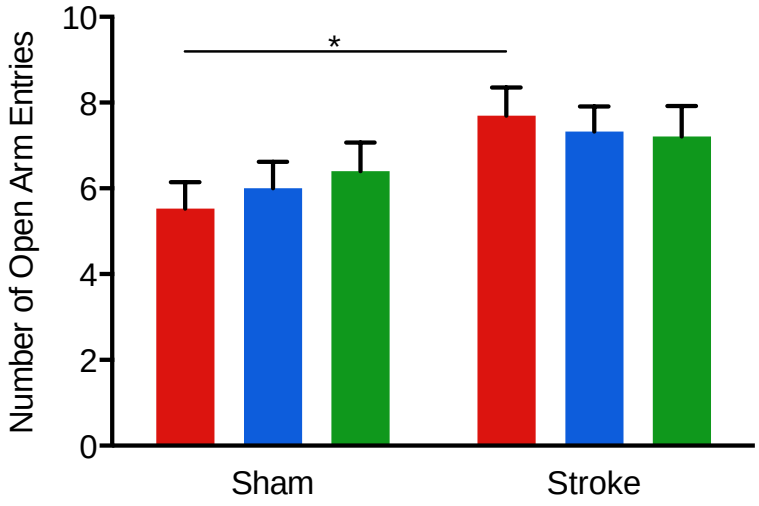
1.4.2. Open Arm Entries

In week one, stroke animals made more open arm entries than shams (two-way ANOVA, $p<0.01$, Figure 3.9 A). Stroke DE animals (7.70 ± 0.65) made more entries than sham DE (5.53 ± 0.62, $p<0.05$) animals. This is consistent with the results for time spent in the open arms. In week four, there were no differences found, similarly to the results for time spent in the open arms. These results indicate that while stroke and DE may cause decreased anxiety one week after stroke, these effects are reduced by four weeks after stroke.

Overall, animals made less entries in week four compared with week one (two-way ANOVA, $p<0.001$, Figure 3.9 B). Multiple comparisons showed that all groups decreased their number of entries from week one to four (sham DE $p<0.001$; sham EE $p<0.01$; sham EEE $p<0.05$; stroke DE $p<0.001$; stroke EE $p<0.01$; stroke EEE $p<0.01$).

In week one, animals made more entries into the open arm in round one than round two (two-way ANOVA, $p<0.05$, Appendix A Figure A7 A). Individual groups to make more entries in round one were sham DE ($p<0.01$) and sham EEE ($p<0.001$). On the other hand, the stroke DE group made more entries in round two ($p<0.05$). In week four, animals also made more entries into the open arm in round one (two-way ANOVA, $p<0.05$). Individual groups that made more entries in round one were sham EEE ($p<0.01$) and stroke EEE ($p<0.05$, Appendix A Figure A7 B). These results, combined with the round one and four comparisons of time spent in the open arms, indicates that animals in round two of testing had higher anxiety levels, especially by week four, and especially in EEE groups.

A) Week 1



B) Week 4

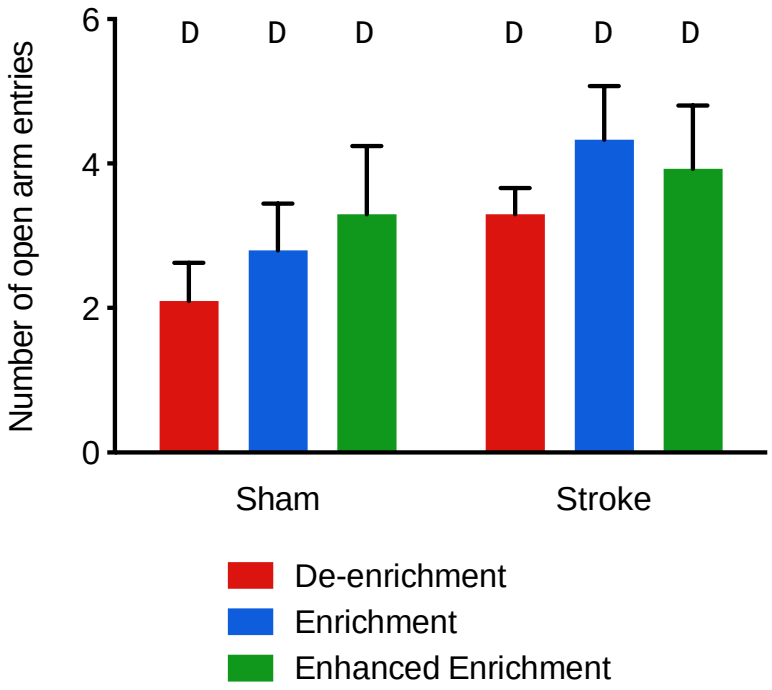


Figure 3.9. The number of entries made into the open arms of the elevated plus maze. * = $p<0.05$. Δ = significantly different from week one.

1.5 Light-Dark Box

The light dark box measures anxious behaviour. More anxious animals spend less time in the light section and make fewer entrances into the light section.

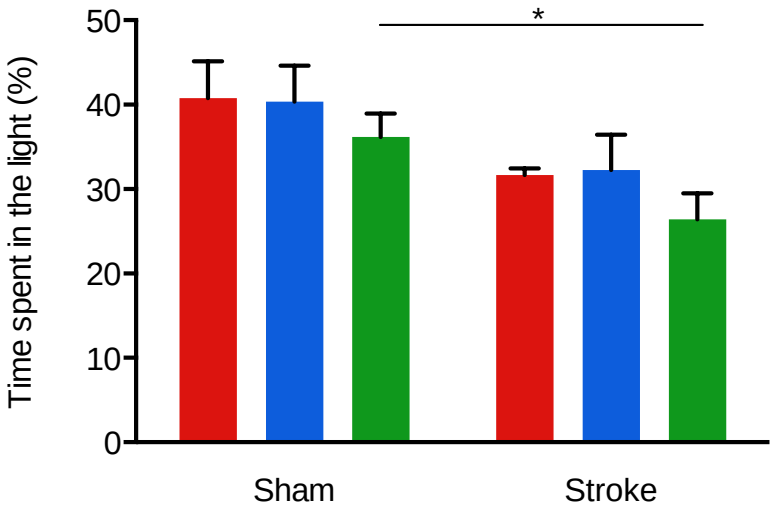
1.5.1 Time Spent in the Light

In week one, stroke animals spent less time in the light section (two-way ANOVA, $p<0.01$, Figure 3.10 A). Multiple comparisons revealed that sham EEE mice (36.20 ± 2.74 %) spent more time in the light than stroke EEE mice (26.39 ± 3.09 %, $p<0.05$). These results indicate that stroke increases anxiety, which is the opposite of the results in the elevated plus maze.

In week four, there were no differences found (Figure 3.10 B). There were no data for stroke EE mice as the light-dark box test was only carried out in round two, and these animals were removed due to injuries.

In week four, animals spent less time in the light section than in week one ($p<0.01$). The sham DE group spent less time in the light section in week four ($p<0.05$, Figure 3.10 B).

A) Week 1



B) Week 4

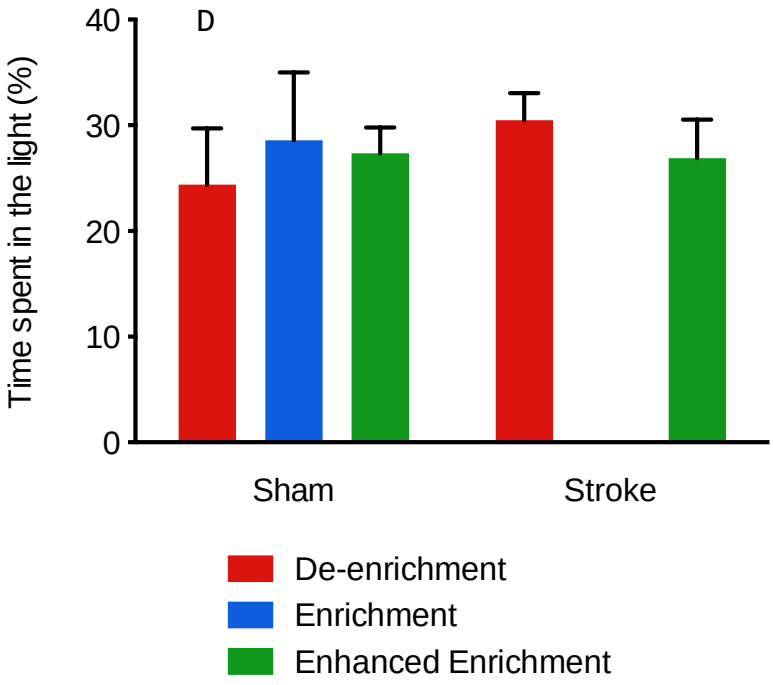


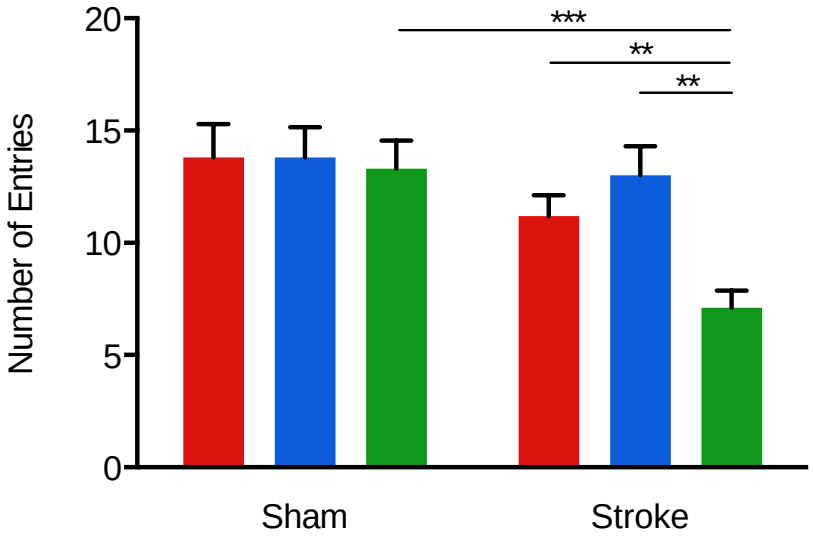
Figure 3.10. The percentage of time spent in the light section of the arena of the light-dark box test. * = $p<0.05$. Δ = significantly different from week one.

1.5.2 Entries into the Light Section

In week one, stroke animals made fewer entries to the light than shams (two-way ANOVA, $p<0.01$, Figure 3.11 A), and there was a main effect of housing where EEE reduced the number of entries ($p<0.05$). Stroke EEE animals (7.11 ± 0.77) made fewer entries than sham EEE animals (13.30 ± 1.27, $p<0.001$). Among stroke groups, EEE animals made fewer entries than both DE (11.20 ± 0.93, $p<0.01$) and EE animals (13.00 ± 1.30, $p<0.001$). In week four, there were no significant differences.

Overall, there were no differences between weeks one and four. The stroke EEE group increased the number of entries into the light in week four ($p<0.05$, Figure 3.11 B). These results, combined with the amount of time spent in the light section, indicate that while stroke increases anxiety one week after stroke, especially in EEE animals, this effect is absent by four weeks after stroke. This contrasts with the results of the EPM, in which stroke decreased anxious behaviour, at both one and four weeks post-stroke.

A) Week 1



B) Week 4

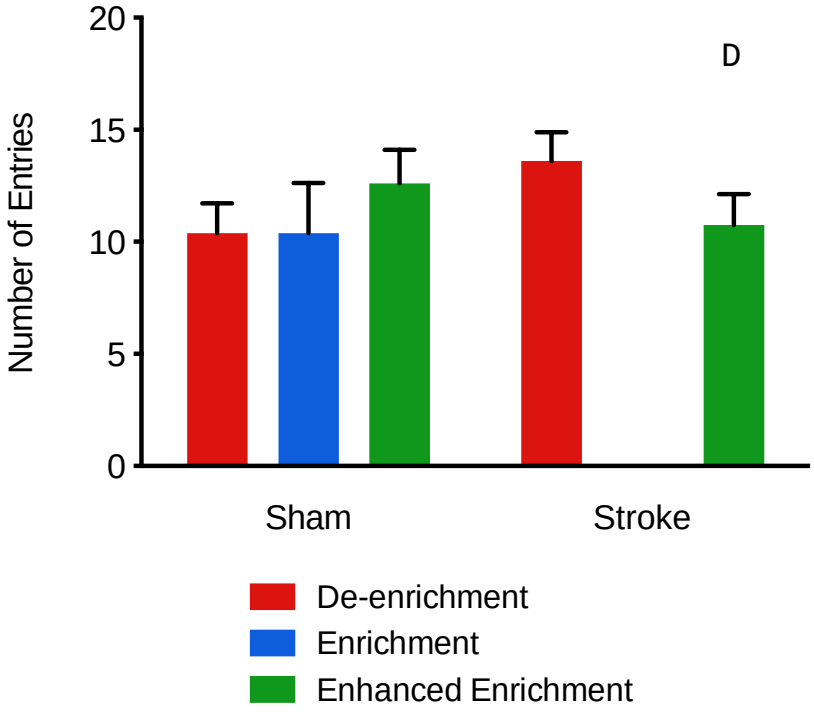


Figure 3.11. The number of entries made into the light section of the arena of the light-dark box test. ** = p<0.01, *** = p<0.001. Δ = significantly different from week one.

1.6. Novel Object Recognition

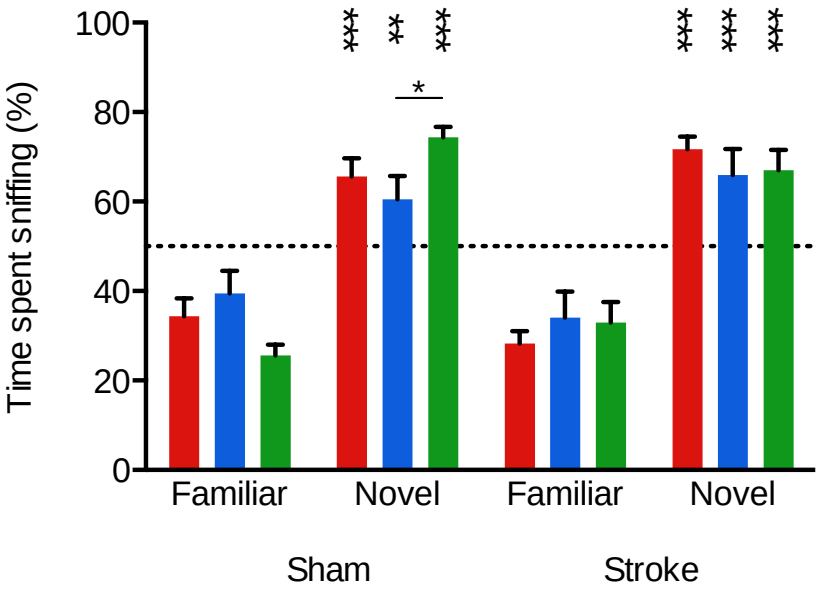
The novel object recognition task assesses visual recognition memory. In the test run, animals that recognise the new object spend more time investigating it than the familiar object. In week one, all animals spent more time sniffing the novel object, indicating that all groups recognised it as new (Figure 3.12 A). There was a main effect of housing (two-way ANOVA, $p<0.001$). Sham EEE mice (74.34 ± 2.39 %) preferred the novel object more than the sham EE mice (60.58 ± 5.11 %, $p<0.05$).

In week four, all groups recognised the novel object. Two-way ANOVA showed a main effect of housing ($p<0.001$, Figure 3.12 B). Stroke EE animals (84.65 ± 2.47 %) preferred the novel object more than the sham EE animals (68.88 ± 5.21 %, $p<0.05$).

Overall, animals spent more time investigating the novel object in week four than in week one (two-way ANOVA, $p<0.001$, Figure 3.12 B). Individual comparisons showed this was true for the sham EE animals ($p<0.01$). This may indicate an improvement in recognition memory.

There were no significant differences between rounds one and two in either week one or week four (Appendix A Figures A8 A and B).

A) Week 1



B) Week 4

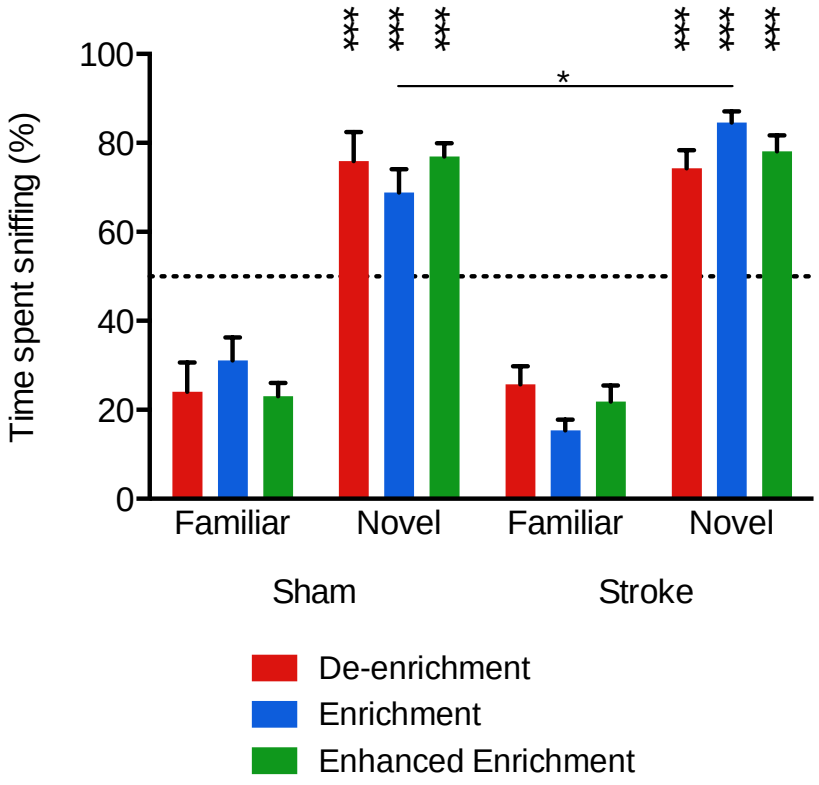


Figure 3.12. The percentage of time spent investigating both the familiar object and the novel object. * = $p<0.05$. ** = $p<0.01$. *** = $p<0.001$. Vertical asterisks indicate significantly different from time spent sniffing familiar object. Δ = significantly different from week one. The dotted lines at 50% indicate the amount of time expected if neither object was preferred.

1.7. Object Location Recognition

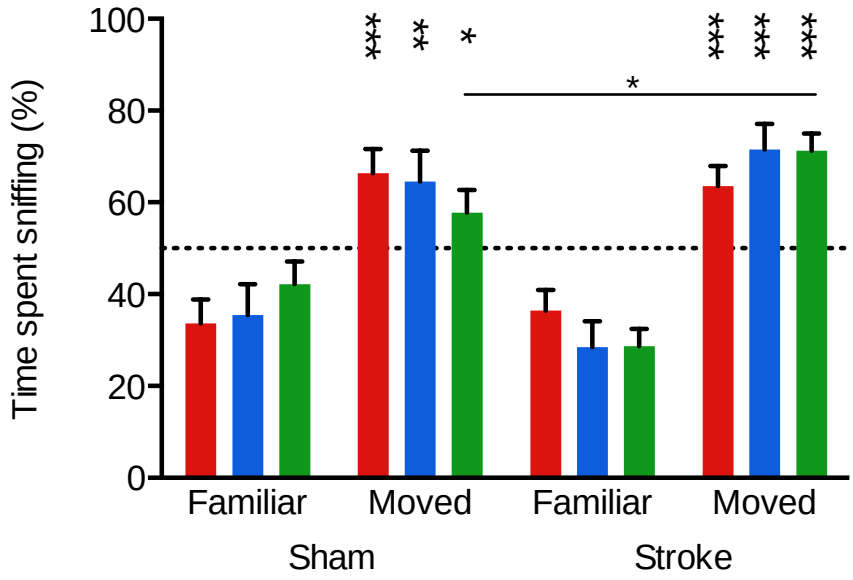
The object location recognition task measures the amount of time animals spend investigating a moved familiar object. This is a measure of simple spatial memory performance. In week one, all groups spent more time investigating the moved object than the familiar object (Figure 3.13 A). This shows that all groups recognised that the moved object was in a new location. Two-way ANOVA revealed a main effect of housing ($p<0.001$). Stroke EEE animals (71.30 ± 3.75 %) preferred the moved object more than the sham EEE animals (57.79 ± 4.88 %, $p<0.05$).

