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Changes in components of the brain extracellular matrix after experimental ischemic stroke

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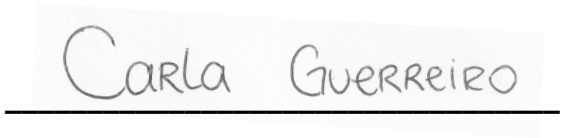
I, Carla Guerreiro, hereby declare to be the author of this original and unique work. Authors and references in use are properly cited in the text and are all listed in the reference section.



Carla Guerreiro

To my beloved family

Family is one of nature's masterpieces.

– George Santayana

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Abstract

Stroke is the 3rd cause of death in the world. During stroke, there is a disruption in the blood supply to the brain leading to rapid loss of brain function. Ischemic strokes are caused by obstruction of the blood supply, while hemorrhagic strokes results from rupture of a blood vessel. Eight-five percent of the strokes are ischemic. The only treatment recommended for acute ischemic stroke is the recombinant tissue activator of plasminogen but only a few percentages of patients are eligible for rtPA administration. Approximately 30% of the ischemic stroke victims die and 30% become severely disabled, resulting in among others deficits in motor function in the contralateral musculature.

Spontaneous recovery occurs during weeks to months following injury. There are many physiological and anatomical examples of cortical brain plasticity and one of the most potent modulators of cortical structure and function is behavioral experience. Functional recovery after stroke can be enhanced by physical training in stroke patients. In the animal settings, physical training can be accomplished by enriched environment (EE). EE refers to housing conditions, either home cages or exploratory chamber, that facilitate enhanced sensory, cognitive and motor stimulation relative to standard housing conditions.

The extracellular matrix (ECM) is important in the regulation of brain plasticity but is also a potential hampering factor for recovery after stroke. It is known that EE affects chondroitin sulfate proteoglycans (CSPGs) present in ECM, leading to functional recovery. Matrix metalloproteinases (MMPs) are able to cleave ECM components. There are some evidences that beta-dystroglycan (β-DG) is a MMP-9 target. After the degradation of β-DG, there is a 30 kDa product.

The aim of this work is to explore how EE affects β-DG and gelatinases over 1 week of recovery after experimental stroke, performed as photothrombosis (PT). We show that EE does not affect the infarct size and improves tactile/proprioceptive response to limb stimulation. We found that β-DG is mostly present in vessels across the brain cortex and animals housed in an EE had a higher degradation than STD animals when comparing to sham non-operated animals.

β-DG can be related with changes in the ECM that leads to brain plasticity, promoting functional recovery after experimental stroke, possibly due to MMPs enzymatic activity.

Keywords: ischemic stroke, infarct, photothrombosis, enriched environment, extracellular matrix, matrix metalloproteinases, beta-dystroglycan

Resumo

O Acidente Vascular Cerebral (AVC) consta como a terceira causa de morte no Mundo e a primeira em Portugal. Num AVC, uma disrupção no fluxo sanguíneo no cérebro leva a uma rápida perda da função cerebral. Os AVCs podem ser qualificados em isquémicos ou hemorrágicos. Os AVCs isquémicos são causados por uma obstrução no fluxo sanguíneo, enquanto que os AVCs hemorrágicos são causados por uma ruptura do vaso sanguíneo. Cerca de 85% dos AVCs são isquémicos. O único tratamento recomendado para a fase aguda do AVC isquémico é o ativador plasminogénio tecidual recombinante (rtPA) mas apenas uma pequena percentagem de pacientes é elegível para a administração de rtPA. Sendo que maioria dos pacientes não chegam nas primeiras 4,5 horas do AVC, após esse intervalo de tempo para o tratamento não há nenhum tratamento alternativo. Existem vários fatores de risco para o AVC, inclusive hipertensão arterial e aterosclerose que normalmente advém de obesidade. Ainda a base genética é conhecida como um importante fator de risco, como por exemplo, polimorfismos e mutações em genes que regulam a tensão arterial e a fibrilação nos vasos.

As vítimas do AVC isquémico podem morrer ou ficar severamente inválidos. O AVC leva a deficits na função motora da musculatura contralateral à lesão cerebral. Porém, recuperação espontânea pode ocorrer nas semanas a meses após a lesão. Há vários exemplos fisiológicos e anatómicos de plasticidade no córtex cerebral e um dos maiores moduladores da estrutura e função cortical é a experiência comportamental de cada paciente.

Assim, a recuperação funcional após AVC pode ser aumentada através de um treino físico em pacientes com AVC. Em experimentos animais, o treino físico é chamado de "enriquecimento ambiental" (*enriched environment*: EE). EE refere-se a condições habitacionais, quer em gaiolas que em câmara exploratórias, que facilitam o melhoramento sensorial, cognitivo e estimulação motora relativamente a condições habitacionais standard.

A matriz extracelular é importante na regulação da neuroplasticidade mas também é um factor que poderá potencialmente impedir a recuperação após o AVC. Visto que a matriz extracelular a estabilizar EE afecta proteoglicanos de sulfatos de condroitina (CSPGs) que se encontram na ECM. A diferença encontrada nestes proteoglicanos ajuda na recuperação funcional cerebral. Apesar de não se saber que proteína poderá ajudar nesta situação, sabe-se que metaloproteinases da matriz são enzimas que têm a capacidade de clivar componentes da matriz extracelular. Existem evidências que beta-distroglicano, que faz

parte do complexo distroglicano, é um substrato alvo de MMP-9. Após a degradação de β-DG, obtém-se um produto de 30 kDa.