In week four, two-way ANOVA showed a main effect of housing where EE and EEE decreased the time spent sniffing the moved object ($p<0.01$, Figure 3.2). Not all groups spent more time investigating the moved object. Among shams, DE and EE animals spent more time with the moved object ($p<0.05$ and $p<0.05$, respectively), but EEE animals did not (60.08 ± 4.72 % time with the moved object). Among stroke animals, sham DE mice preferred the moved object ($p<0.05$), but EE (54.03 ± 6.41 %) and EEE mice (58.18 ± 3.34 %) did not. This indicates that these groups did not recognise that the object had been moved.

There were no overall differences between week one and week four. The stroke EEE group spent less time investigating the moved object in week four than week one ($p<0.05$, Figure 3.13 B). Although some of these differences between the time spent sniffing in weeks one and week four were not significant, the stroke EEE group and the stroke EE group did recognise the moved object at week one, then failed to do so at week four, indicating that there may be some deterioration of spatial memory over the four weeks following stroke.

In week one, animals in round two spent more time investigating the moved object than those in round one (two-way ANOVA, $p<0.001$, Appendix A Figure A9 A). Only the sham DE group showed the same relationship in multiple comparisons ($p<0.05$). In week four, there were no differences found between rounds one and two.

A) Week 1



B) Week 4

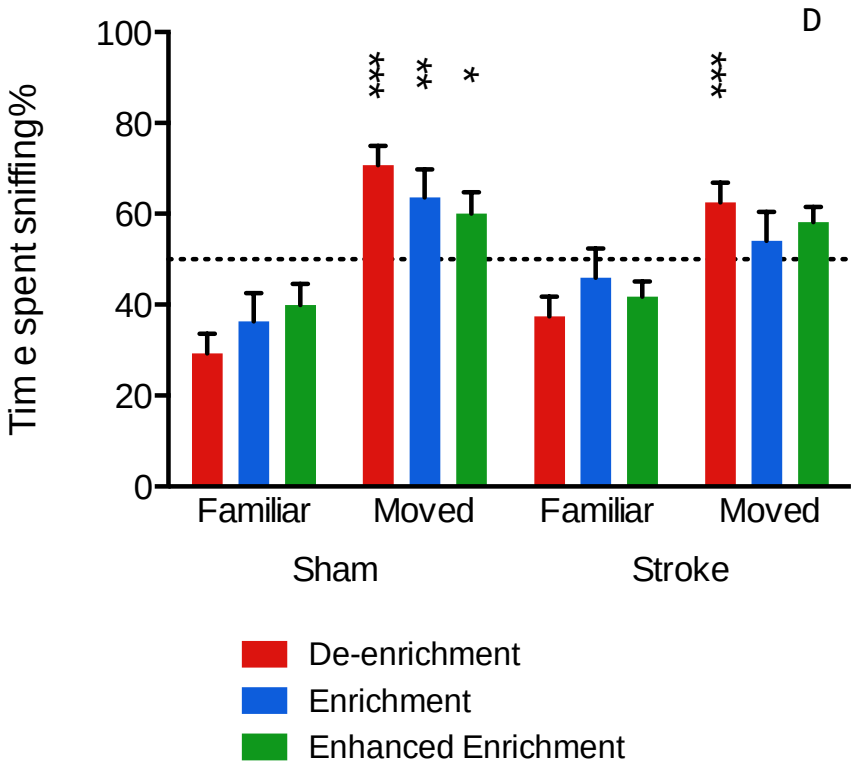


Figure 3.13. The percentage of time spent investigating both the familiar object and the moved object in the object location recognition test. * = $p<0.05$. ** = $p<0.01$. *** = $p<0.001$. Vertical asterisks indicate significantly different from time spent sniffing familiar object. Δ = significantly different from week one. The dotted lines at 50% indicate the amount of time expected if neither object was preferred.

2. Histology

2.1 Stroke Volume

A representative diagram of infarct volume is shown in Figure 3.14. Lesions damaged the pre-motor, or secondary motor cortex (M2) and the (ACC). There were no differences between the stroke volumes of the left and right hemispheres at either one or four weeks post-stroke so the volumes were combined for further analysis. One week after surgery, DE animals (1.95 ± 0.24 mm^3) had significantly smaller strokes than EE (3.36 ± 0.21, $p<0.001$) and EEE (3.20 ± 0.36, $p<0.001$) animals (Figure 3.15 A). There was no difference between EE and EEE groups. Four weeks after stroke there were no differences in total volume between the three groups (Figure 3.15 B).

There was a significant interaction (two-way ANOVA, $p<0.001$,, Figure 3.15 C) where stroke volume decreased from weeks one to four among EE and EEE groups but not the DE group. There was a main effect of time where stroke volume decreased from weeks one to four ($p<0.001$), a main effect of housing ($p<0.001$). There were significant decreases from weeks one to four for the EE ($p<0.001$) and EEE ($p<0.01$) groups but not the DE group. This shows that immediately following stroke, DE protected against increased infarct size, but by four weeks post-stroke both enrichment groups had decreased infarct sizes to the same amount.

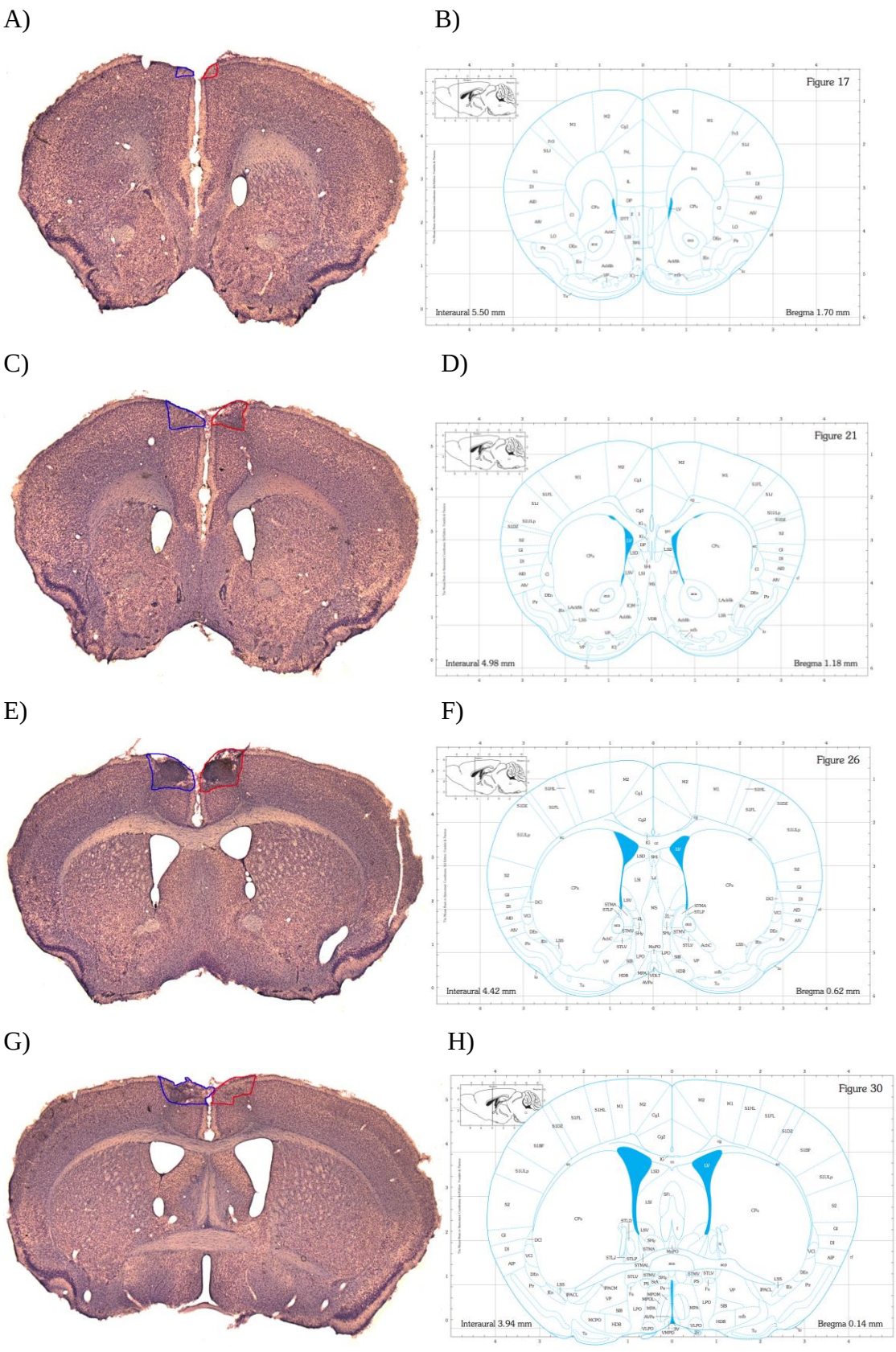
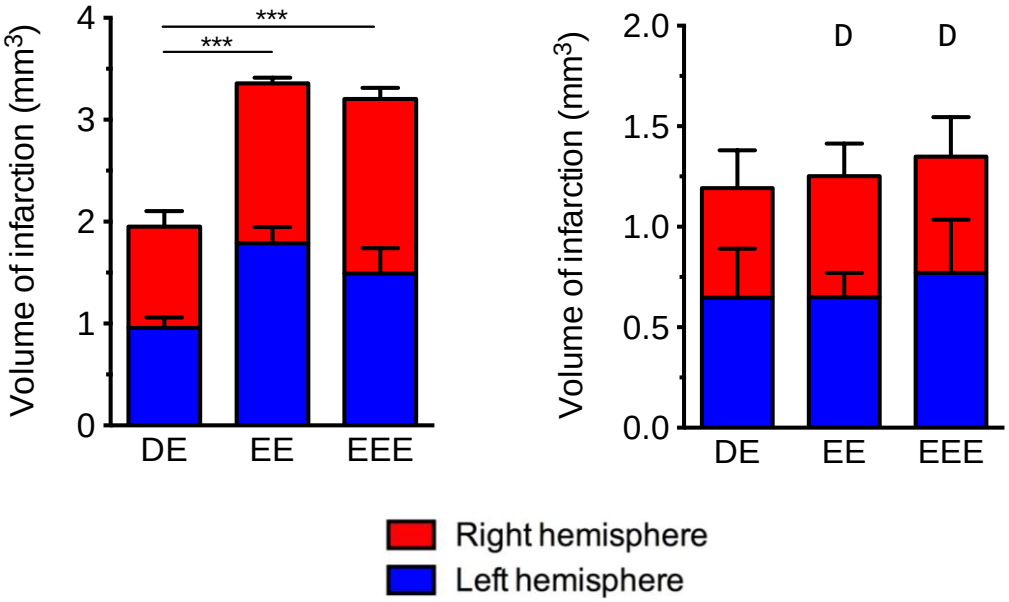


Figure 3.14. A), C), E) and G) Representative photomicrographs of Cresyl violet stains showing stroke location and size in an EE animal one week after stroke. Blue lines show the damaged area of the left hemisphere. Red lines show the damaged area of the right hemisphere. The infarct volume is 3.00 mm^3. B), D), F) and H) show the locations in a mouse brain atlas (Franklin & Paxinos, 2007).

A) Week One

B) Week Four



C)

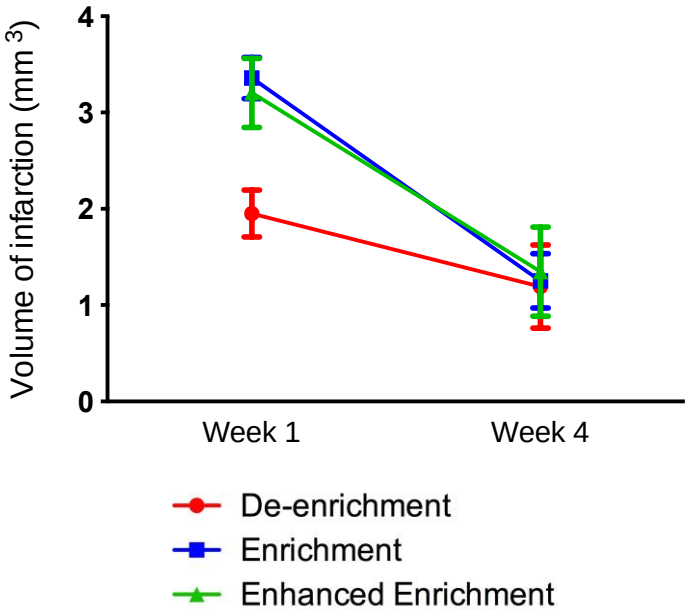


Figure 3.15. Infarct volume analysis. A) the infarct volume in each hemisphere at one week post-stroke. *** = $p<0.001$. B) the infarct volume at four weeks post-stroke. Δ = significantly different from week one. C) shows data from both hemispheres combined for each group at both time periods.

2.2 IBA1

IBA1 is a marker of microglial activation and inflammation. Staining results from four brain regions are presented here – the v3V, M2, Thalamus and SNr. These regions were selected because they showed the most variation between sham and stroke animals. Results for the other four regions (S1, Hipp, RSC and LV) were more homogenous and are shown in Appendix B.

The cell count method may have been more accurate than the thresholding method. There are two main reasons for this. Firstly, as the cell count was done by hand, the different levels of background staining had no effect on results. Thresholding, while still corrected for different levels of background staining, may have been more affected by these differences. Secondly, images analysed were darker in the corners than in the centre. This meant that a balance was needed between false positive darker background pixels in the corners, and false negative lighter microglial pixels in the centre. Although every effort was made to ensure this balance was consistent across all images, there may have been some variability.

2.2.1 Ventral Third Ventricle

The results of the IBA1 qualitative analysis are shown in Table 3.1. Shams show even, moderate staining in most areas, with dense IBA1 positive cells in the v3V, and among de-enriched animals, in the SNr. Among stroke animals it is clear that around the lateral ventricles, in the SNr and in the thalamus there is only sparse IBA1 staining, and less staining than shams in the v3V. The only region in which stroke animals have denser staining than shams is the secondary motor cortex (M2) where the infarct was present.

There was a significant interaction (two-way ANOVA, $p<0.05$, Figure 3.16 E) where EE and EEE decreased staining in stroke groups but not sham groups. There was a main effect of stroke where stroke animals had less staining than shams ($p<0.001$). Stroke EE animals (1.82 ± 0.23) had less staining than sham EE animals (2.80 ± 1.33, $p<0.01$). Stroke EEE animals (2.23 ± 0.17) had less staining than sham EEE animals (3.00 ± 0.00, $p<0.001$). Among stroke animals, the EE group had less staining than the DE group (2.53 ± 0.13, $p<0.01$).

Table 3.1. Quantitative analysis of the amount of staining in various areas. M2 refers to the secondary motor cortex, and S1 the primary somatosensory cortex. The dense staining in the M2 area among stroke animals is due to the infarct itself.

	Sham			Stroke		
Brain Regions	DE	EE	EEE	DE	EE	EEE
M2	++	++	++	+++	+++	+++
S1	++	++	++	++	++	++
LV	++	++	++	+	+	+
v3V	+++	+++	+++	+++	++	++
Hipp.	++	++	+	+	+	+
SNr	+++	++	++	+	-	+
RSC	++	++	++	++	++	++
Thal.	++	++	++	++	+	+

-	No staining
+	Occasional cells
++	Medium density
+++	High density

Cell counting data showed that overall, stroke animals had fewer IBA1 positive cells (two-way ANOVA, $p<0.001$). Stroke reduced the number of cells for DE animals (146.6 ± 10.4 and 112.5 ± 11.3 cells for sham and stroke, respectively; $p<0.05$), EE animals (145.5 ± 4.6 and 90.8 ± 9.9, $p<0.001$) and EEE animals (158.8 ± 11.1 and 84.2 ± 45.2, $p<0.001$, Figure 3.16 C).

Thresholding data showed an interaction effect (two-way ANOVA, $p<0.05$, Figure 3.16 D) where EE and EEE increased staining in shams but decreased staining in stroke animals. There was a main effect where stroke animals showed reduced staining ($p<0.001$). Stroke reduced the number of above-threshold pixels in the EE (286223 ± 60718 and 122310 ± 20166 for sham and stroke, respectively; $p<0.05$) and EEE groups (365522 ± 46139 and 152565 ± 32005, $p<0.001$). Among shams, EEE animals had more staining than DE animals (212076 ± 42847, $p<0.05$).

There were no significant differences between weeks one and four for the qualitative data (Figure 3.16. F). Figure 3.17. shows the same comparison for A) cell count data and B) threshold data. For the cell count data, there was a significant interaction effect (two-way ANOVA, $p<0.01$) where stroke EEE animals had lower staining than stroke EE animals at one week, but more at four weeks. At week one, EEE animals (43.2 ± 9.1) had fewer positive cells than EE animals (101.8 ± 19.0, $p<0.05$). EEE mice showed increased cell counts from week one (43.2 ± 9.1) to four (113.4 ± 12.4, $p<0.01$). Thresholding data showed that the EEE group increased staining from week one (43193 ± 8862) to four (207251 ± 36235, $p<0.05$). Overall, animals had increased staining at four weeks ($p<0.01$).

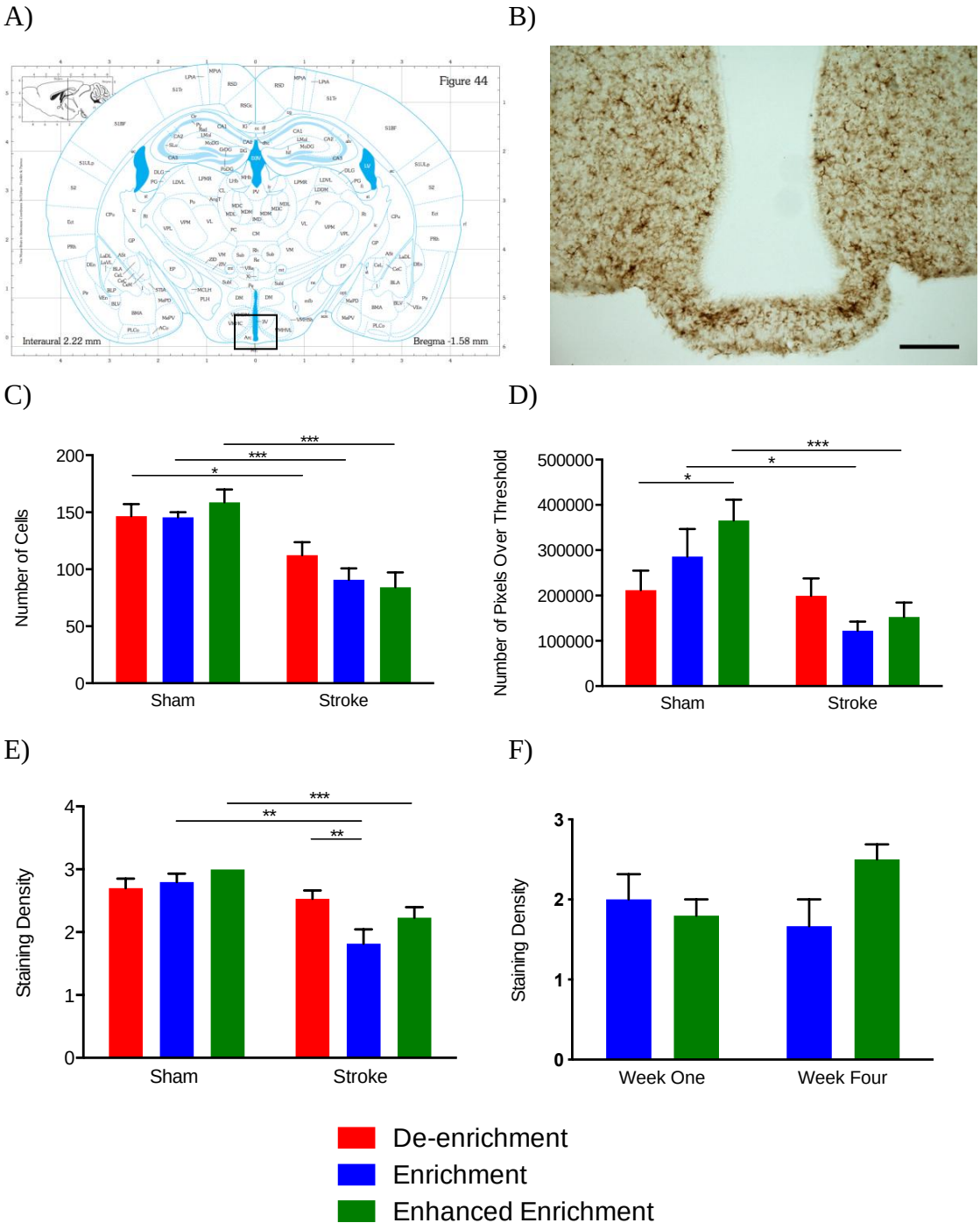


Figure 3.16. Analysis of IBA1 positive staining around the ventral third ventricle. A) The region of interest (Diagram from Franklin & Paxinos (2007)). B) A representative photograph of staining in the area. The scale bar indicates 100 µm. (Additional photographs can be found in Appendix C). C) Cell count data. D) Thresholding data. E) Qualitative analysis. F) Comparison of stroke animals at weeks one and four. De-enriched animals were not included as there was incomplete data due to removal of some animals from the study. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

While these methods differ in specific significant results, the trend is the same – for EE animals, there is no change from weeks one to four post-stroke, whereas EEE animals show an increase from one to four weeks. Table 3.2. shows this comparison for the qualitative data for all brain regions. At a glance, enrichment groups either have no change or a decrease in the amount of IBA1 staining, while EEE groups have either no change or an increase in staining (with the exception of the secondary motor cortex).