O objetivo desta tese tem como observar se EE afecta β-DG durante uma semana de recuperação após AVC experimental, neste caso, foi usado uma técnica chamada fototrombose.

Neste estudo, mostramos que EE não afecta o tamanho do enfarte e que melhor a resposta táctil/propriocetiva após estimulação dos membros afetados. Vimos ainda que β-DG está maioritariamente presente em vasos sanguíneos no córtex cerebral. Animais em gaiolas EE tiveram uma maior degradação de beta-distroglicano do que animais STD quando comparando com animais sham não operados. Esta degradação pode dever-se a diferenças na actividade enzimática das gelatinases MMP-2 e MMP-9.

O beta-distroglicano pode estar relacionado com alterações na matriz extracelular que levam à plasticidade sináptica cerebral, que ajuda na recuperação funcional após AVC experimental.

Palavras-chave: AVC isquémico, enfarte, fototrombose, enriquecimento ambiental, matriz extracelular, metaloproteinases da matriz, beta-distroglicano

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List of abbreviations

ANOVA	analysis of variance
AU	arbitrary unit
AVC	acidente vascular cerebral
BBB	blood brain barrier
cc	corpus callosum
Cg	cingulate cortex
CNS	central nervous system
CSPG	chondroitin sulfate proteoglycan
DG	dystroglycan
ECM	extracellular matrix
EE	enriched environment
GABA	gamma-aminobutyric acid
MCAO	middle cerebral artery occlusion
MMP	matrix metalloproteinase
OGD	oxygen-glucose deprivation
PBS	phosphate buffered saline
PFA	paraformaldehyde
PNN	perineuronal net
PP	paw placement
PSD-95	postsynaptic density protein 95
PT	photothrombosis
PV	parvalbumin
RT	room temperature
SD	standard deviation
SS	somatosensory cortex
STD	standard
Syn	synaptophysin Ab 4
TBI	traumatic brain injury
β-DG	beta-dystroglycan

Chapter 1
Introduction

1.1 Ischemic stroke

Worldwide, stroke is amongst the leading causes of death and serious disability (Go et al., 2014), being the third cause in the Western world (Lo et al., 2003) (Figure 1). In Portugal, nearly 20.000 cases are reported each year (World Health Organization, 2014).

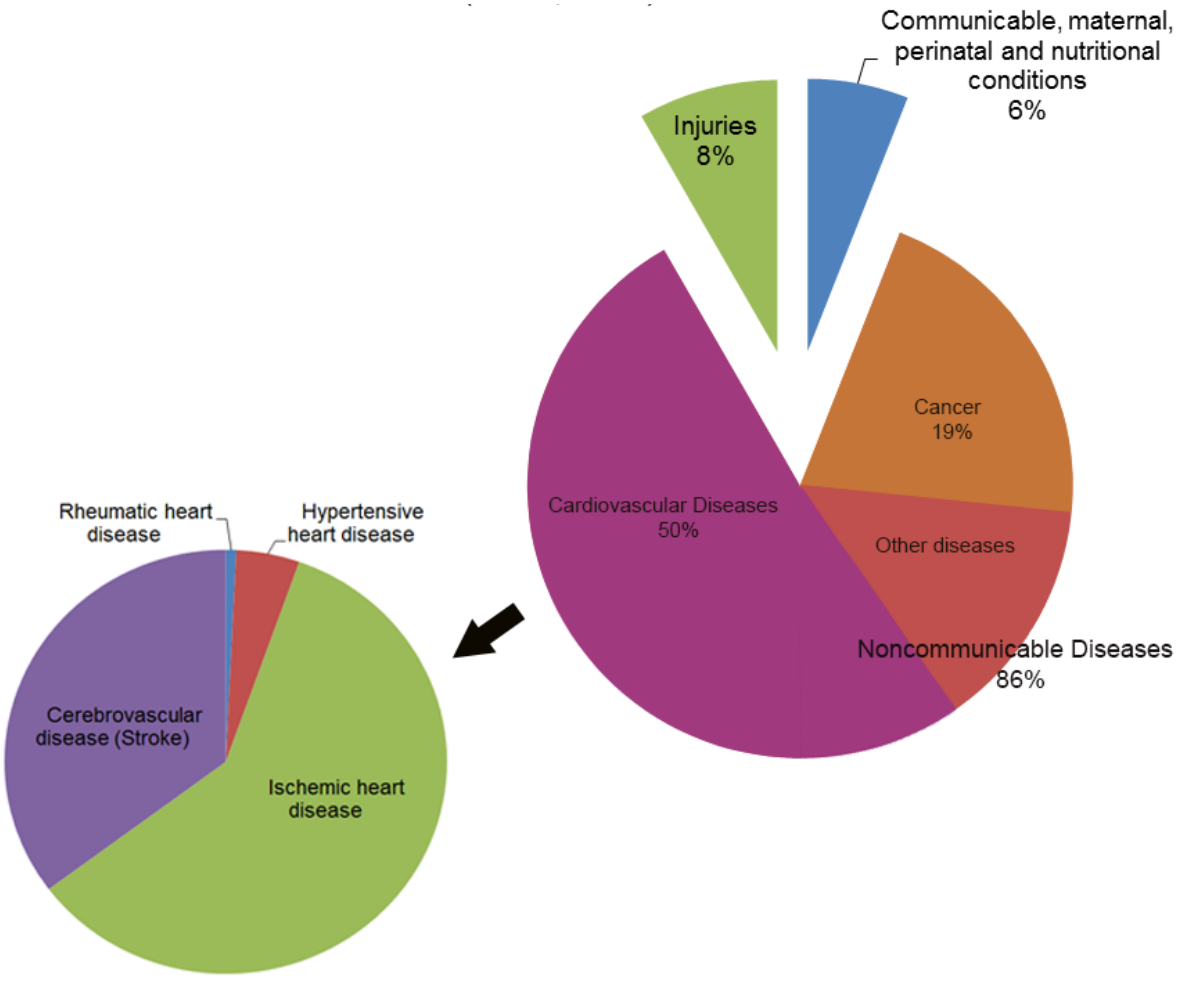

Figure 1. Estimated percentage of causes of death in Europe in 2004 (WHO). In 2004, 50% of deaths occurred due to cardiovascular diseases, among them Cerebrovascular Disease, such as Stroke (cause of 14% of all deaths). Stroke is the third cause of death in Europe, after Ischemic Heart Disease (24%) and Cancer (19%). Data analyzed from World Health Organization, 2004.

During stroke, there is a disruption in the blood supply to the brain leading to rapid loss of brain function (Chaturvedi and Kaczmarek, 2013; Lo et al., 2003).

Strokes are classified as ischemic or hemorrhagic (Figure 2). Ischemic strokes are caused by obstruction of the blood supply, while hemorrhagic strokes results from rupture of a blood vessel. About 85–90% of the strokes are ischemic (Chaturvedi and Kaczmarek, 2013; Sicard and Fisher, 2009) and the treatment is still unsatisfying. Until today, the only treatment recommended for acute ischemic stroke is the recombinant tissue activator of plasminogen (rtPA), which aims to breakdown the blood clots. Nevertheless, only a few percentages of patients are eligible for rtPA administration (Chaturvedi and Kaczmarek, 2013; Kurzepa et al., 2014).

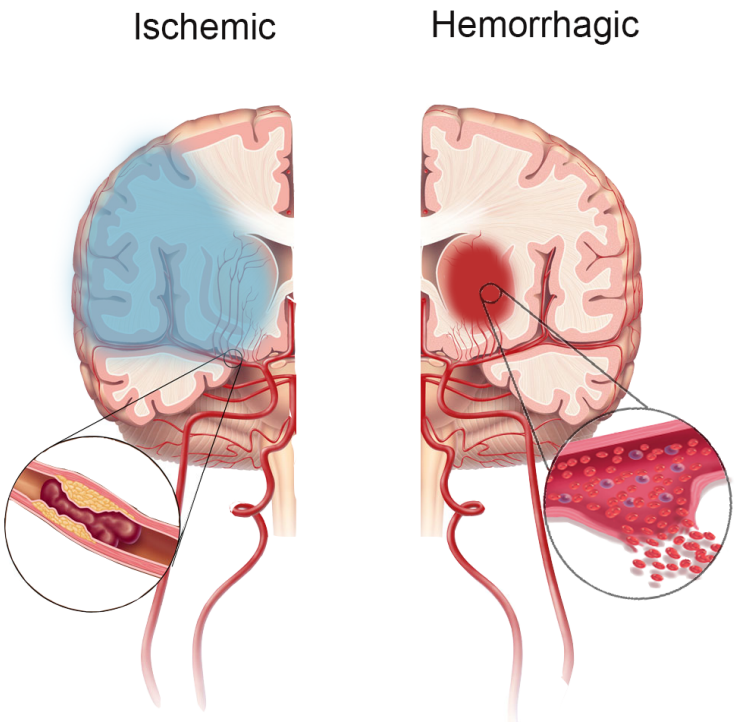

Figure 2. Illustration of stroke pathology. Left: Representation of an ischemic stroke caused by a blood clot obstruction. **Right**: Representation of a hemorrhagic stroke caused by a rupture of a blood vessel. (Adapted from http://www.strokecenter.org/patients/about-stroke/ischemic-stroke/ and http://www.compelvisuals.com/compel_blog/basic-anatomy-of-the-brain-injuries-and-pathologies-an-illustrated-review/).

Many factors can increase the risk of stroke. Elevated arterial blood pressure is a notable risk factor that normally is related to obesity (Zhao et al., 2006). Angiotensin-converting enzyme (ACE) inhibitors and diuretics lower the risk of stroke, in addition to other antihypertensive therapies (Lo et al., 2003). Another important risk is atherosclerosis. Treatment with statins reduces stroke risk (Lo et al., 2003). Genetic background is also acknowledged as an important stroke risk factor, wherein several genes can interact with the environment and affecting the risk of an individual. For instance, polymorphisms in genes such as ACE, endothelial nitric oxide synthase (eNOS), Apolipoprotein E (APOE) and beta-fibrinogen (FGB) apparently increase the risk of stroke (Lo et al., 2003).

Brain ischemia is the condition or state wherein the brain is subjected to a lack of nutrients and to hypoxia or low oxygen because of an obstruction of the arterial blood supply or inadequate blood flow; and it can be divided into two classifications:

- Global ischemia: the blood flow to the entire brain is transiently blocked, leading to a selective neuronal death and arises commonly as a consequence of cardiac arrest, open-heart surgery or carbon monoxide poisoning (Mohr et al., 2011)
- Focal ischemia: also known as cerebral infarction, in which a specific area of the brain tissue undergoes injury; most strokes are caused by clots that form at the site of occlusion in a cerebral artery or move there from the heart (Mohr et al., 2011).