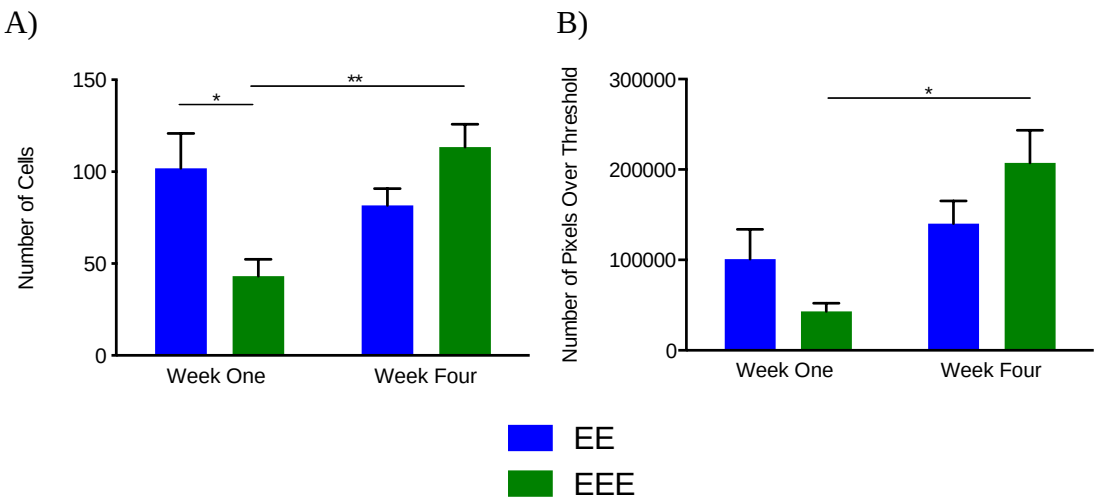


Figure 3.17. IBA1 staining in the v3V at weeks one and four post-stroke. A) Cell count data. B) Thresholding data. * = $p<0.05$, ** = $p<0.01$.

Table 3.2. Qualitative data showing the change in staining from week one to week four post-stroke for the EE and EEE groups.

	EE		EEE	
Brain Regions	1 week	4 weeks	1 week	4 weeks
M2	+++	+++	+++	++
S1	++	++	+	++
LV	++	+	-	+
v3V	++	++	++	+++
Hipp.	+	+	-	+
SNr	-	-	-	+
RSC	++	++	++	++
Thal.	++	-	+	+

-	No staining
+	Occasional cells
++	Medium density
+++	High density

2.2.2. Secondary Motor Cortex

The secondary motor cortex was the only region where stroke animals showed higher levels of staining than shams, due to the stroke being in this area. Overall, stroke animals had more intense staining (two-way ANOVA, $p<0.001$, Figure 3.18. E). Stroke animals had more staining than shams in DE housing (2.00 ± 0.00 and 2.80 ± 0.14 for sham and stroke, respectively; $p<0.001$), EE housing (2.00 ± 0.00 and 2.73 ± 0.14, $p<0.001$) and EEE housing (1.80 ± 0.13 and 2.54 ± 0.14, $p<0.001$).

Cell counting showed that while stroke animals had more variability in the number of stained cells, means were comparable to shams (Figure 3.18. C). Thresholding showed that stroke animals had more staining than shams (Figure 3.18. D). These results combined may indicate that following stroke, the number of activated microglia does not increase, but these cells become more activated, expressing more IBA1 and possibly increasing in size. Due to the small number of animals used for cell counting and thresholding, statistics were not performed on these data.

Figure 3.18. F shows the comparison between animals sacrificed at weeks one and four post-stroke. There were no significant differences.

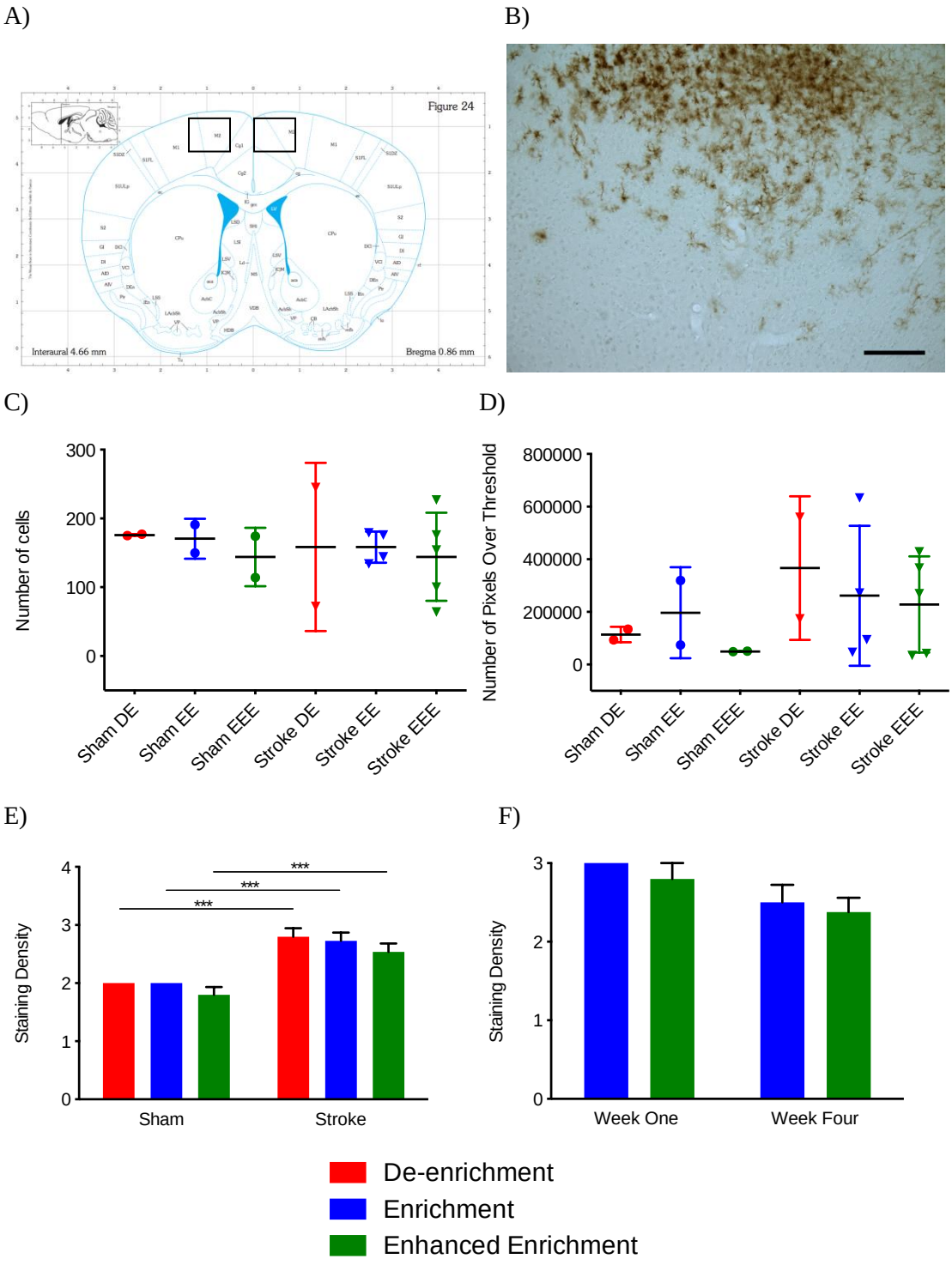


Figure 3.18. Analysis of IBA1 positive staining in the secondary motor cortex. A) The region of interest (Diagram from Franklin & Paxinos (2007)). B) A representative photograph of staining in the area. This photo comes from a stroke animal. The scale bar indicates 100 μm (Additional photographs can be found in Appendix C). C) Cell count data. D) Thresholding data. E) Qualitative analysis. F) Comparison between weeks one and four post-stroke. *** = $p<0.001$.

2.2.3. Thalamus

In the thalamus, stroke animals showed less staining than shams (two-way ANOVA, $p<0.01$), and there was a main effect of housing where EE and EEE reduced staining ($p<0.05$). Stroke EE animals (0.82 ± 0.33) showed less staining than sham EE animals (1.90 ± 0.18, $p<0.05$) and stroke EEE animals (0.78 ± 0.26) showed less staining than sham EEE animals (1.70 ± 0.30, $p<0.05$, Figure 3.19 E). Among stroke animals, the DE group (1.80 ± 0.28) showed more staining than either the EE ($p<0.05$) or EEE groups ($p<0.05$).

Cell count and thresholding data show the same trends as qualitative data – lower average staining among stroke animals. Stroke animals are more variable, with several animals showing high IBA1 levels. These outliers may be due to some stroke animals having dense clusters of stained cells in the thalamus. One of these clusters is shown in Figure 3.19. B. Generally, around the clusters there is a low level of staining, whereas the sham animals have an even, moderate distribution of stained cells.

In the week one and four comparison, there was a significant interaction effect (two-way ANOVA, $p<0.01$, Figure 3.19 F) where stroke EE animals had more microglial activation than stroke EEE animals at one week, but less at four weeks. There was a main effect where staining in the thalamus decreased from one to four weeks post-stroke ($p<0.05$). For the EE group, staining decreased from week one (1.80 ± 0.37) to four (0.00 ± 0.00, $p<0.05$).

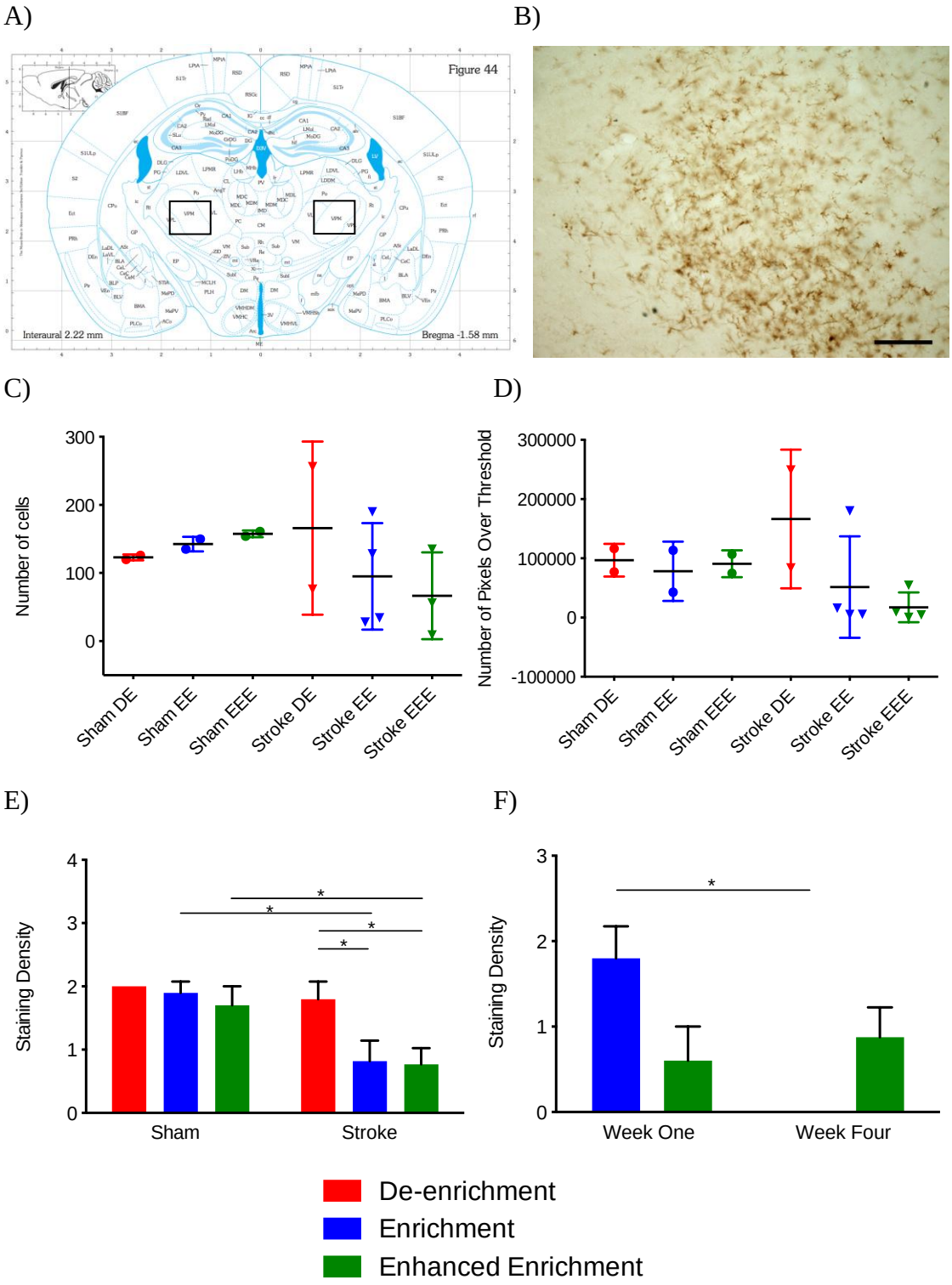


Figure 3.19. Analysis of IBA1 positive staining in the thalamus. A) The region of interest (Diagram from Franklin & Paxinos (2007)). B) A representative photograph of staining in the area. This photo shows a cluster of stained cells in a stroke animal. The scale bar indicates 100 µm (Additional photographs can be found in Appendix C). C) Cell count data. D) Thresholding data. E) Qualitative analysis. F) Comparison between weeks one and four post-stroke. * = $p<0.05$.

2.2.4. Substantia Nigra pars Reticulata

There was a main effect where stroke animals had less staining (two-way ANOVA, $p<0.001$, Figure 3.20 E), and there was a main effect of housing where EE decreased staining ($p<0.01$,). Stroke decreased the amount of IBA1 staining in DE animals (2.60 ± 0.16 and 1.20 ± 0.24, sham and stroke respectively, $p<0.001$), EE animals (1.80 ± 0.25 and 0.27 ± 0.14, $p<0.001$), and EEE animals (0.27 ± 0.14 and 0.85 ± 0.25, $p<0.01$). EE animals had less staining than DE animals in both shams ($p<0.05$) and strokes ($p<0.05$).

Cell counts and thresholding showed similar trends, with stroke animals again showing decreased staining (Figure 3.20 C and D), however there do not seem to be lower scores for EE animals.

There was an overall increase in staining from one to four weeks post-stroke (two-way ANOVA, $p<0.05$, Figure 3.20 F). Multiple comparisons revealed that EEE animals had higher staining at week four (1.25 ± 0.26) than week one (0.20 ± 0.20, $p<0.05$), and that at week four, EEE animals had more staining than EE animals (0.33 ± 0.21, $p<0.05$).

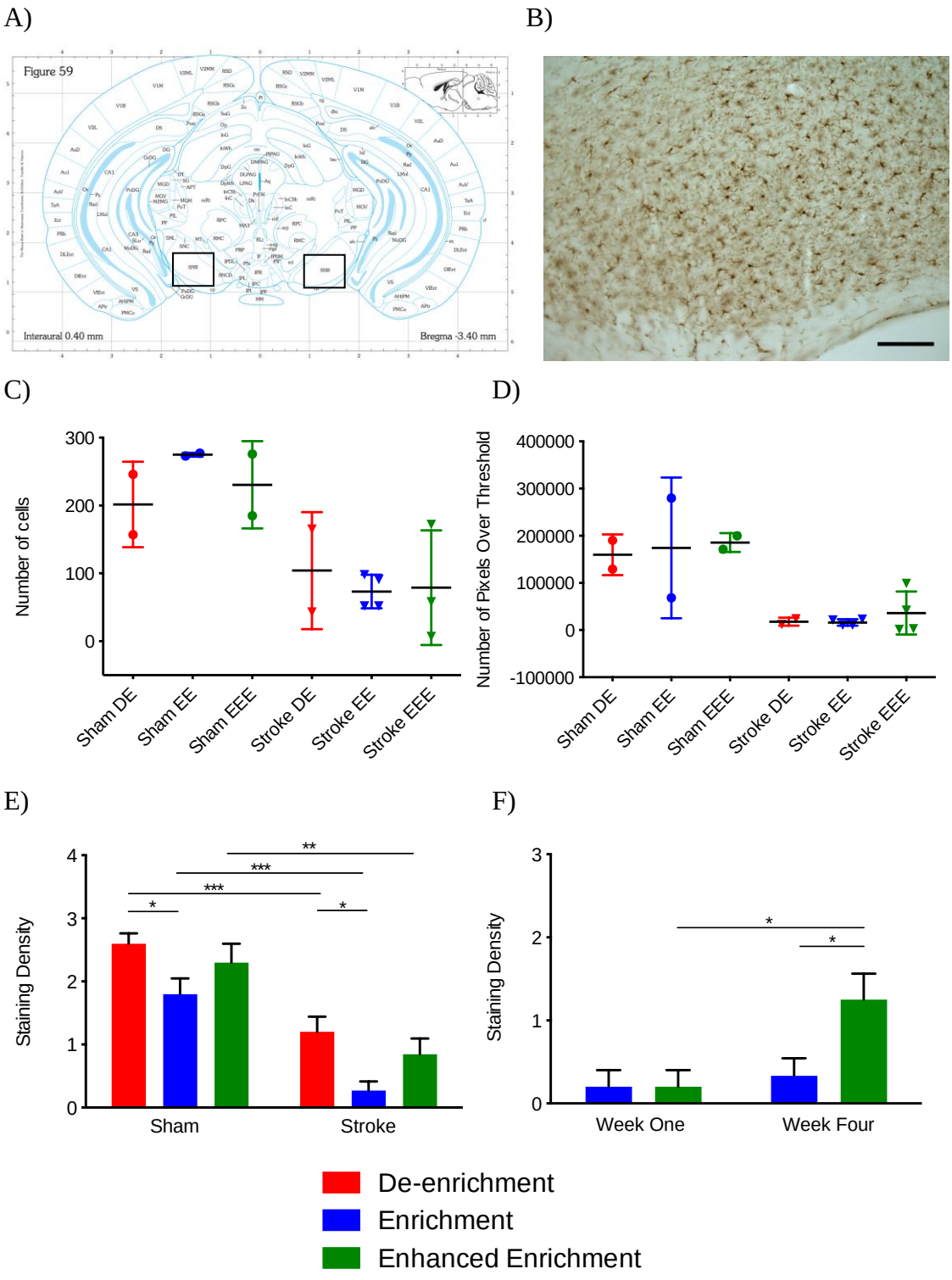


Figure 3.20. Analysis of IBA1 positive staining in the substantia nigra pars reticulata. A) The region of interest (Diagram from Franklin & Paxinos (2007)). B) A representative photograph of staining in the area. The scale bar indicates 100 µm (Additional photographs can be found in Appendix C). C) Cell count data. D) Thresholding data. E) Qualitative analysis. F) Comparison between weeks one and four post-stroke. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

Chapter 4. Discussion

1. Brief Summary of Results

We found that mPFC stroke had several effects on histology and behaviour. Stroke decreased IBA1 staining in several brain regions (except for some dense cores in the thalamus and v3V). Stroke caused motor impairment and increased the number of bursts one week post, but not at four weeks post. Stroke had mixed effects on anxiety in different tests, and had no effect on object memory. Consistent with our previous observation in the laboratory, we observed a delayed impairment in spatial memory in some of the stroke groups. Overall, initial impairment at one week post-stroke had resolved by four weeks post-stroke. The interesting exception to this was delayed spatial impairment at four weeks but not one week post-stroke.