In focal ischemia, the victims may die or become severely and permanently disabled, leading to paralysis, abnormalities in motor strength, coordination, sensory function and neurologic deficits including impaired cognition, visual disturbance and loss of sensation (Mohr et al., 2011). The ischemic tissue includes the *infarct* or the core of the stroke, which contains cells that receive essentially no blood and rapidly die, and the *penumbra* or surrounding region, which contains cells that receive some blood from other arteries and may be salvaged (Figure 3). Cells in the infarct die from several causes and probably cannot be salvaged by any treatment short of immediate clot removal (Mohr et al., 2011) and also neurological functions related to this area is lost (Wieloch and Nikolich, 2006). During the next months following a stroke certain neurological functions are recovered specially during the first 30 days but also for the next 6 months (Duncan et al., 2000). This functional recovery depends on the degree of tissue loss and engagement of neuronal networks that are involved in the lost brain functions (Wieloch and Nikolich, 2006).

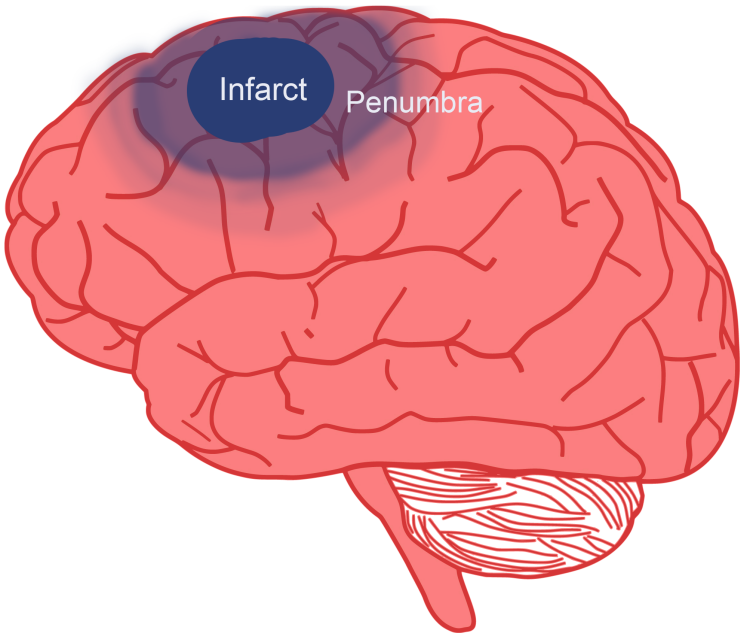

Figure 3. Affected areas on focal ischemic stroke. The infarct (core of the stroke) is the most affected area and receives essentially no blood supply, leading to neuronal death. The surrounding area, called penumbra, receives some blood flow, being less affected.
(Adapted from http://www.clipartbest.com/clipart-di8xnor5T).

1.2 Recovery after brain injury

After injury in the cerebral cortex, as often occurs in stroke or traumatic brain injury (TBI), the sensorimotor area located in forebrain may be affected, resulting in deficits in motor function in the contralateral musculature. However, spontaneous functional recovery occurs in the weeks to months following injury (Nudo, 2013). It has been shown many examples of cortical plasticity and one of the most effective modulators of cortical structure and function is behavioral practice (Karni et al., 1998; Kleim et al., 1998; Nudo and Milliken, 1996; Nudo, 2013).

To help understand plasticity in adult brains, developmental neuroscience can be of help. During brain development, guidance clues for axonal sprouting are activity-dependent. In a later phase, cortical activity guides axonal sprouting within the cerebral cortex, leading to a connectivity patterns (Nudo, 2013).

If there is a brain injury, an environment for axonal sprouting can be re-initiated, although it

was once thought that it was non-existent in adult animals (Carmichael and Chesselet, 2002).

Anatomical alterations occur in adult animals as a consequence of behavioral training. Dendritic and synaptic morphology of motor cortex neurons are altered by specific motor learning tasks (Kleim et al., 2002). For instance, after 1 hour of limb task performance, dendritic spine formation occurs on pyramidal neurons (primary excitatory neurons) in the motor cortex contralateral to the limb (Nudo, 2013). Structural changes are highly specific to the neurons relevant for the trained task (Wang et al., 2011).

The adult CNS holds an impressive capacity to recover and adapt following injury, such as spinal cord injury, TBI and stroke, called spontaneous functional recovery (Nudo, 2013). Much of the recovery that occurs after injury may be due to behavioral compensation. For instance, in a rat model of TBI, injured in the primary motor cortex, the forelimb area was affected. In the absence of rehabilitative training, leading to spontaneous recovery, behavioral performance on a pellet-reaching task improved over time, but at 5 weeks post-injury, rats still have significant motor deficits (Nishibe et al., 2010).

For development of recovery there is a process of both reinstatement and relearning of lost functions. Hence, it is thought that the neural plasticity that support learning in the intact cortex also mediate motor relearning and adaptation in the injured brain (Nudo, 2013).

Data also has been shown that contiguous intact regions of the injured cortex might be involved in recovery after injury. For instance, motor cortex infarcts resulted in the somatosensory area to emerge in the undamaged motor cortex near the lesion, adding believability to the notion that uninjured regions help in this recovery (Nudo, 2013).

Studies in human surviving stroke patients suggest that the peri-infarct cortex, still intact, may play a role in neurological recovery (Jaillard et al., 2005; Teasell et al., 2005). Fourteen to sixty days after stroke, synaptophysin staining is elevated, showing increased synaptogenesis. Local sprouting occurs in the peri-infarct area (Carmichael, 2006). New capillaries are also formed in the ischemic border (Wei et al., 2001). And after several weeks of rehabilitation, motor representations in the injured hemisphere are enlarged relative to the initial post-injury map (Carey et al., 2002).

Some data show that there is dendritic damage in the ischemic core after photothrombotic stroke model, but this damage was limited to 300 μm around the ischemic border. Beyond this area, dendrites were intact. Hence, at least in this model of experimental stroke, a substantial substrate for structural and functional plasticity exists in the peri-infarct cortex (Nudo, 2013). In the same model, there is also an evidence of functional reorganization such as axonal sprouting, suggesting functional plasticity in this area (Clarkson et al., 2013).

In addition to the peri-infarct cortex, there is functional recovery in remote regions after focal injury. Injury of the motor cortex results in a disruption of sensorimotor networks, leading to a loss of fine motor control. It is possible to observe upregulation of a glutamate receptor (NMDA - N-methyl-D-aspartate receptor), in an excitatory level, and downregulation of $GABA_A$ receptors, inhibitory level, either in the ipsilesional and contralesional hemisphere (Redecker et al., 2000).

Since the development of compensatory behaviors and commitment of undamaged primary motor cortex are thought to contribute to functional recovery, it can be that intact motor areas outside of primary motor cortex may also contribute to recover. Hence, after an injury in the primary motor cortex, the remaining intact motor areas provide some role in functional recovery, for instance via intracortical connectivity with other cortical regions and/or their direct corticospinal projection pathways (Nudo, 2013).

Some studies show that somatosensory area near the primary motor cortex injured is also affected. Basically, the motor cortex cannot be considered solely as a motor structure. There are deficits resulting from sensorimotor disconnection. After primary motor cortex injury, there is a reduction of somatosensory input to motor areas, interfering with motor behavior (Nudo, 2013).

Plasticity during recovery after brain injury is not only evident in the injured area, but also its surrounding areas are involved in this process, such as peri-infarct, somatosensory cortex (SS) and also the contralateral hemisphere.

1.3 Recovery after stroke

Stroke causes significant tissue damage, disruption of internal intricate circuits of the brain and its external neuronal connections that are involved in cognitive, sensory and motor functions (Nygren and Wieloch, 2005; Wieloch and Nikolich, 2006). During and immediately after stroke, neurological functions associated to the infarcted area are lost. During the subsequent months certain neurological functions recover but most patients have several disabilities. Recovery is most prominent during the first 30 days but continues for at least 6 months and is dependent on the degree of tissue loss (Wieloch and Nikolich, 2006).

Recovery of function involves three distinct phases: first, reversal of diaschisis and activation of cell repair, second, functional cell plasticity, that means changing the properties of existing neuronal pathways, and third, neuroanatomical plasticity leading to the formation of new connections. This sequence of events of brain plasticity is considered to be the basis for

spontaneous functional recovery after stroke (Wieloch and Nikolich, 2006).

By this mechanism, stroke reorganizes cognitive maps in cortex in patterns that correlate with functional recovery (Carmichael, 2006). First, there is an induction of a bilateral cortical activation to sensory or motor stimulation of the affected limb. Later on during recovery, this bilateral pattern becomes more restricted, to a reorganized and expanded sensorimotor activation in the ipsilateral cortex of the stroke and an increase in activity in the connected regions of another motor and premotor areas of the contralateral side (Calautti et al., 2001; Carmichael, 2006; Tombari et al., 2004).

It has been shown that this neurological functional recovery after stroke can be enhanced by external interventions, such as physical training in stroke patients.

The American Heart Association/American Stroke Association states that physical training is "a subset of physical activity that is planned, structured and repetitive and has as a final objective the improvement or maintenance of physical fitness", showing evidences that this training after stroke leads to a improvement of the cardiovascular fitness, walking ability and upper-extremity muscle strength (Billinger et al., 2014). The view of the world stroke researchers/professionals about physical training commencing early after stroke is consensual; however how early remains controversial (Billinger et al., 2014; Brethour et al., 2012). Recent clinical trials have tested the beginning of the physical training within 24 to 72 hours of stroke onset, yet with inconclusive results (Billinger et al., 2014).

The exercises included in the physical training for stroke survivors are aerobic – including large-muscle activities, muscular strength, flexibility by stretching and neuromuscular exercises – such as balance activities (Billinger et al., 2014).

In the animal settings, physical training can be accomplished by enriched environment (EE).

1.3.1 Enriched environment

Enriched environment refers to housing conditions, either home cages or exploratory chambers, that facilitate enhanced sensory, cognitive and motor stimulation relative to standard housing conditions, having larger cages with toys and tools (as tunnels, nesting material and food location changed often) or by training them 2-5 days after stroke (Figure 4). Enrichment can also include increased social stimulation through housing the animals with several other individuals together (Nithianantharajah and Hannan, 2006; van Praag et al., 2000; Wieloch and Nikolich, 2006).