Housing conditions also affected histology and behaviour. EE and EEE increased stroke volume one week after stroke, but not at four weeks. EE and EEE reduced IBA1 staining compared with DE in several regions. EE and EEE animals showed deterioration in spatial memory from one to four weeks post-stroke.

2. Discussion of Results

2.1 Stroke Volume

Average stroke volumes ranged from 1.19 mm^3 to 3.36 mm^3. This is similar to what has been found previously with similar photothrombotic lesions (Clarkson et al., 2013; Zhou et al., 2015; Zhao et al., 2005).

De resulted in smaller infarct volumes one week post-stroke compared with EE and EEE. By four weeks post-stroke the infarct sizes of the EE and EEE groups had decreased to be the same as the DE animals. This is consistent with the finding of Janssen and colleagues' (2010) meta-analysis that enrichment increases infarct volume, although we saw a considerably larger difference than the average 8% they reported. Interestingly, DE appears to have a protective effect on lesion volume early (one-week) post-stroke. This effect correlates with the effect of DE on microglial activation, which is further discussed in section 2.2 of the Discussion. However, DE does not decrease stroke volume from one to four weeks post-stroke. This indicates that DE has impaired

recovery over this time period compared with EE and EEE. This is a novel finding, as previous studies directly comparing enrichment with impoverished housing have found no difference in infarct size (Dahlqvist et al., 2004; Hirata et al., 2011; Olsson et al., 1995).

This larger infarct size may indicate that EE and EEE early (less than one week) after stroke have negative effects on recovery, while having positive effects later on. Our animals were placed into EE or EEE very soon (approximately 30 minutes) after recovering from surgery. This may have caused added activity and stress that adversely affected early recovery. Previous studies that have not found differences in stroke volume have used longer post-stroke recovery periods from one to four days before placing animals in EE (Dahlqvist et al., 2004; Hirata et al., 2011; Olsson et al., 1995). DE animals were placed immediately into individual housing, and this may have allowed them faster recovery of the infarct volume.

This finding may be relevant to the timing of intervention following stroke in human patients. Recently there has been a move towards beginning treatment early after stroke, especially when treating motor deficits (The AVERT Trial Collaboration group, 2015). This has shown mixed results where intervention within 24 hours has caused slight decreases in favourable outcomes (The AVERT Trial Collaboration group, 2015), while intervention within 48 hours has shown improved outcomes (Liu et al., 2014). It may be that the stress of suffering a stroke has a negative effect on treatment outcomes (Walker et al., 2014). There is an increase in circulating ACTH and cortisol soon after stroke that lasts for several days and is associated with larger infarct volume and worse functional outcomes (Fassbender et al., 1994). This supports the idea that de-enrichment has positive effects on infarct size through a reduction in stress soon after stroke. This idea is further explored in section 3.3 of the Discussion. It may be that early interventions could be more effective when targeted to those patients with lower levels of stress hormones.

2.2 IBA1-positive Microglia

2.2.1 Effects of Stroke on Microglia

Generally, stroke decreased the amount of IBA1 positive staining, except in the secondary motor cortex around the infarct. An increase in IBA1 staining immediately around the infarct is commonly found after stroke or other brain injury (Gerhard et al., 2005; Jones et al., 2015). Even in areas containing dense cores of staining such as the v3V and thalamus, stroke resulted in an overall decrease in the number and staining intensity of microglia. In the secondary motor cortex, even though there was dense staining immediately adjacent to the infarct, there was a decrease in microglia in nearby areas. These are unusual results, as microglial staining is generally either unchanged globally or increased in certain areas connected to the damaged region such as ipsilateral striatum (Planas et al., 1996) and cortex (Planas et al., 1996; Gerhard et al., 2005). In the thalamus, despite a general decrease in staining among stroke groups, in some stroke animals we found dense cores of activated microglia. This is consistent with other studies that have found activated microglia in the thalamus between two and four weeks after MCAo (Rupalla et al., 1998) and four weeks after motor cortex photothrombosis (Jones et al., 2015). Dense areas of inflammation may indicate that stroke is causing secondary degeneration in areas remote from the lesion site. Conversely, loss of a standard level of activation may be detrimental to recovery.

Stroke may be reducing activation through a change in inflammatory signalling molecules. These findings challenge the dogma that stroke would increase inflammatory signalling systemically in the brain. Perhaps in areas not adjacent or connected to the infarct these molecules have the opposite effect, or perhaps there is a reactionary dampening mechanism that prevents immune over-activation which is in effect by one or four weeks post-stroke.

2.2.2 Effects of Housing on Microglia

In shams, EEE increased staining in the v3V. In some of the other areas we looked at (M2, S1, Thal., Hipp., RSC, LV), housing had no effect on the IBA1 staining of shams. This is consistent with previous results where enrichment either caused no difference in microglia (Ehninger & Kempermann, 2003), or increased expression (Williamson et al., 2012). However, in the SNr, EE decreased staining compared with DE. This

matches the pattern seen in stroke animals, indicating that in the SNr, EE is having the same dampening effect in shams as in stroke animals.

In stroke animals, EE and EEE decreased staining relative to DE in the v3V, hippocampus, SNr, and thalamus. In other areas, housing had no effect on staining. De-enrichment seems to have mitigated the reduction of staining caused by stroke. The DE results are consistent with previous studies showing that social isolation increases microglial staining after stroke (Karelina et al., 2009) and after global ischemia caused by cardiac arrest (Weil et al., 2008). The reduction of staining in EE and EEE animals is unusual, as enrichment has been previously found to have no effect on microglial expression after stroke (Auriat et al., 2010). As mentioned above, microglial activation can have beneficial effects. It seems that EE and EEE have exacerbated the reduction of activation caused by stroke. The reduction in microglial activation in stroke EE and stroke EEE groups may be detrimental to recovery, and seems to coincide with larger stroke volumes one week after stroke. It is not clear why EE and EEE reduce IBA1 expression. It may be through causing low-level chronic stress, as this combined with photothrombosis can cause similar staining patterns to ours (Jones et al., 2015. This is further discussed in section 3.3.3.

In most brain regions, IBA1 staining stayed constant from one to four weeks post-stroke in stroke EE and stroke EEE animals. This is consistent with the results of Rupalla and colleagues (1998) who showed no change from 15 to 30 days post-stroke. However, in the v3V, thalamus, SNr, and lateral ventricles, IBA1 staining decreased or did not change from weeks one to four in stroke EE animals, but increased from weeks one to four in stroke EEE animals. This may indicate a delayed inflammatory effect in enhanced enrichment animals. This seems especially likely in the thalamus, where some stroke animals developed dense cores of IBA1 positive microglia. However, the DE group also developed these dense cores, so it doesn't seem to be the effects of EEE that are causing this. The increase in staining in EEE animals may also indicate a recovery of normal microglial levels following an early post-stroke decrease. It has previously been reported that microglial activation can be neuroprotective in some circumstances (Neumann et al., 2006; Lalancette-Hebert et al., 2007). It may be that EE and EEE caused a decrease in beneficial activation that contributed to neuronal loss, and that EEE was better than EE in recovering this activation at four weeks. Future studies could examine neuronal loss in the peri-infarct area as well as the

thalamus to investigate this idea.

Based on the IBA1 staining and infarct volume analysis, it would appear that activation of microglia are having a protective effect acutely (week one post-stroke), yet with prolonged activation, impairing the resolution of the stroke by four weeks. This may account for differences in behavioural assessments observed and discussed below.

2.3 Behavioural Results

2.3.1 Motor Impairments

Among sham animals, the DE group showed impaired motor performance on the grid-walking task one week post-stroke, indicating that DE without stroke may be harmful. This is consistent with studies of impoverishment and stroke (Hirata et al., 2011). Stroke impaired motor performance on the grid-walking task one week post-stroke, especially in EEE animals. Motor performance had recovered to pre-stroke levels by week four, especially in the EEE animals, who performed better than either the stroke DE and stroke EE groups at four weeks. This is consistent with the positive effect on motor recovery found in many enrichment studies (e.g. Janssen et al., 2010; Biernaskie et al., 2004). The increased stimulation of EEE relative to EE may have contributed to both the larger increase in motor impairment at week one (Zhang et al., 2012), and the larger improvement at week four.

While photothrombotic stroke in the motor or sensorimotor cortex usually causes motor impairments (e.g. Lee et al., 2007; Clarkson et al., 2013), this is less likely when the infarct is medial or anterior to these areas, as in this study. Motor skills were intact following lesions to either the medial wall of the mPFC (Deacon et al., 2003), or the ACC (Hewlett et al., 2014). Further, we recently reported using this model without any changes in environment that stroke had no effect on gross motor function when assessed on the grid-walking task (Zhou et al., 2015). Conversely, cortical impact injury to the mPFC has been shown to result in gross motor deficits (Moritz et al., 2014). This indicates that the degree by which the stroke impacts on secondary motor regions dictates whether or not gross motor impairments, albeit transient, would be

observed.

The pattern of motor impairment generally corresponds to the pattern of infarct volume. In stroke EE and stroke EEE groups, animals had larger infarct volumes and motor impairments were observed at week one. As these strokes resolved over the four-week period, we saw an improvement in motor performance. This may indicate that larger stroke volumes extended further into the pre-motor cortex causing this impairment in motor function. However, the stroke DE group had a smaller stroke volume at week one but still showed motor impairments. In addition, the sham DE animals showed the same pattern of impairment as the stroke animals. It may be that among EE and EEE animals, larger stroke volume contributed to motor impairments, while in sham DE and stroke DE animals, housing conditions caused the impairments. Impoverishment has previously been found to worsen motor impairments after stroke (Hirata et al., 2011).

The stroke EEE group showed increased IBA1 staining from weeks one to four in the v3V, thalamus, SNr, and lateral ventricles, while the stroke EE group did not. This coincides with a decrease in foot faults in the grid-walking test in the stroke EEE group, but not the stroke EE group. This may indicate that a return towards normal IBA1 levels (similar to shams) by four weeks post-stroke is beneficial for motor recovery, especially in motor-related areas such as the thalamus and SNr.

2.3.2 Activity Levels

Stroke had mixed effects on activity levels in the open field, increasing high-speed bursts at week one (especially in EE animals) but not changing the total distance travelled. The increase in speed bursts is consistent with the increase in activity found in several studies (Zhou et al., 2015; Deacon et al., 2003; Hewlett et al., 2014). This does not seem to support the findings that PFC stroke can cause depressive symptoms in the forced swim and sucrose consumption tests (Moritz et al., 2014). The increase in speed bursts without an increase in total distance travelled may indicate increased impulsivity in lesioned animals, which is consistent with the idea that the secondary motor cortex is responsible for action selection (Sul et al., 2011) and initiation (Murakami et al., 2014). At week four, stroke increased both speed bursts and total

distance travelled among EEE animals. Again this is consistent with the above findings that stroke can increase activity levels. This effect seems to have grown stronger among EEE animals from week one to four. This may be partly due to the reduction of motor impairment seen over the same time frame, or the worsening of impulsivity due to pre-motor lesion. However, the lesion size decreased over this time period, indicating that some recovery occurred in the pre-motor cortex.

Housing had no significant effects on activity levels at week one. At week four, enhanced enrichment decreased activity (both distance travelled and high-speed bursts) among shams, but increased activity in stroke animals. The explanation for this is unclear. It may be that among stroke animals, EEE worsens recovery of the secondary motor cortex (although there is no difference in lesion volume between EE and EEE groups) and enhances the effects of the lesion, increasing impulsivity and activity levels.

Changes in IBA1 staining may relate to changes in activity levels. As activity levels (both distance travelled and high-speed bursts) increase in stroke EEE animals from one to four weeks compared with stroke EE animals, there is a coinciding increase in IBA1 staining in some areas related to motor function such as the SNr and the thalamus. This may indicate that a return towards normal IBA1 levels (similar to shams) by four weeks post-stroke is beneficial for motor recovery, and that in the stroke EE group, the further decrease or failure to increase may hinder motor recovery.

2.3.3 Anxiety

There were mixed results in tests of anxiety. Stroke had no effect on anxious behaviour at weeks one or four in the open field, but decreased anxiety in the elevated plus maze, and increased anxiety in the light-dark box. This is consistent with the literature which shows increased (Blanco et al., 2009) and decreased (Deacon et al., 2003) anxiety, as well as no difference (Zhou, 2013). Other studies have also seen mixed results between different anxiety tests (Lacroix et al., 1998; Maaswinkel et al., 1996).

Housing had no effect on the anxiety of shams but had mixed effects on stroke animals. De-enrichment increased anxiety among stroke animals in week one of the open field, but strongly decreased anxiety in week one of the EPM, and had no effect in the LDB. Stroke EE and Stroke EEE groups showed the same levels of anxiety, except in week one of the LDB, where the EEE animals made less entries into the light section than all other groups. De-enrichment among stroke animals increased anxious behaviour in the open field in week one, which is consistent with previous isolation experiments (Weiss et al., 2004). However, stroke DE animals showed greatly decreased anxious behaviour in the EPM in week one. There was no effect of standard EE. This is counter to previous results, which generally find that enrichment reduces anxiety (Ravenelle et al., 2013; Sampedro-Piquero et al., 2013), although not always after brain injury (Moritz et al., 2014). Increased or decreased anxiety in one test without the same effect in other tests is difficult to explain. It may be that groups are reacting to different stimuli in different ways (height exposure in the EPM, and light or openness exposure in the LDB and open field). The LDB and open field results are more similar, which supports this idea. In any case, the effects of stroke and housing are largely absent by week four post-stroke in all tests of anxiety.

In week one of the EPM, stroke DE animals spend more time in the open arm than other stroke groups. They also had much smaller lesion volumes than EE or EEE groups. In week four, there was no difference between stroke groups in either time spent in open arm or lesion volume. It seems that among stroke groups, increased lesion volume coincides with more time spent in the open arm, possibly indicating lower anxiety.

2.3.4 Visual Recognition Memory

mPFC stroke had little effect on object memory in the novel object test, which is consistent with some other results using larger infarcts (Markowitz et al., 2011). However, this contradicts results after vasoconstrictive lesions to the peri-ventricular white matter (Blasi et al., 2014). The one exception was among EE animals, where stroke actually improved performance at week four. This is similar to findings that lesions to the sensory cortex increase novel object preference (Liguz-Lecznar et al., 2014).

Housing also had minimal effects - in week one, EE improved performance among shams. When analysed together, the animals improved performance from weeks one to four, although stroke EE was the only individual group to do this. This is consistent with the results of Doulames and colleagues (2013), who found that social and enriched groups improved NOR performance over one month of differential housing, while individually housed animals did not. EE has been shown to improve object exploration and episodic memory in animals (Gobbo & O'Mara, 2004; Diniz et al., 2010).

2.3.5 Spatial Memory

One week after stroke, both stroke and sham animals recognised the moved object. These results are consistent with Deacon and colleagues' (2003) findings that mPFC lesions did not affect spatial memory, although other groups have found deficits (Moritz et al., 2014). Animals showing stroke-induced spatial memory deficits one or two days post-stroke have been shown to recover to sham levels by three and four days post (Hewlett et al., 2014), which supports our finding after one week.

However, by four weeks, stroke EE and stroke EEE groups did not recognise the moved object. This indicates that among these groups, there is a deterioration in spatial memory over four weeks after stroke. These results confirm earlier findings of our group using the same stroke model with standard housing (Zhou et al., 2015). A similar decline in the alternating Y maze from two to seven weeks after stroke was found following MCAo in mice by Doyle and colleagues (2015). Our results contradict several studies that show that enrichment improves spatial memory performance in naive animals (Frick et al., 2003; Diniz et al., 2010) as well as after MCAo (Dahlqvist et al., 2004; Xu et al., 2009) and chronic cerebral hypoperfusion (Zhu et al., 2011).

Interestingly, stroke DE animals did not show any impairment at four weeks, suggesting that de-enrichment was protective. Again, this contradicts previous studies that have found negative effects of impoverishment on spatial tasks (Monteiro et al., 2013; Doulames et al., 2013). It is likely that the effects of housing on stroke volume are connected to the spatial memory results. In week one, animals have larger stroke volumes and can recognise the moved object. In week four, their stroke volumes have reduced to the same size as DE mice but they now fail to recognise the moved object.

Although this seems counter-intuitive, this may indicate that having a larger lesion at week one post-stroke causes delayed secondary degeneration. This may impair spatial memory performance at week four post-stroke, even though the initial stroke region has recovered somewhat. This could also explain why DE protects against spatial impairment at four weeks post, as the DE animals had much lower lesion volumes one week post, and possibly less secondary degeneration as a result.

IBA1 staining may also be related to performance in the OLR. EE and EEE animals show lower staining than DE animals in the thalamus and hippocampus. Normally, this can indicate inflammation, but as mentioned above, staining is much lower than sham levels, and may be harmful. A moderate level of microglial activation may be protective of neuronal loss in these areas, and shield the DE animals from secondary degeneration and spatial impairments.

3. General Discussion

3.1 Microglia

The observation of lower microglial levels in stroke EE and stroke EEE animals, especially in the thalamus, combined with the decline in spatial memory from two to five weeks after stroke in these groups, may indicate that a moderate level of microglial activation is beneficial, and that decreased activation to well below sham levels may be harmful.

There are many studies showing the positive effects of microglial activation. Microglia can become alternatively activated (M2) and release growth factors such as BDNF, TNF-α and IGF-1, while releasing less inflammatory cytokines (Gomes-Leal, 2012; Lalancette-Hebert et al., 2007; Lambertsen et al., 2009). While inflammatory cytokines are important in removing debris acutely after stroke, prolonged increases in cytokine levels is detrimental to recovery (Gomes-Leal, 2012). M2 microglia also increase neurogenesis (Thored et al., 2009), phagycytose neutrophils and increase neuronal survival following oxygen and glucose deprivation (Neumann et al., 2006), and clear apoptotic tissue (Zhao et al., 2007). These and other positive effects mentioned in chapter 1 section 6.2 may be lost when microglial activity is reduced.

There is an association between decreased microglia and impaired spatial memory in stroke EE and EEE groups. Despite this, the observation of dense cores of activated microglia in the thalamus and v3V in stroke animals may indicate inflammation that could be a sign of secondary neuronal degeneration.

3.2 Remote Inflammation

As mentioned in section 7 of chapter 1, after stroke, secondary neuronal loss can affect brain areas distal but connected to the infarct, such as the thalamus, hippocampus and SNr. Microglial activation is often seen as a sign that neuronal damage is occurring.

3.2.1 Thalamus

Neuronal loss in the thalamus is frequently seen after MCAo (Rupalla et al., 1998; Dihne et al., 2002) as well as photothombotic stroke (Block et al., 2005). Microglial activation is associated with neuronal loss in this area (Block et al., 2005) and occurs before signs of apoptosis and neuronal death (Rupalla et al., 1998). Secondary damage in the thalamus after stroke is associated with spatial learning deficits (Block et al., 2005; Kumon et al., 1996). Influx of B-lymphocytes (which is partly mediated by microglia) is associated with spatial memory decline, which is prevented when treated with a B-lymphocyte-blocking antibody, and also prevented in B-lymphocyte knockout mouse strains (Doyle et al., 2015).

The dense areas of activated microglia we saw in the thalamus may be signs of neuronal stress in this area. However, these were most commonly seen in DE animals, who did not have spatial memory impairments. It may be that DE animals experience a moderate inflammatory response that is beneficial, whereas EE and EEE animals have less activation and more neuronal damage.

3.2.2 Ventral Third Ventricle

Similarly to the thalamus, we saw an overall decrease in IBA1 staining among stroke animals in the v3V, especially in EE and EEE groups. We also observed areas of dense

activated microglia in all groups, including shams. As in the thalamus, this may indicate inflammation and the potential for neuronal damage, but because of the higher levels of staining in shams, this does not seem to be the case. Inflammation in this area (particularly the median eminence) may indicate disruption to the neuroendocrine system. This is a possibility, as the mPFC has connections to the hypothalamus and both areas are involved in the regulation of the HPA axis and stress response (Ulrich-Lai & Herman, 2009). Damage to the mPFC may disrupt this system, especially if animals experience stress during the post-stroke period.

3.3 Stress

3.3.1 Causes of Stress

The surgery and stroke may have induced stress in the animals. Certainly, human patients experience stress after stroke (Walker et al., 2014), but the symptoms to animals in this study were minimal. The process of recovering from anaesthesia and reintegrating back into a housing group after surgery may have contributed more stress than the strokes. Our animals had a relatively short recovery period after surgery – approximately 30 minutes before being placed into their new differential housing. Therefore, animals quickly had to interact with others very soon after stroke, possibly involving fighting. Due to the lack of symptoms caused by our stroke method and location, animals are able to quickly re-enter group housing. However, among EE and EEE groups, this may have increased stress, compared with the DE animals that were placed in individual housing immediately after surgery. This may explain the increased lesion volume of the EE and EEE groups two weeks post-stroke.