The standard condition (STD) of an EE is "a combination of complex inanimate and social stimulation" which means that no single variable can account for the consequences of

enrichment (van Praag et al., 2000). For example: alone neither an EE with active participation nor EE with social interaction can lead to the effects of EEs (Rosenzweig et al., 1978).

Housing injured animals in an EE, 2-5 days after stroke, is an efficient way to stimulate functional recovery (Johansson, 2004). The effect of EE is not due to a decrease in infarct volume, since the size of the infarct area is the same in animals housed in different conditions (Johansson, 2004; Nygren and Wieloch, 2005). The effect of EE on functional recovery declines with time, and is not effective if started 30 days after stroke (Dahlqvist et al., 2003). Also, if initiated earlier than 2 days post-stroke it might be detrimental (Dahlqvist et al., 2003).

Enriched environment induces biological effects in the brain that could account for the positive effect in recovery (Wieloch and Nikolich, 2006) and activates brain plasticity mechanisms such as increased spine density, dendritic arborization, cell genesis, gliogenesis and angiogenesis (Johansson, 2004; Nithianantharajah and Hannan, 2006; Nygren and Wieloch, 2005; Wieloch and Nikolich, 2006). In addition, there are changes on the contralateral pyramidal neurons, showing that the contralateral hemisphere is involved in the recovery process (Johansson, 2004). Animals housed under EE showed enhanced astrocytogenesis in the ipsilateral peri-infarct area (Wieloch and Nikolich, 2006) and an increase in the number of neuronal stem cells and precursor cells in the subventricular zone and striatum but not the number of mature neurons (Komitova et al., 2005; Matsumori et al., 2006). However, some data suggest that the recovery-enhancing effect on sensorimotor function by an EE is not due to generation of new neurons to the infarct or remote cortical areas (Wieloch and Nikolich, 2006).

The extracellular matrix (ECM) is important in the regulation of brain plasticity (Frischknecht and Gundelfinger, 2012) but is also a potential hampering factor for recovery after stroke (Lo et al., 2003; Rosell and Lo, 2008; Soleman et al., 2013).

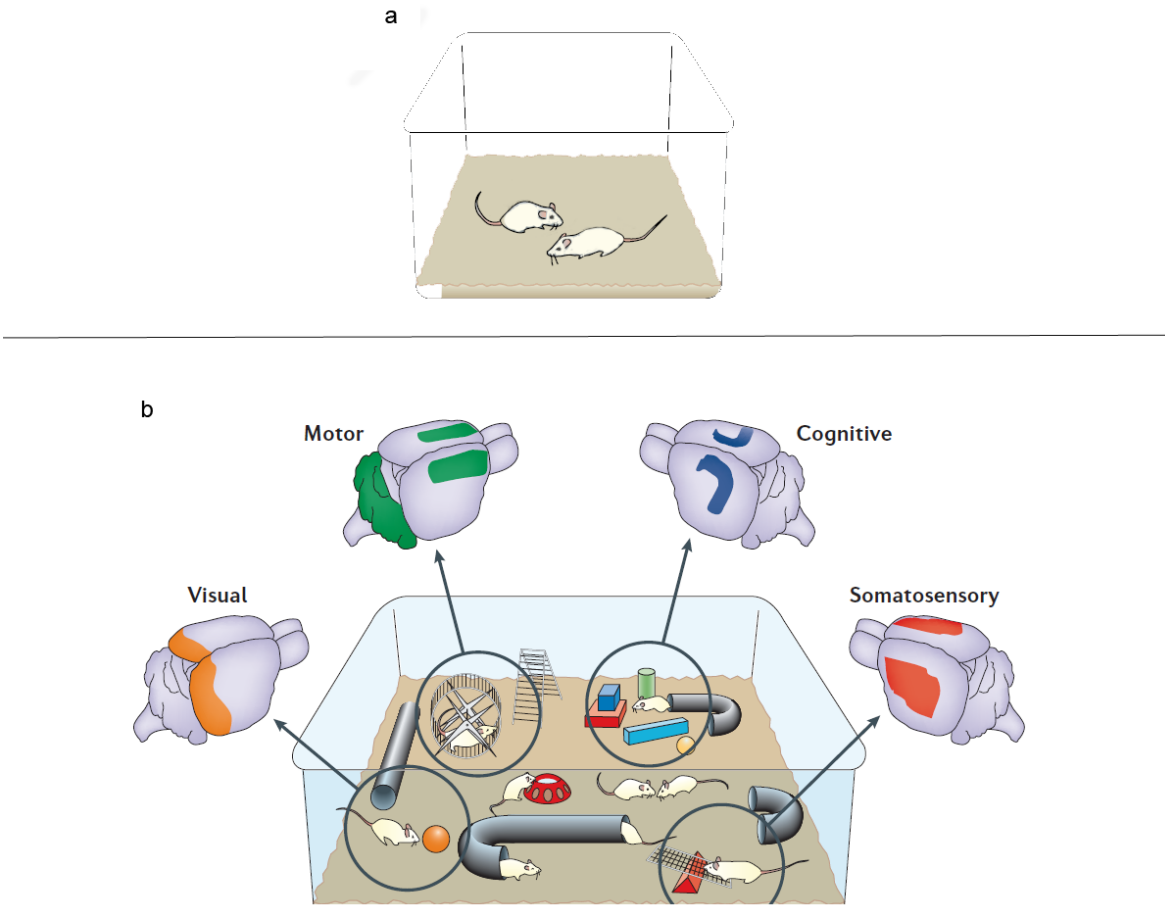

Figure 4. Housing conditions in different experimental groups and the effects of enhanced sensory, cognitive and motor stimulation in the brain. (a) A standard cage. (**b)** Cage for enriched environment with social interaction, stimulation of exploratory behavior with toys and tunnels. Enhanced sensory stimulation activates somatosensory (red) and visual (orange) cortices. Enhanced cognitive stimulation – as spatial maps – activates the hippocampus (blue). Enhanced motor activity – wheels and ladders – stimulates areas such as the motor cortex and cerebellum (green). (Adapted from van Praag et al., 2000 and Nithianantharajah and Hannan, 2006).

1.3.2 Role of the extracellular matrix in stroke

The extracellular matrix (ECM) provides a microenvironment that regulates neural cell activity. It occupies the space between neurons and glial cells and restricts major reorganization of processes and axonal outgrowth, through the appearance of cartilage-like structures called perineuronal nets (PNNs) (Soleman et al., 2013; Wang and Fawcett, 2012).

Perineuronal nets are a highly condensed form of the ECM of the central nervous system (CNS) distributed extensively in the brain and spinal cord (Hobohm et al., 2005; Karetko-Sysa et al., 2011; Wang and Fawcett, 2012). PNNs surround cell bodies, proximal dendrites and initial axonal segments of some populations of neurons (Celio et al., 1998; Karetko-Sysa et al., 2011; Soleman et al., 2013; Wang and Fawcett, 2012), that are often parvalbumin-expressing GABAergic (PV/GABA) interneurons (Härtig et al., 1994; Karetko-Sysa et al., 2011).

The accumulation of chondroitin sulfate proteoglycans (CSPGs) is the main feature of the PNNs (Hobohm et al., 2005; Karetko-Sysa et al., 2011). CSPGs comprise mainly aggrecan, brevican, neurocan and versican but they also contain hyaluronic acid, tenascins and link proteins (Bandtlow and Zimmermann, 2000; Galtrey and Fawcett, 2007; Karetko-Sysa et al., 2011; Wang and Fawcett, 2012). These macromolecules form a dense, negatively charged net around the cell that is attached to the cellular surface, with some holes left at the sites of synaptic contacts on the neuronal membrane (Karetko-Sysa et al., 2011; Wang and Fawcett, 2012). Hence, the PNNs appear to stabilize synapses but the inhibitory CSPGs may block the formation of new synaptic contacts and reduce the neuronal plasticity (Hobohm et al., 2005; Soleman et al., 2013).

ECM components may be affected by brain injury in different ways and their expression might be upregulated or reduced, which leads to function impairment; for instance in the regulation of synaptic plasticity (Hobohm et al., 2005).

The number of PNNs is reduced after stroke induced by MCAO (Hobohm et al., 2005) and PT (Karetko-Sysa et al., 2011; Madinier et al., 2014). Earlier this year, Madinier et al. showed that this reduction is beneficial for recovery after stroke (Madinier et al., 2014).

Stroke is followed typically by the development of glial scars, forming barriers that may protect the surrounding tissue but may also hamper reorganization and functional recovery and the deposition of CSPGs contributes to this hampering (Hobohm et al., 2005).

Some CSPGs are highly expressed in the glial scar. In addition to upregulation of different matrix components in the glial scar, matrix remodeling after stroke is characterized by

increased expression of metalloproteinases and their tissue inhibitors, both involved in the proteolytic cleavage of ECM constituents (Hobohm et al., 2005; Planas et al., 2001).

1.3.2.1 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) comprise a family of zinc endopeptidases associated with extracellular matrix remodeling involved in multifactorial actions in CNS physiology and pathology (Chaturvedi and Kaczmarek, 2013; Lee et al., 2004; Rosell and Lo, 2008; Zhao et al., 2006). They are secreted in a latent (inactive) form designated a pro-MMP or zymogens that require an activation and then they are able to cleave ECM components (Bode and Maskos, 2003; Planas et al., 2001; Snoek-van Beurden and Von den Hoff, 2005).

In general, MMPs have common structural elements:

- the signal peptide
- propeptide (blocking of active centre in proenzymes)
- catalytic domain with the zinc-containing active center
- hinge region
- hemopexin-like domain (binds to the inhibitors) (Kurzepa et al., 2014).

By far, the most important common feature of those listed above is the Zn^{2+} in the active center, necessary for catalysis (Kurzepa et al., 2014).

To this date, more than 20 MMPs have been identified and they are categorized based on the variations in the amino acid structure and affinity to substrates into the following groups (Kurzepa et al., 2014; Snoek-van Beurden and Von den Hoff, 2005):

1. collagenases (MMP-1, MMP-8, MMP-13)
2. gelatinases (MMP-2, MMP-9)
3. stromelysins (MMP-3, MMP-10, MMP-11)
4. matrilysins (MMP-7, MMP-26)
5. membrane-type matrix metalloproteinases – MT-MMP (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, MMP-25)
6. others (MMP-12, MMP-19, MMP-20, MMP-21, MMP-23, MMP-27, MMP-28)