Although some studies show that EE reduces stress responses (e.g. Ravenelle et al., 2013; Wright & Conrad, 2008), and some show no changes (Dahlqvist et al., 2003), there is evidence that enrichment can have negative effects on stress regulation. Social housing causes stress among subordinate animals (Singhal et al., 2014), and EE worsens response to acute and chronic injury (Shum et al., 2007). Stress may also counter the positive effects of enrichment such as reducing BDNF levels (McKlveen et al., 2015).

In intact animals, EE is a mild stressor that can have positive effects (Crofton et al., 2015). In our study, several factors may have combined to increase stress compared with what is generally found in enrichment studies, possibly preventing enrichment from having positive effects, and contributing to the spatial memory decline. EEE and EE groups had more disturbances to the cage environment than DE groups. As well as the weekly change of flooring and bedding material, food and water, and behavioural testing that all groups experienced, there was a weekly object change for the EE group and four changes each week for the EEE group. Cage changes, new objects and handling can cause increases in plasma CORT and heart rate for over 30 minutes (Balcombe et al., 2004). Several days after stroke, animals were moved from open-topped breeder cages in groups of five, to smaller IVC housing in groups of two or three. IVC housing is known to increase stress (Shan et al., 2014) and adrenal weights (David et al., 2013) in rodents. Transfer into IVC cages caused an increase in fecal immunoglobulin A (IgA, a marker of stress) concentrations, which took six weeks to return to normal (Bundgaard et al., 2012). Animals switched between DE and EE had lower brain weights than those in consistent environments (Klippel et al., 1978). Although there were only two or three animals per cage in EE and EEE groups, the enrichment objects took up a reasonable amount of space, which may have caused stress. High social density can increase CORT and negatively affect weight gain, behaviour and the immune system (Laber et al., 2008).

Although impoverishment can cause stress through isolation and lack of enrichment, the combination of all these factors may have combined to increase stress in the enrichment groups more than the DE group, and thus prevented positive effects of enrichment, and possibly had negative effects on spatial memory.

3.3.2 Stress and the mPFC

In section 12.4 of chapter 1, the relationship of the mPFC to stress regulation is discussed. The mPFC is connected to the PVN (Radley & Sawchenko, 2011) and helps to regulate the release of CRH and CORT in response to stress (McKlveen et al., 2015; Diorio et al., 1993). Damage to the mPFC disrupts this regulation, increasing ACTH and CORT release in response to stress (Ondicova et al., 2012; Figueiredo et al., 2003). The cingulate gyrus and secondary motor cortex (both damaged by our lesion) have

further connections to the mediodorsal nucleus of the thalamus and the basolateral nucleus of the amygdala, which are involved in the fear response circuit (Matyas et al., 2014). ACC stroke caused adrenal hypertrophy and enhanced suppression of the HPA axis, and when combined with chronic mild stress, increased novelty-induced defecation in the EPM (Hewlett et al., 2014). In addition to the effects of the lesion, the mild stress of enrichment may have caused further disruption of the HPA axis. These factors may have combined to prevent EE and EEE from having beneficial effects.

3.3.3 Stress and Microglia

Most commonly, stress increases microglial activation after stroke. Chronic stress increased the staining intensity and number of microglia in the mPFC and hippocampus, among other areas (Tynan et al., 2010; Hinwood et al., 2012), which was associated with impaired spatial memory (Hinwood et al., 2012). Stress before cardiac arrest and resuscitation increased microglial activation, hippocampal neuronal damage, and anxiety (Neigh et al., 2009). When microglia were blocked with minocycline, the damage and anxiety were prevented (Neigh et al., 2009). Stress, through changes in CORT and NE concentration, causes remodelling of microglia and increases microglial release of inflammatory cytokines (Walker et al., 2013). In contrast, Jones and colleagues (2015) found that after motor cortex photothrombosis microglial activation was significantly increased. Chronic stress reduced microglial activation and exacerbated neuronal loss in the thalamus and hippocampus after motor cortex photothrombosis. This is similar to our findings, although in our study stroke decreased microglial activation. If our enrichment protocol caused chronic stress, this may have had a similar effect and dampened the microglial response to stroke. The idea that microglia are protective of neuronal loss (Jones et al., 2015) is supported by the correlation we saw between the decrease in IBA1 staining in EE and EEE groups in several brain regions with the delayed spatial impairments.

4. Limitations

4.1 Differences Between Rounds One and Two

Due to the number of animals used in this study and the time required to carry out surgery, behavioural tests and perfusions, all procedures were carried out using two

cohorts of mice eight weeks apart. This meant that the second group was two months older and had experienced two months more EE prior to stroke. Since animals had already experienced three months of enrichment, we do not think that this extra two months has affected our data. The rationale for housing the mice in enriched environments for at least three months was that we believed that this is long enough to fully change the intrinsic memory of the cells, that is, changes in epigenetics caused by the enrichment. Epigenetic changes, once triggered, can last from weaning into adulthood (Branchi et al., 2011), and even from one generation to the next (Kiyono et al., 1985). Therefore the additional two months of housing for the second cohort is unlikely to have any further effects on this. In support of this hypothesis, enrichment has previously been shown to induce long-lasting epigenetic changes, such as increasing histone acetylation at multiple sites including on the BDNF gene in both young and aged animals (Branchi et al., 2011; Morse et al., 2015), genes involved in regulating LTP, and several gene sites in the hippocampus (Arai & Feig, 2011).

A difference that may have affected our results between the two cohorts of mice is the presence of a female experimenter during round one of behavioural testing. During round two of testing only one male experimenter was present. The presence of one male experimenter was recently shown to increase stress and anxiety-related behaviours in the open field, compared with one female experimenter, or one male and one female (Sorge et al., 2014). This is consistent with our open field results, with animals from multiple groups spending less time in the middle in round two. Animals also spent less time in the open arm of the EPM in week four, round two, especially animals in the EEE groups. This also indicates anxiety and supports the idea that EEE causes stress. The combination of increased stress from housing and acute stress from a male experimenter may have caused the increased anxiety in the EPM.

4.2 Missing Data

In round two, five animals in the stroke EE group were placed into individual housing four days after stroke due to excessive fighting and injuries. This meant their data could not be used. Because the remaining stroke EE animals were sacrificed at one week post-stroke, there were no stroke EE week four data in round two.

4.3 Behavioural Tests

There may have been a factor biasing object preference in the NOR and OLR tests. Figure A19, Appendix G shows the combined habituation data from NOR and OLR from weeks one and four. Although some groups did not show a preference, overall in round one animals spent more time investigating object one, and in round two they spent more time with object two.

This bias doesn't seem to have had an effect on the NOR task, as there is no difference between rounds one and two in the test round (Appendix B, Figure A8). However, in the OLR test, there is a trend for animals in round two to have a higher preference for the moved object than those in round one (Appendix B, Figure A9). This is consistent with the object bias seen in the habituation phase, as the moved object was on the same side as object two. Despite this bias towards object two, stroke EEE animals in round two do not spend more time with the moved object at week four, which indicates that there is a real effect on spatial memory. Despite these potential biases these NOR and OLR results agree with previous work by our group (Zhou et al., 2015) as well as a drug study also completed by Zhou & Clarkson (Zhou & Clarkson, personal communication, 2015). It is likely that this variation is due to natural variation among small numbers of animals in each group, and when combined the data is normalised and there is no overall bias.

5. Future Directions

The obvious next step is to examine whether EE and EEE cause stress. A likely candidate here for assessment would be to measure changes in CORT levels in the blood across the test period and assess whether this is related to behavioural changes and microglial activation. This would answer some key questions, such as whether our enrichment paradigm increases CORT either chronically or acutely after a further stressor. We would also confirm what effect mPFC stroke combined with enrichment has on CORT levels, and if this is related to changes in microglial activation.

We used a microglial stain to check for inflammation that may indicate neuronal loss. As there is marked controversy surrounding microglial activation as being protective

or detrimental (Cherry et al., 2014; Gomes-Leal, 2012), further stains should also be undertaken. It would be useful to use another antibody that directly targets neurons (such as NeuN), or markers of neuronal injury such as astroglial activation (GFAP), inflammatory cytokines (IL-1b or TNF-α) or apoptosis (cleaved caspase-3). Would changes in these markers be associated with increases or decreases in microglial staining, showing that reductions in microglia can be detrimental or beneficial? Based on the data that we have collected, we predict that loss of IBA1 labelling is detrimental and contributes to the delayed impairment in spatial memory. Would changes in these other markers that we outline above also be associated with behavioural changes such as delayed spatial impairment? Quantifying the number of amoeboid cells could also be a useful addition to our study, as this can distinguish between anterograde and retrograde degeneration (Sorensen et al., 1996).

Using additional spatial memory tests would further confirm our results in the OLR task. Would we see the same pattern of decline in tests such as the MWM, or radial arm water maze? And would the deficit also occur in spatial working memory in an alternating Y maze? These are studies that have been proposed and Lisa Zhou in the laboratory of Dr Clarkson is establishing some of these for her PhD.

The implementation of EE and EEE could be changed to moderate the potential induction of stress. Using larger, non-IVC cages may reduce stress and allow the positive effects of enrichment to be seen. Animals could also be given one or two days to recover alone after stroke, before differential housing, which may reduce the increase of infarct volume caused by EE and EEE, as well as reduce secondary degeneration.

6. Concluding Remarks

We have found that enrichment causes an increase in stroke volume one week after stroke that is associated with motor impairments one week after stroke and delayed impairment in spatial memory four weeks after stroke. Microglial activation was decreased by stroke, and also by enrichment, and this may be connected to increased lesion volume and secondary neuronal degeneration. It should be noted however, that

these effects might be specific for strokes that affect or disrupt the HPA axis. If we were to combine enrichment with a motor cortex stroke, then these same effects associated with changes in IBA1 staining, stress and enrichment may not be the same.

Our results indicate that very early post-stroke enrichment has negative effects on recovery, and can cause delayed impairments. This may be caused by an increase in stress in the enrichment groups. High-intensity intervention within 24 hours of stroke, while often used in human stroke rehabilitation (especially to treat motor impairments), increases the chance of a negative outcome (The AVERT Trial Collaboration group, 2015). It seems that it may be better to delay intervention for a period of hours or days to maximise recovery. The best time to begin intervention is still a matter of study, and it may be that it varies depending on stroke severity and location, and other individual variables.

Delayed cognitive decline due to secondary neuronal loss is common after stroke, and post-stroke depression and anxiety may also be affected by neuronal loss. Optimising treatment schedules to minimise secondary degeneration could help to reduce impairment and give independence back to stroke survivors, as well as reducing the burden on the health system.

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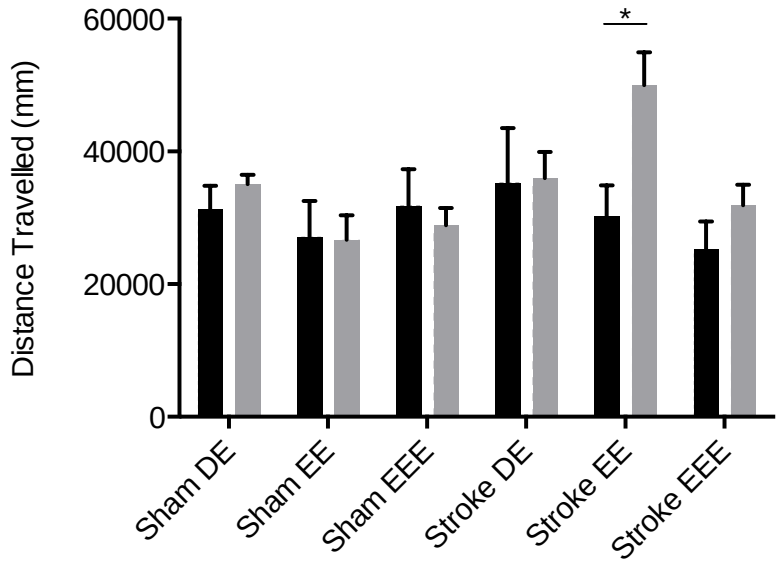
Appendices

Appendix A. Round One and Two Comparisons

A) Week 1



B) Week 4

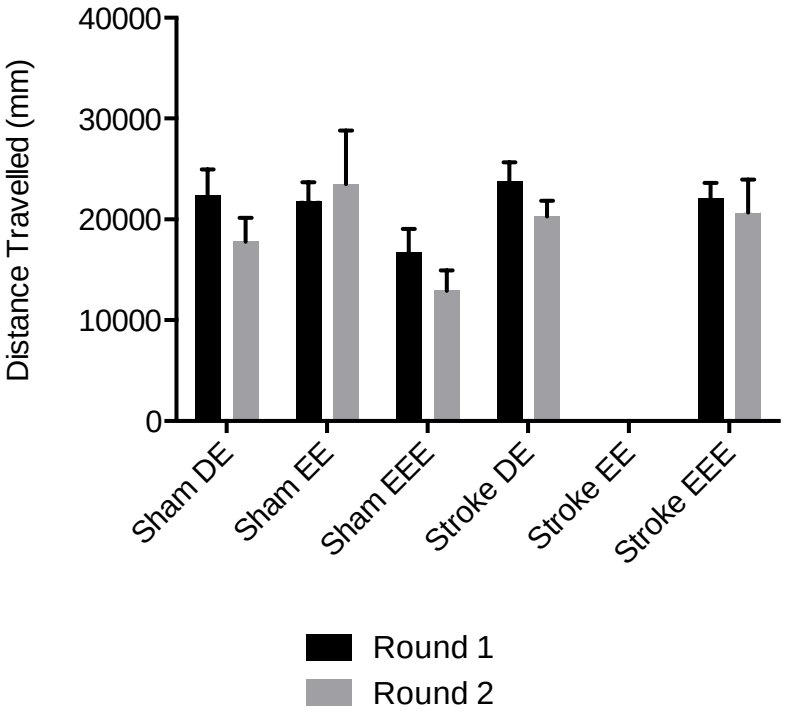


Figure A1. Total distance travelled in the open field test. * = $p<0.05$.

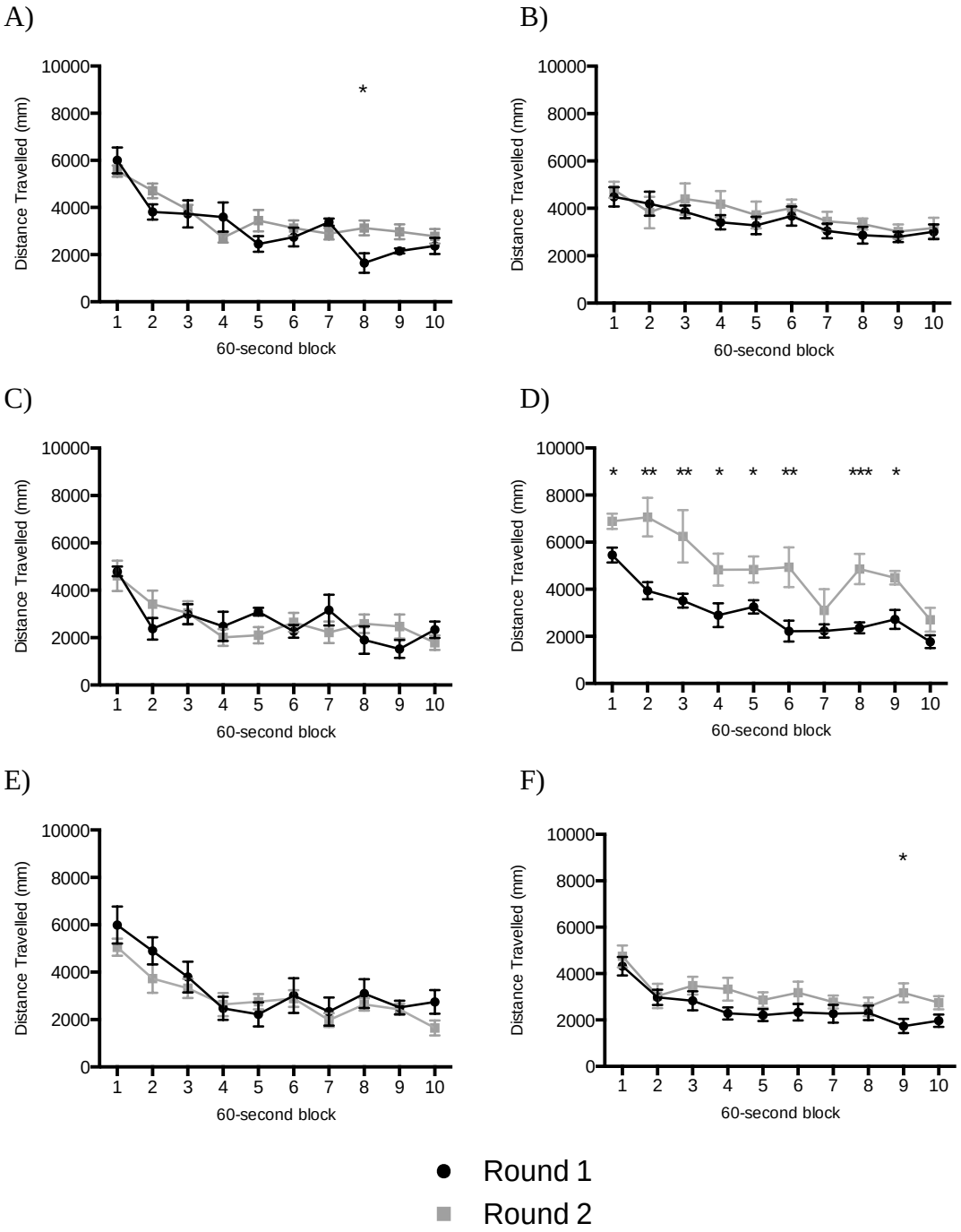


Figure A2. Distance travelled in the open field one week after stroke. Each graph shows the data from rounds one and two for one experimental group. A) sham DE; B) stroke DE; C) sham EE; D) stroke EE; E) sham EEE; F) stroke EEE. Stars above each 60-second block indicate significance level of the t-test comparing round one and four for that block. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$. Two-way ANOVAs revealed significant main effects of round in experimental groups sham DE ($p<0.05$), stroke EE ($p<0.001$) and stroke EEE ($p<0.001$) (A, D and F, respectively).

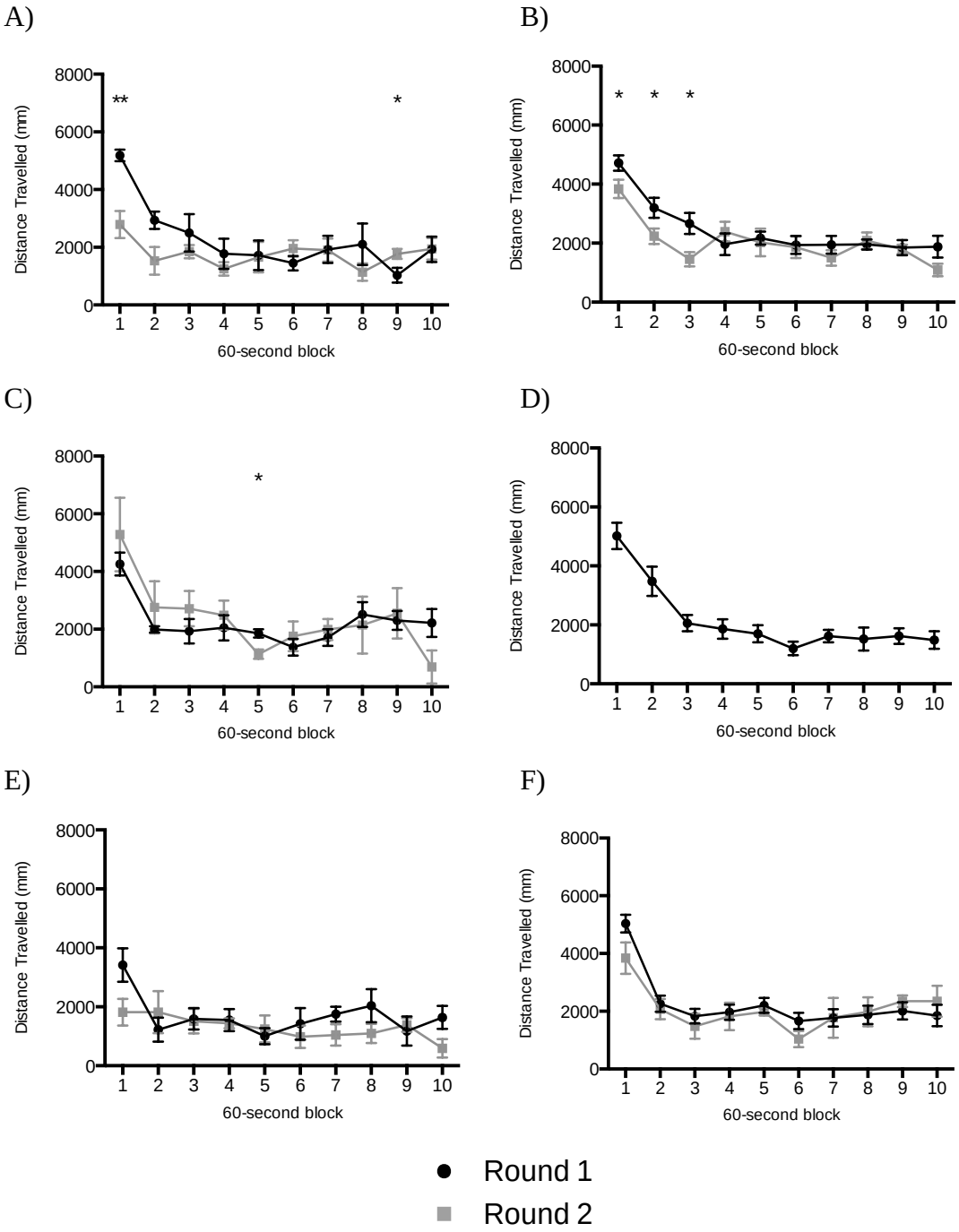
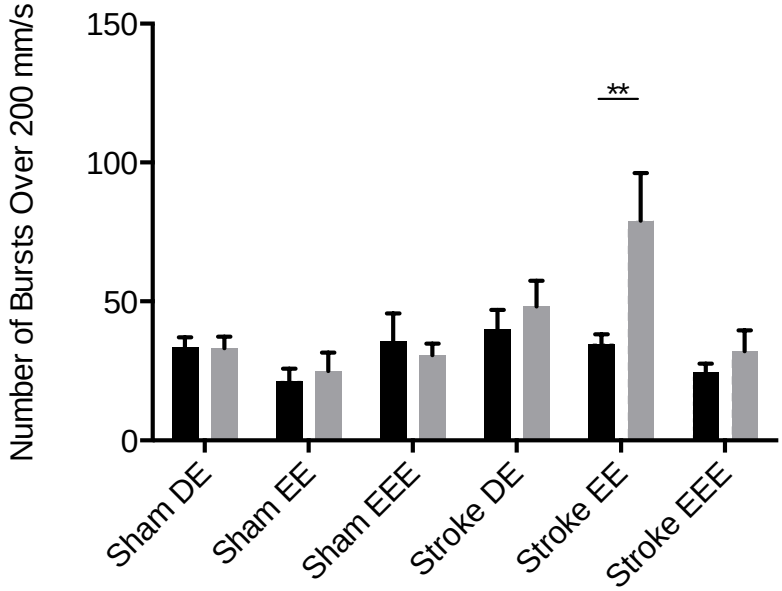


Figure A3. Distance travelled in the open field in week four in 60-second blocks. Each graph shows the data from rounds one and two for one experimental group. A) sham DE; B) stroke DE; C) sham EE; D) stroke EE; E) sham EEE; F) stroke EEE. Stars above each 60-second block indicate significance level of the t-test comparing round one and four for that block. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

A) Week 1



B) Week 4

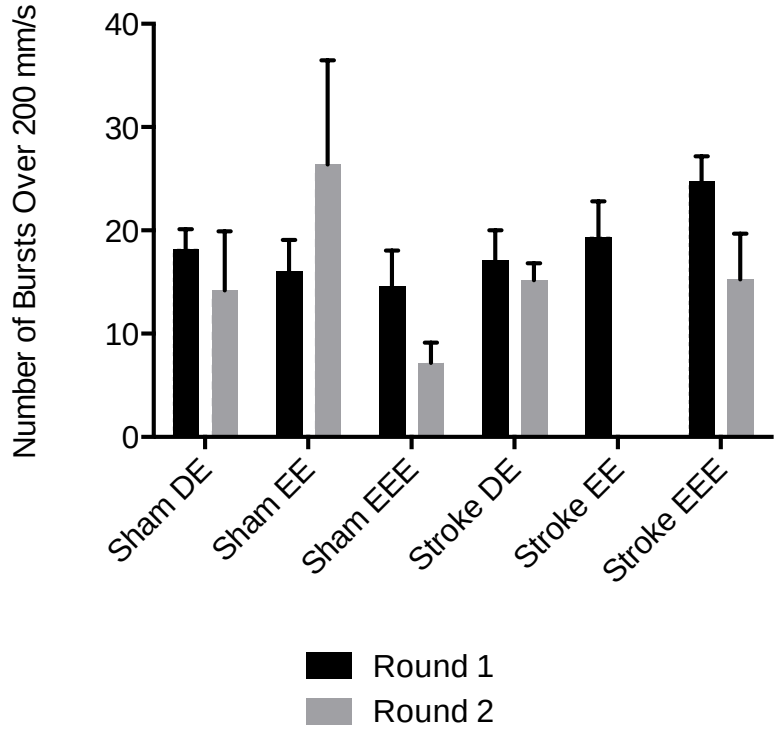
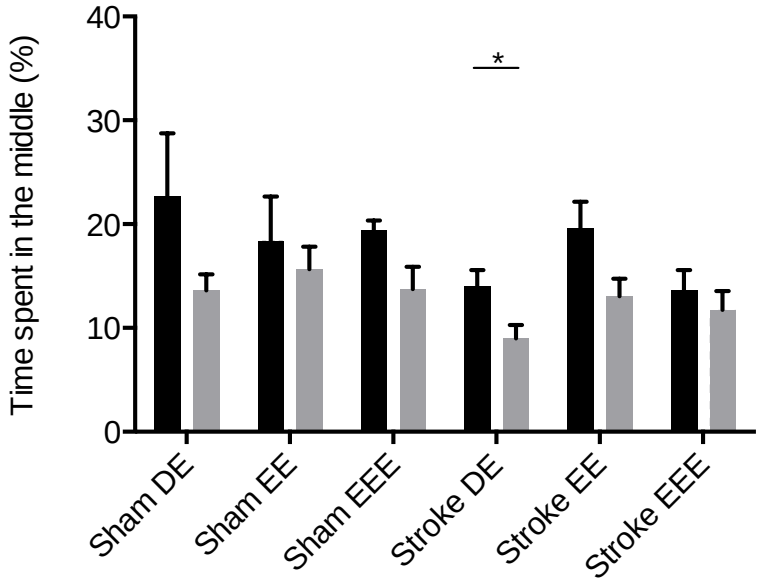


Figure A4. The number of high-speed bursts made in the open field test. ** = $p<0.01$.

A) Week 1



B) Week 4

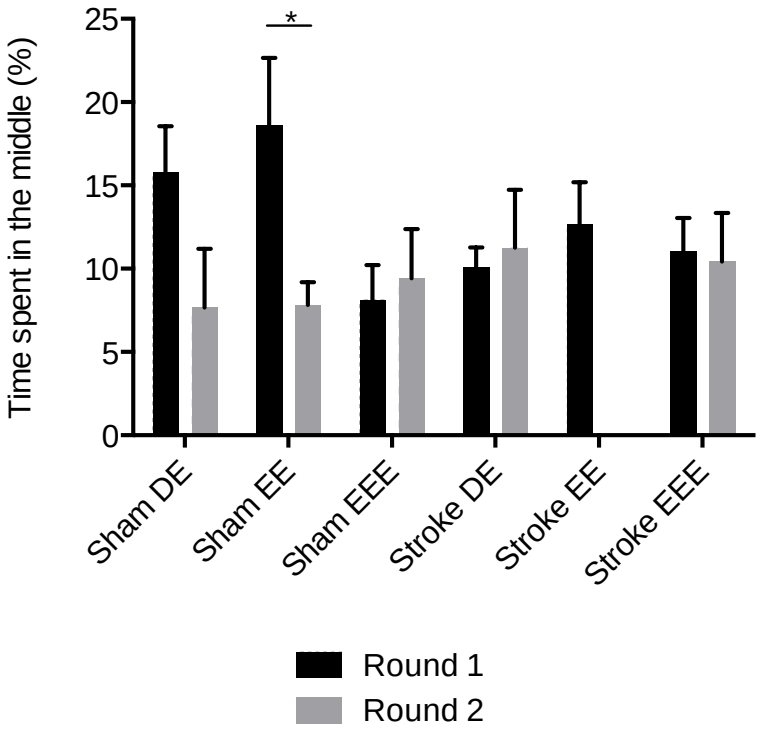
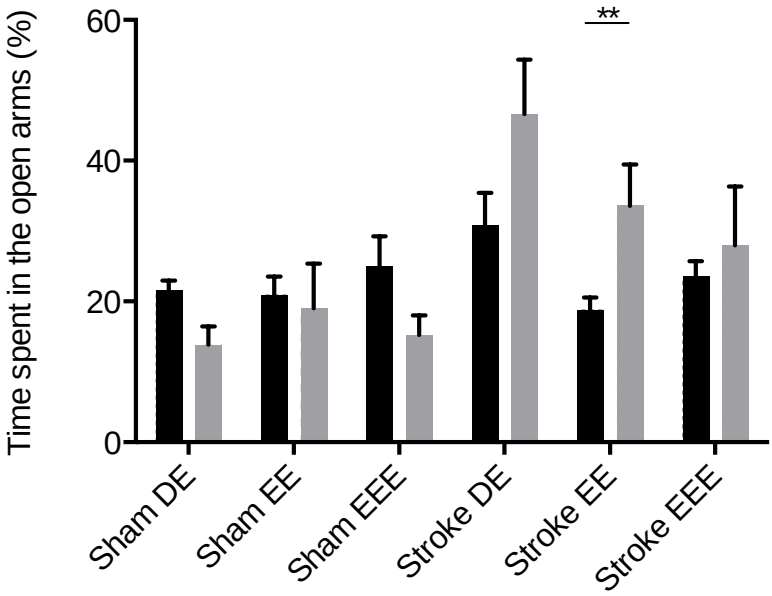


Figure A5. The percentage of time spent in the middle of the open field arena. * = $p<0.05$.

A) Week 1



B) Week 4

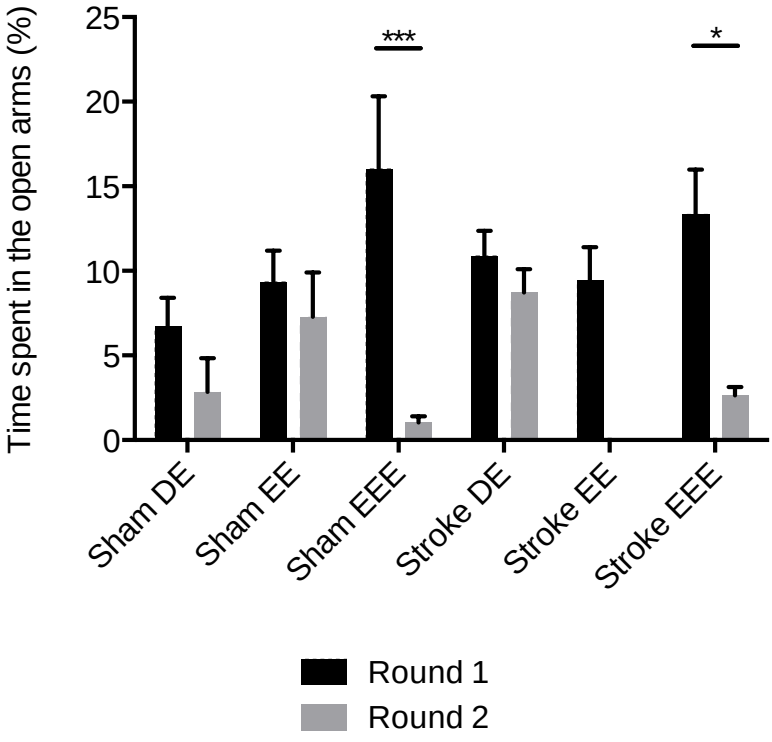
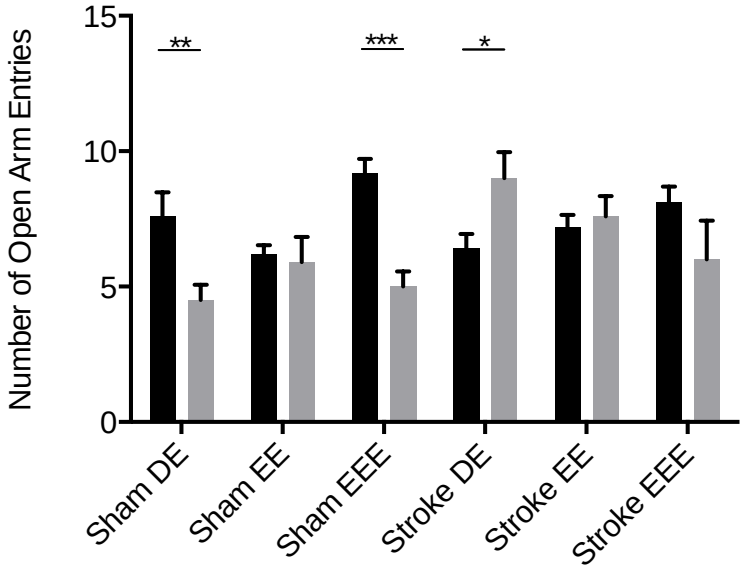


Figure A6. The percentage of time spent in the open arms of the elevated plus maze. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

A) Week 1



B) Week 4

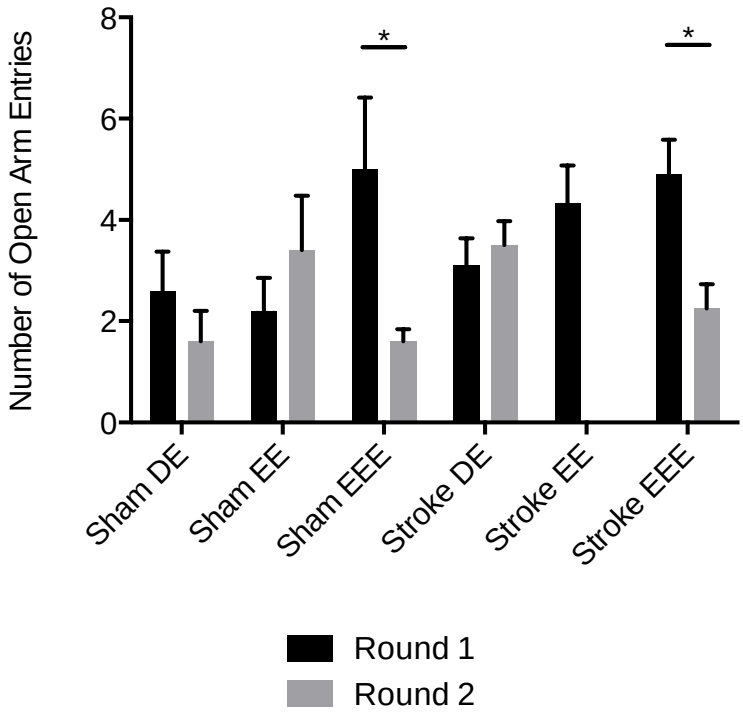
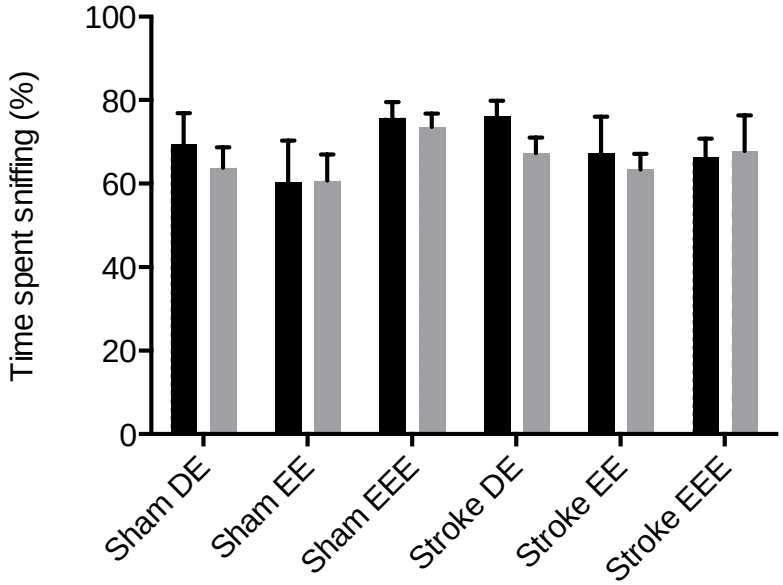


Figure A7. The number of entries made into the open arms of the elevated plus maze. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

A) Week 1



B) Week 4

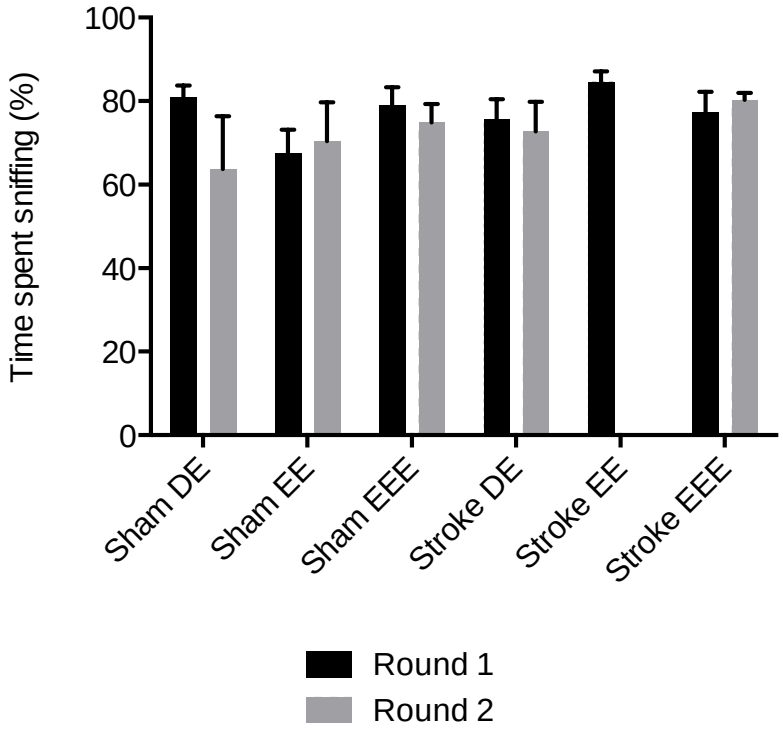
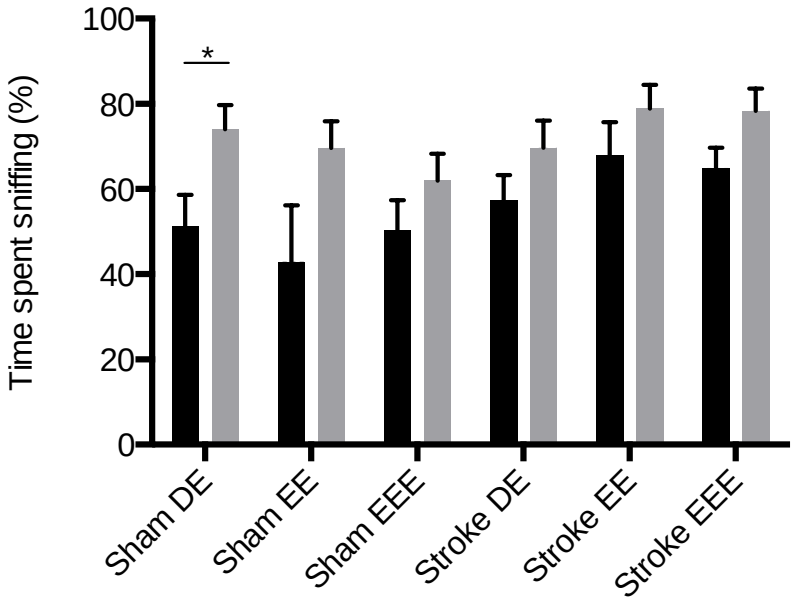


Figure A8. The percentage of time spent sniffing the novel object in the NOR test.

A) Week 1



B) Week 4

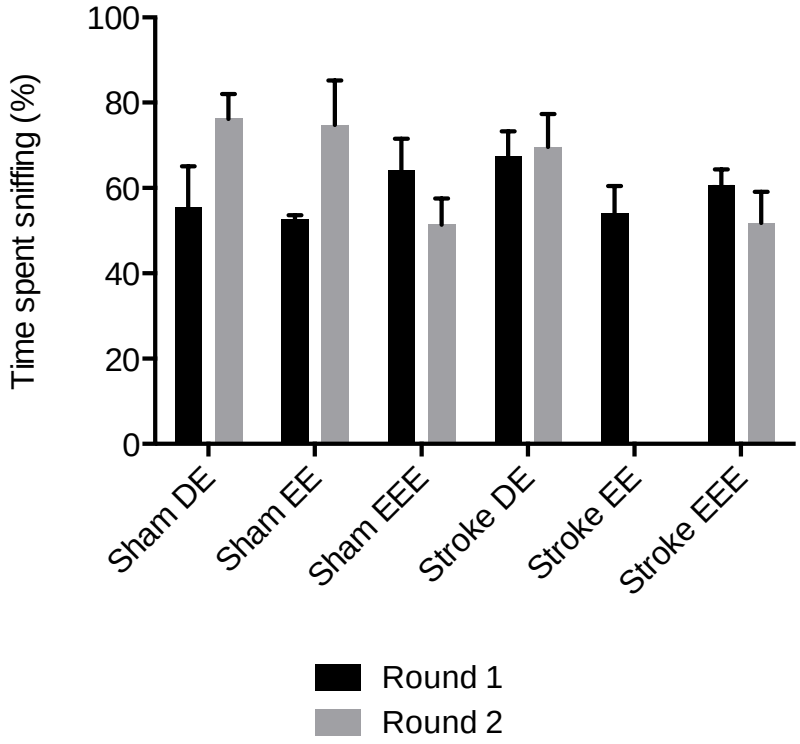


Figure A9. The percentage of time spent sniffing the moved object in the OLR test. * = $p<0.05$.

Appendix B. IBA1 Staining in Other Brain Regions

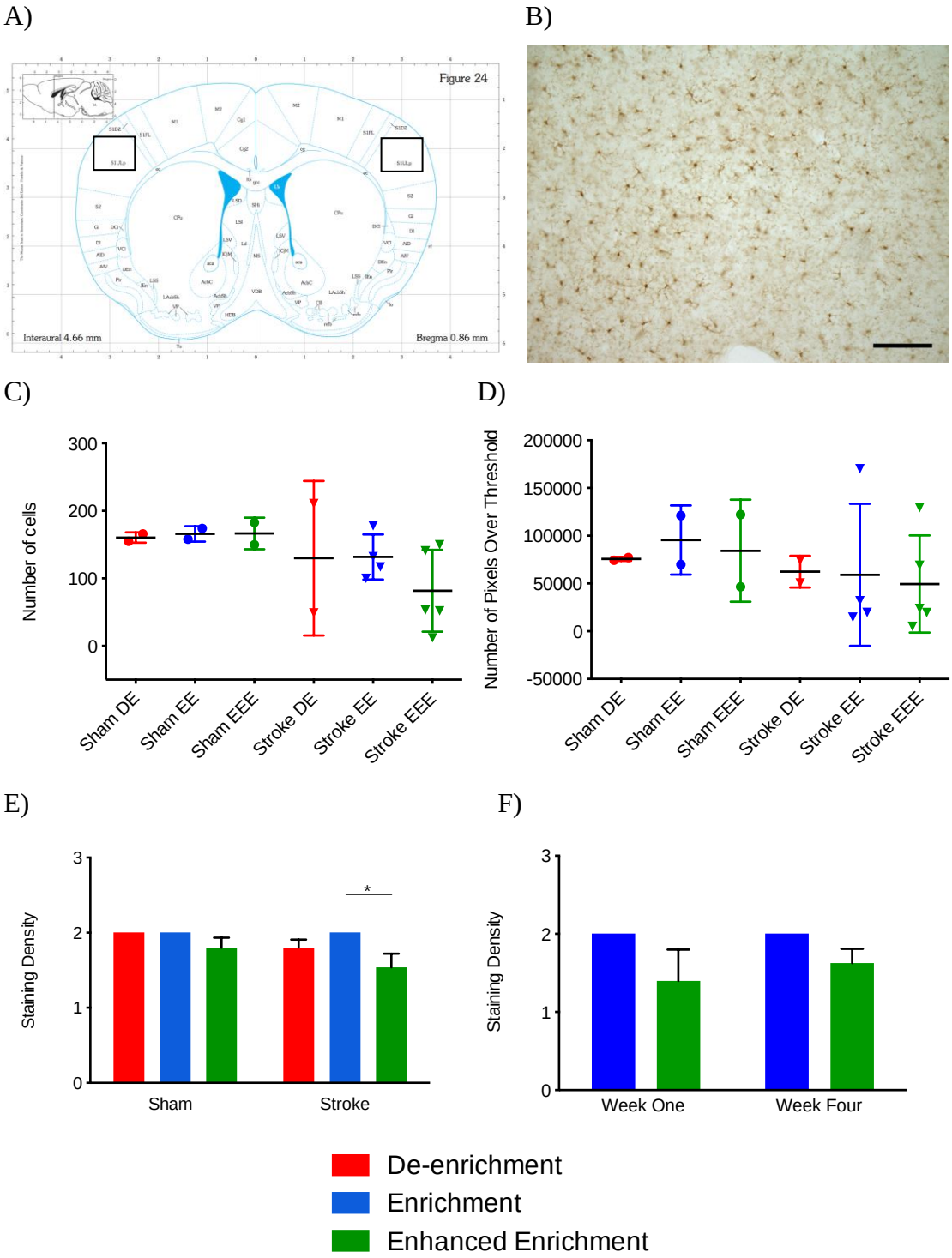


Figure A10 Analysis of IBA1 positive staining in the primary sensory cortex. A) The region of interest (Diagram from Franklin & Paxinos (2007)). B) A representative photograph of staining in the area. The scale bar indicates 100 µm. C) Cell count data. D) Thresholding data. E) Qualitative analysis. * = $p<0.05$.

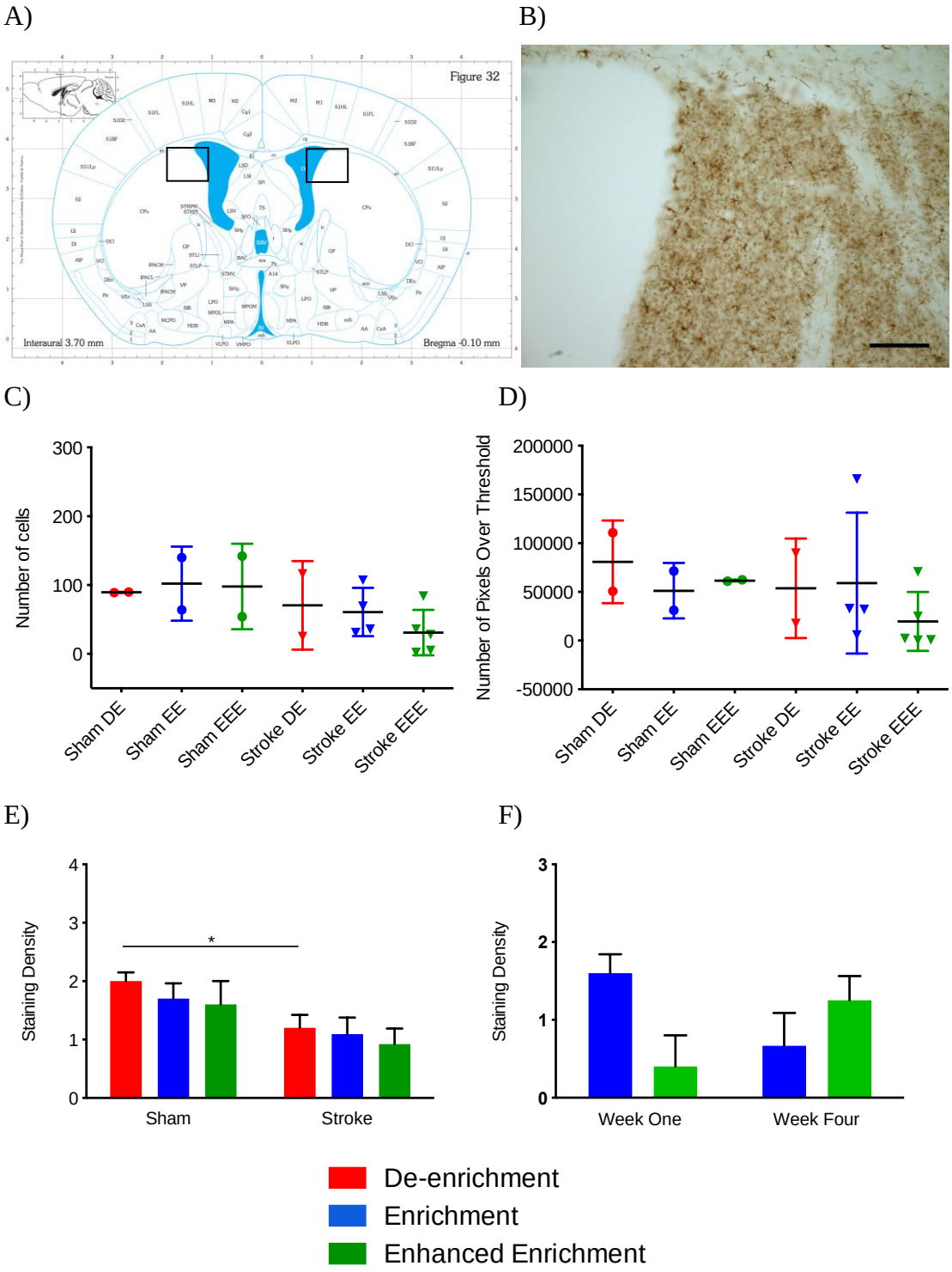


Figure A11. Analysis of IBA1 positive staining adjacent to the lateral ventricles. A) The region of interest (Diagram from Franklin & Paxinos (2007)). B) A representative photograph of staining in the area. The scale bar indicates 100 µm. C) Cell count data. D) Thresholding data. E) Qualitative analysis. * = $p<0.05$.

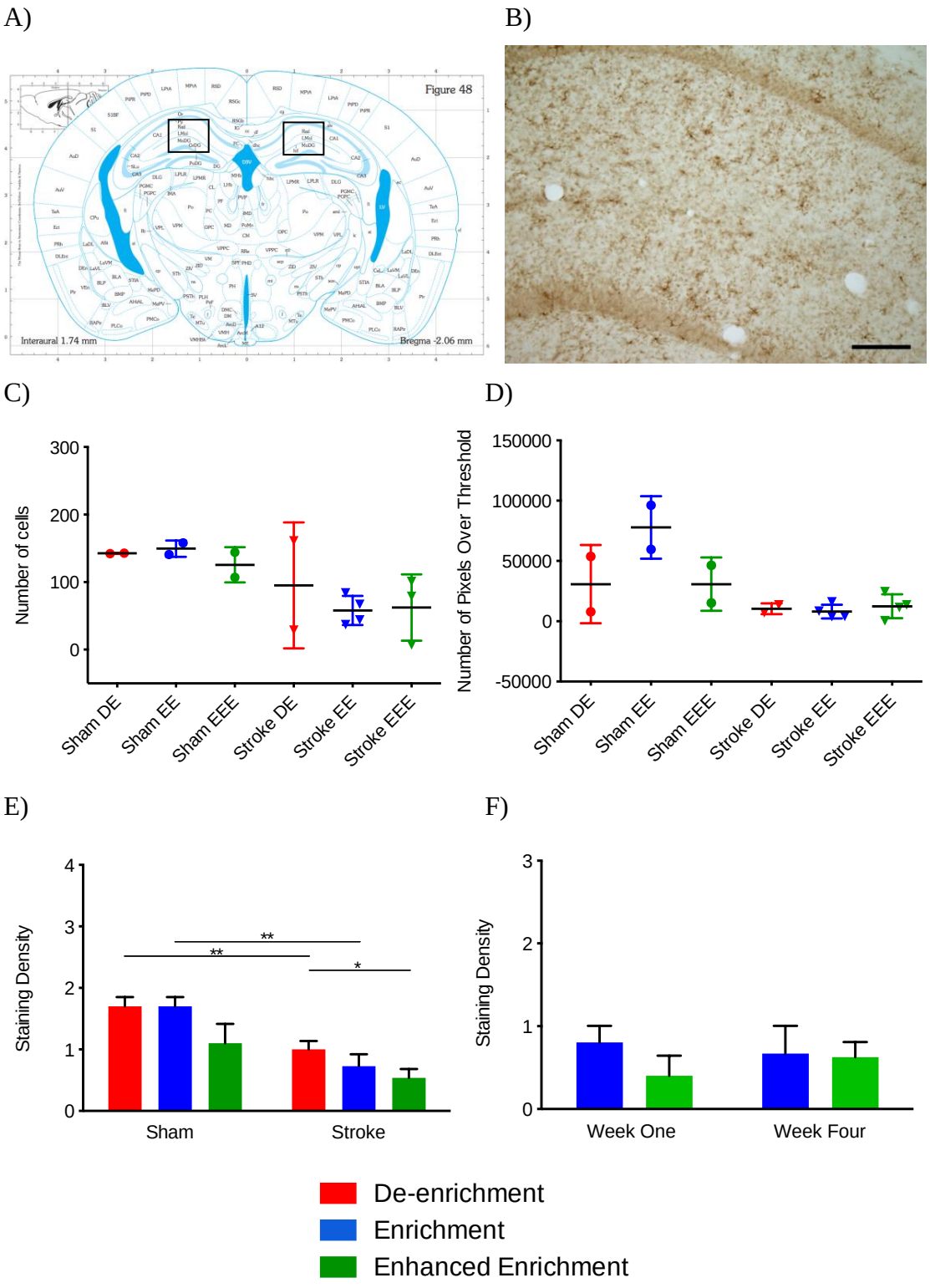


Figure A12. Analysis of IBA1 positive staining in the hippocampus. A) The region of interest (Diagram from Franklin & Paxinos (2007)). B) A representative photograph of staining in the area. The scale bar indicates 100 µm. C) Cell count data. D) Thresholding data. E) Qualitative analysis. * = $p<0.05$, ** = $p<0.01$.

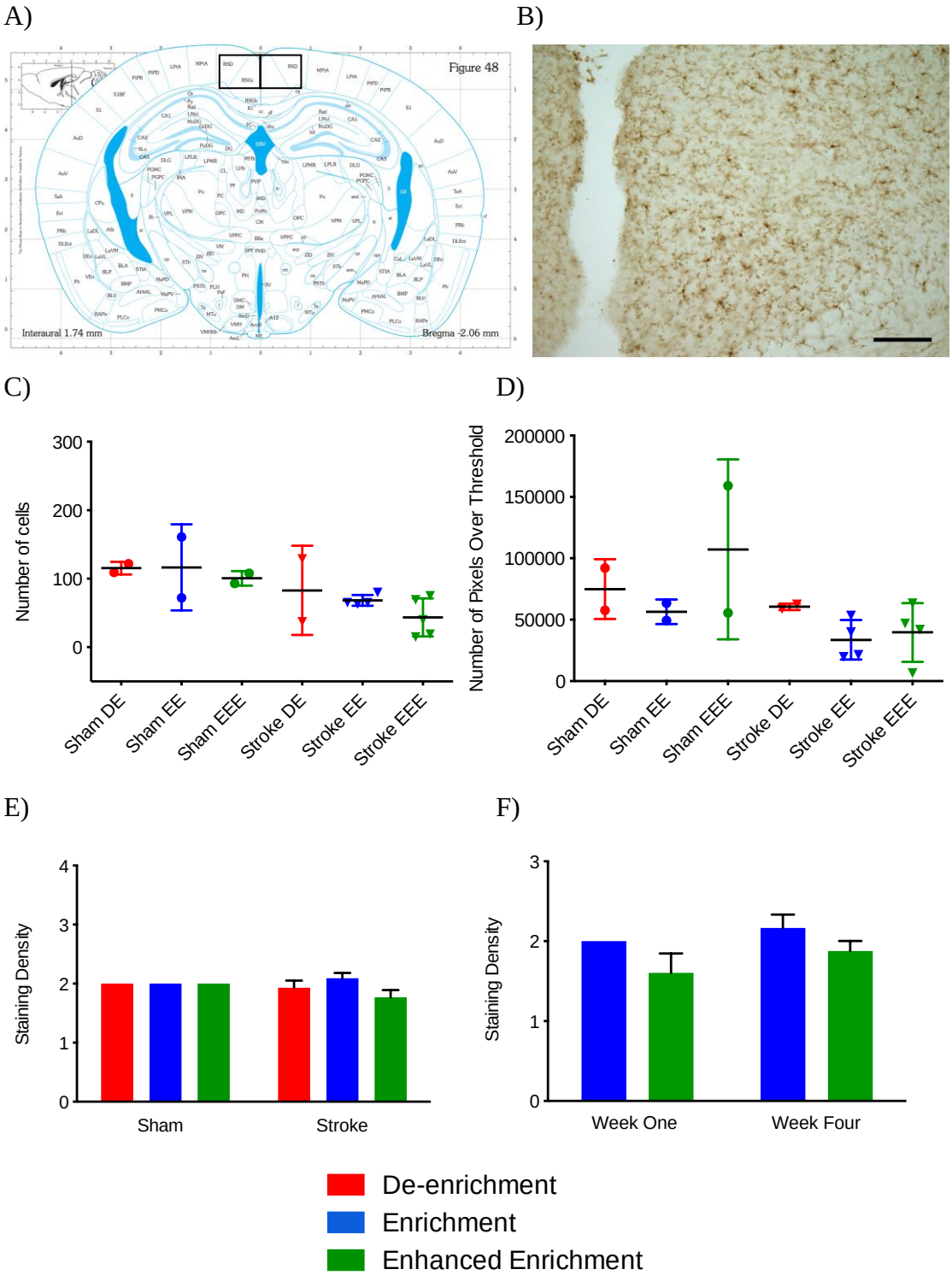


Figure A13. Analysis of IBA1 positive staining in the retrosplenial cortex. A) The region of interest (Diagram from Franklin & Paxinos (2007)). B) A representative photograph of staining in the area. The scale bar indicates 100 µm. C) Cell count data. D) Thresholding data. E) Qualitative analysis.

Appendix C. IBA1 Staining Supplementary Photographs

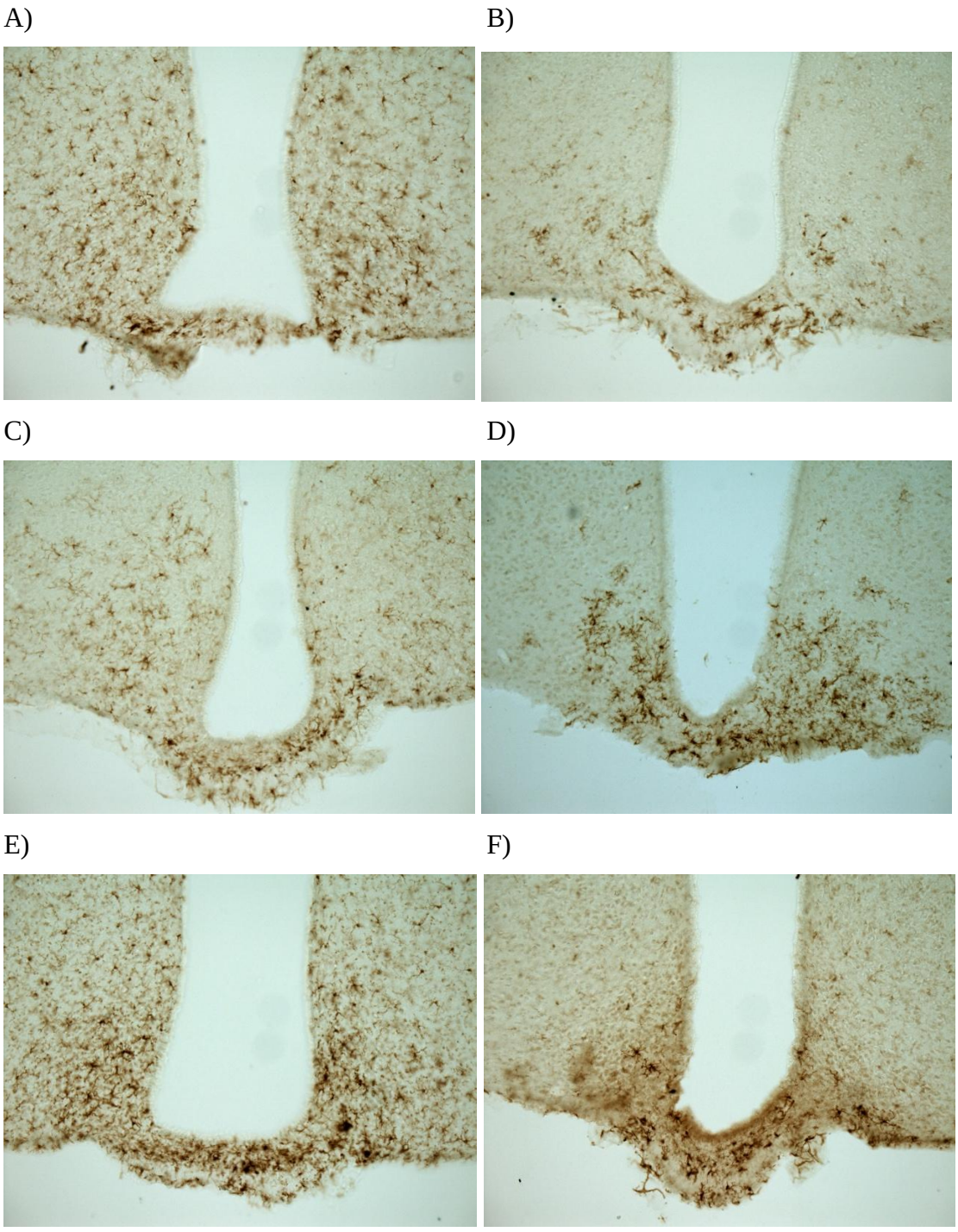


Figure A14. Representative staining from the v3V. A), C) and E) are from sham DE, sham EE and sham EEE animals, respectively. B), D) and F) are from stroke DE, stroke EE and stroke EEE animals, respectively.

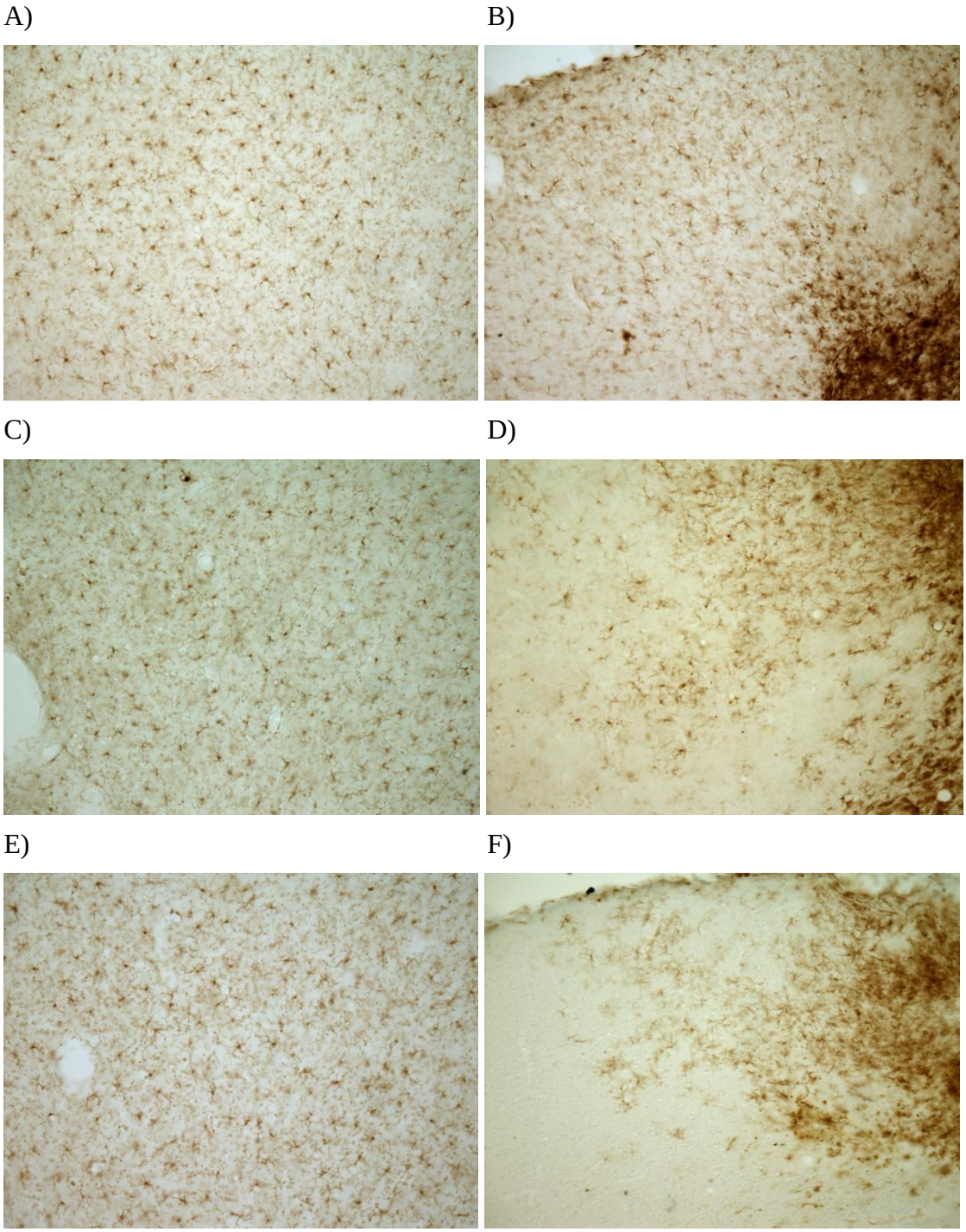


Figure A15. Representative staining from M2. A), C) and E) are from sham DE, sham EE and sham EEE animals, respectively. B), D) and F) are from stroke DE, stroke EE and stroke EEE animals, respectively.

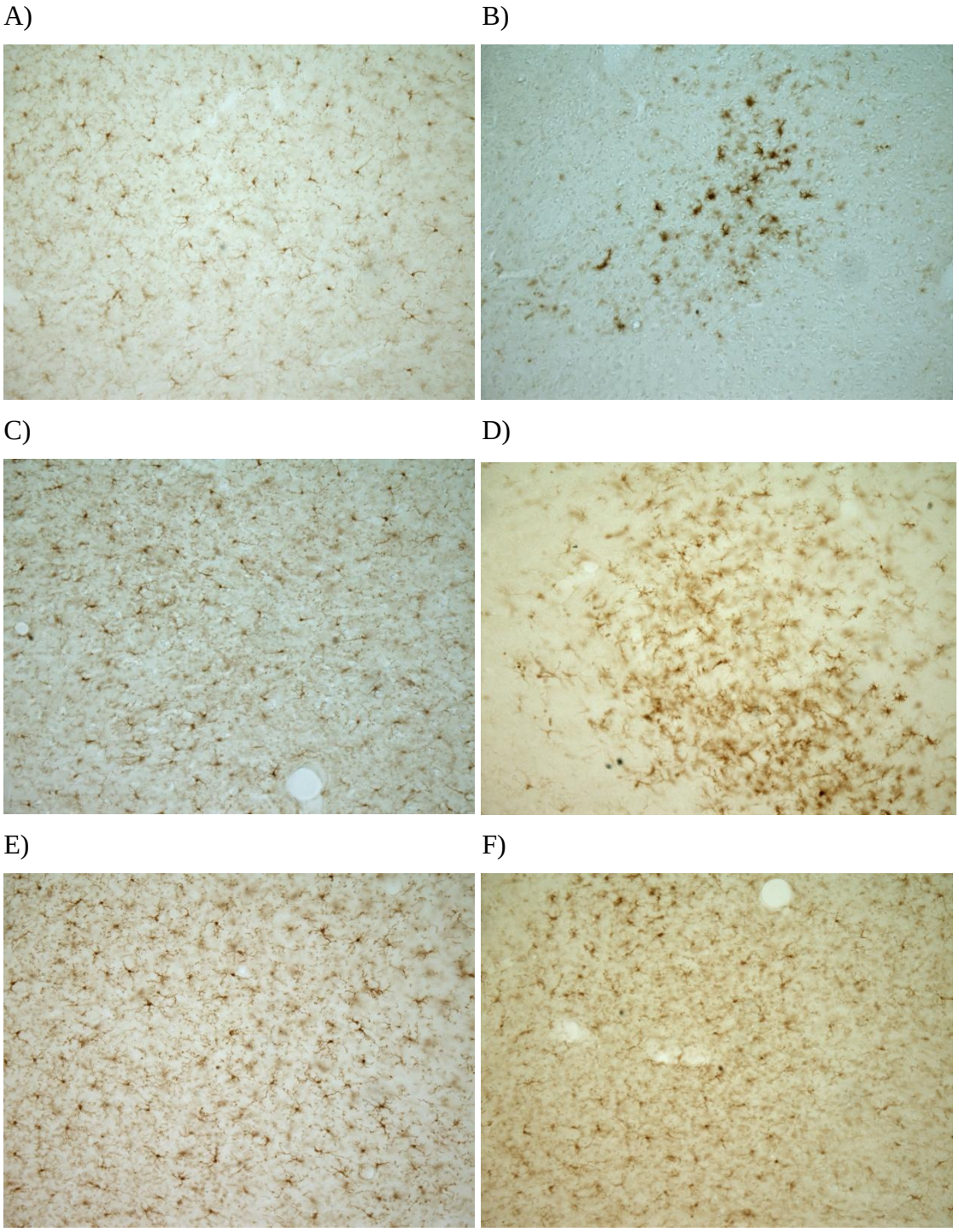


Figure A16. Representative staining from the thalamus. A), C) and E) are from sham DE, sham EE and sham EEE animals, respectively. B), D) and F) are from stroke DE, stroke EE and stroke EEE animals, respectively.

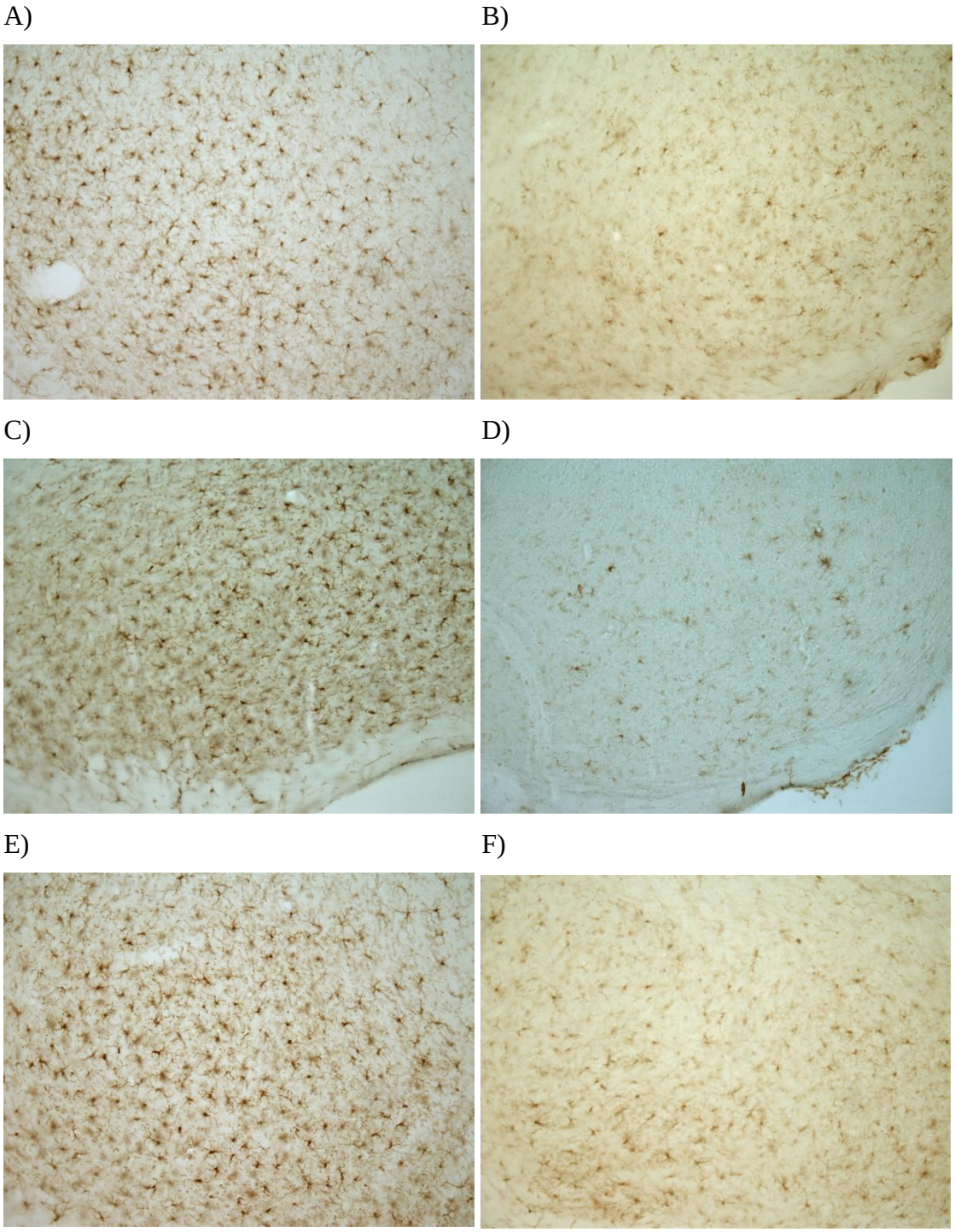


Figure A17. Representative staining from the SNr. A), C) and E) are from sham DE, sham EE and sham EEE animals, respectively. B), D) and F) are from stroke DE, stroke EE and stroke EEE animals, respectively.

Appendix D. High-speed burst analysis

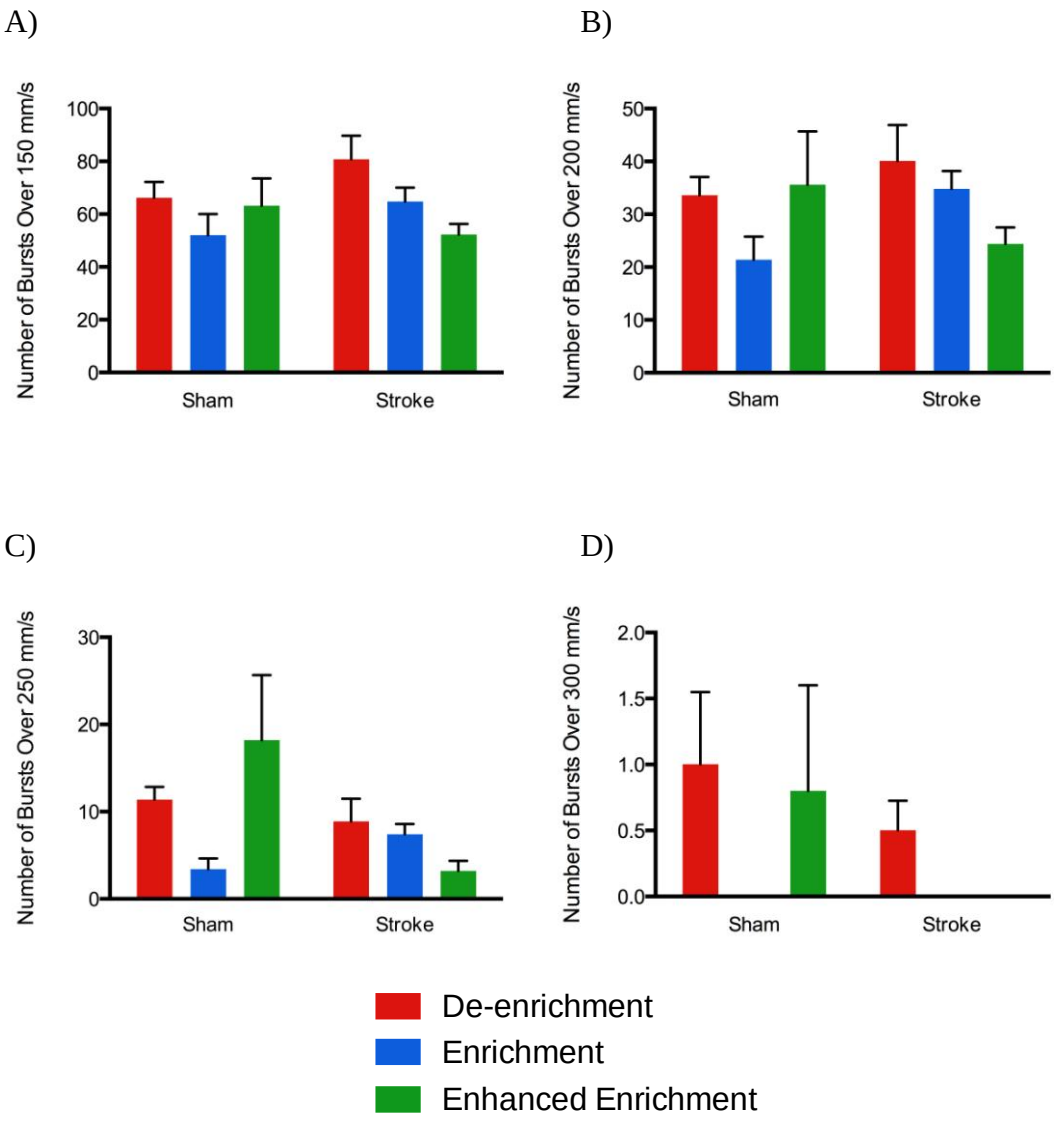


Figure A18. The number of high-speed bursts made over different speed thresholds in the open field. A) 150 mm/s. B) 200 mm/s. C) 250 mm/s. D) 300 mm/s. These data are from testing round one only, at week one post stroke, and are shown to give an indication of numbers and variation. A threshold of 200 mm/s was selected for further analysis as it showed larger differences between groups than 150 mm/s without potential floor effects of higher speeds.

Appendix E. TopScan Analysis

Table A1. Comparison of TopScan analysis with analysis by hand for the novel object task at Week 1. Animals were chosen pseudo-randomly.

	Time spent sniffing object 1	
Animal	TopScan	By hand
4.2 # 1	0.92	0.72
5.1 # 2	2.68	2.62
2.1 # 1	3.68	3.32
4.1 # 1	0.36	0.32
2.2 # 1	0.96	0.8
2.2 # 4	2.24	1.96

Table A2. Comparison of TopScan analysis with analysis by hand for the object location recognition task at Week 1. Animals were chosen pseudo-randomly.

	Time spent sniffing object 1	
Animal	TopScan	By hand
4.2 # 2	5.52	5.4
5.1 # 5	8.24	7.12
5.2 # 4	2.16	1.76
2.1 # 4	4.64	4.14
2.2 # 2	4.56	5.2
3.1 # 5	5.04	4.36

Appendix F. NOR and OLR Habituation

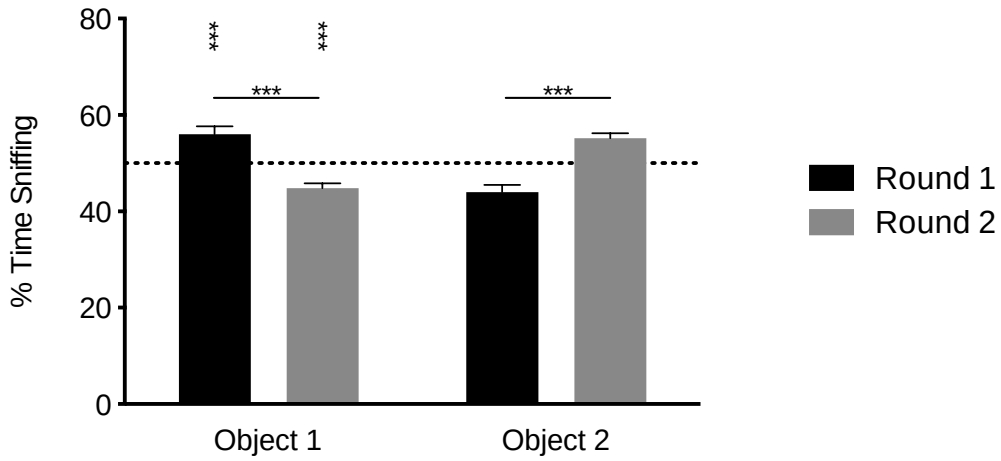


Figure A19. The time spent sniffing each object in the habituation phase. Data from both NOR and OLR has been combined to examine whether or not animals preferred either of the objects in the habituation phase. *** = $p < 0.001$; vertical asterisks indicate significantly different from Object 2.