The MMPs activity is regulated at the level of gene transcription and the synthesis of pro-MMPs (Snoek-van Beurden and Von den Hoff, 2005). In the propeptide domain of the MMPs, a cysteine residue (Cys^{73}) is present, which functions as a stabilizer of the inactive proenzyme (Springman et al., 1990; Van Wart and Birkedal-Hansen, 1990). As referred

above, there is an active Zn^{2+} site in the catalytic domain, which forms a bond with Cys^{73}. When this Cys^{73}-Zn^{2+} is intact, the MMP is inactive. After the disruption of this bond, the MMP will be in the active form (Snoek-van Beurden and Von den Hoff, 2005; Springman et al., 1990; Van Wart and Birkedal-Hansen, 1990). In this dissociation, a water molecule binds to the Zn^{2+} ion replacing the cysteine residue. The noncatalytic zinc is switched to a catalytic one, which results in an intermediate active enzyme (Springman et al., 1990). Then, the pro-domain of the MMP is removed by autolytic cleavage or by other proteases resulting in a fully active enzyme (Snoek-van Beurden and Von den Hoff, 2005; Springman et al., 1990; Van Wart and Birkedal-Hansen, 1990) (Figure 5).

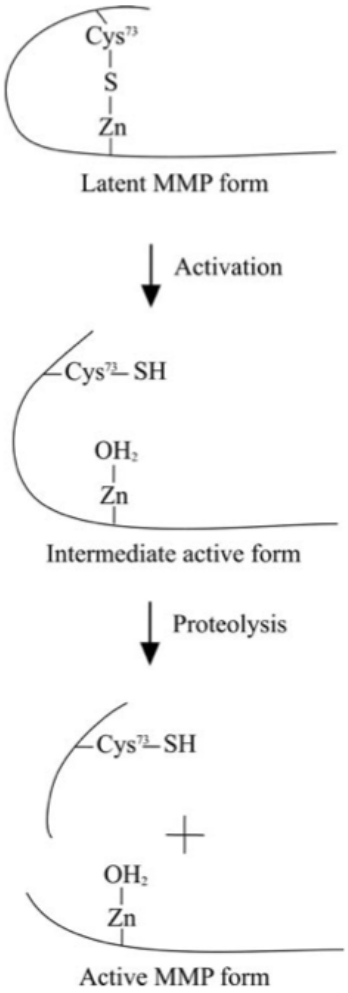

Figure 5. The activation of pro-MMPs. The activation of latent MMPs involves a disruption of the Cys^{73}-Zn^{2+} bond that results in an intermediate active form (from Snoek-van Beurden and Von den Hoff, 2005).

Besides, this activity is also regulated by several inhibitors, mainly by the tissue inhibitors of metalloproteinases (TIMPs) (Kurzepa et al., 2014; Planas et al., 2001; Snoek-van Beurden and Von den Hoff, 2005). TIMPs can bind to the active side of MMPs and block the substrate availability (Kurzepa et al., 2014). There are four forms of TIMPs (1 to 4) (Kurzepa et al., 2014; Snoek-van Beurden and Von den Hoff, 2005). These TIMPs do not seem to differentiate between the various MMPs (Snoek-van Beurden and Von den Hoff, 2005).

Current data suggest that MMPs have a deleterious role in acute stroke by degrading neurovascular matrix contributing to infarct extension and blood brain barrier (BBB) breakdown (Chaturvedi and Kaczmarek, 2013; Clark et al., 1997; Rosell and Lo, 2008). This role of MMPs can trigger brain cell death (Zhao et al., 2006). Hence, there is an effort in research to develop MMP inhibitors for acute stroke therapy (Chaturvedi and Kaczmarek, 2013; Zhao et al., 2006).

On the other hand at delayed stages after stroke, MMPs may have a different role. Since MMPs modulate brain matrix, they may mediate beneficial dendritic plasticity and ECM remodeling during stroke recovery (Zhao et al., 2006) (Figure 6). By degrading inhibitory matrix or glial scars, MMPs may allow axonal extension and recovery (Larsen et al., 2003). Basically, MMPs can have a role for neurovascular remodeling, affecting positively neuroplasticity, vascular recovery that may contribute to functional stroke recovery. Hence, MMPs comprise key molecules for promoting the remodeling of ischemic brain via angiogenesis, vasculogenesis or neurogenesis (Rosell and Lo, 2008).

In particular, the gelatinases (MMP-2 and MMP-9) have been implicated specially in cerebral ischemia (Lo et al., 2002). Some data show that MMP-9 signals emerged in neurons and astrocytes of the peri-infarct cortex at 7-14 days after experimental stroke in rats. These studies suggest that the potentially beneficial actions of MMPs in this area may be mediated through delayed neurovascular remodeling and not through direct neuroprotection (Zhao et al., 2006). In the experimental settings, when they treated rats with two different MMP inhibitors 7 days after stroke, not only the infarction volumes at 14 days were worsened but also behavioral recovery was significantly blocked (Zhao et al., 2006). Regarding the other gelatinase, there is some evidence that MMP-2 plays an important role in the later stage of ischemia as well, during the formation of the glial scar within the damaged area (Kurzepa et al., 2014). Other studies with these gelatinases showed that MMP-2 and MMP-9 promote axonal outgrowth in injured peripheral nerve and were more expressed during maturation of the scar tissue of the injured adult spinal cord (Copin and Gasche, 2007).

For some time, there were no data indicating a synaptic target for enzymatic activity of MMP-9 even though this enzyme is implicated in neuronal/synaptic plasticity. However, one study

has showed that MMP-9 digests the 43 kDa β-dystroglycan (β-DG) to release a 30 kDa product, suggesting that this protein is targeted in response to enhanced neuronal activity (Michaluk et al., 2007).

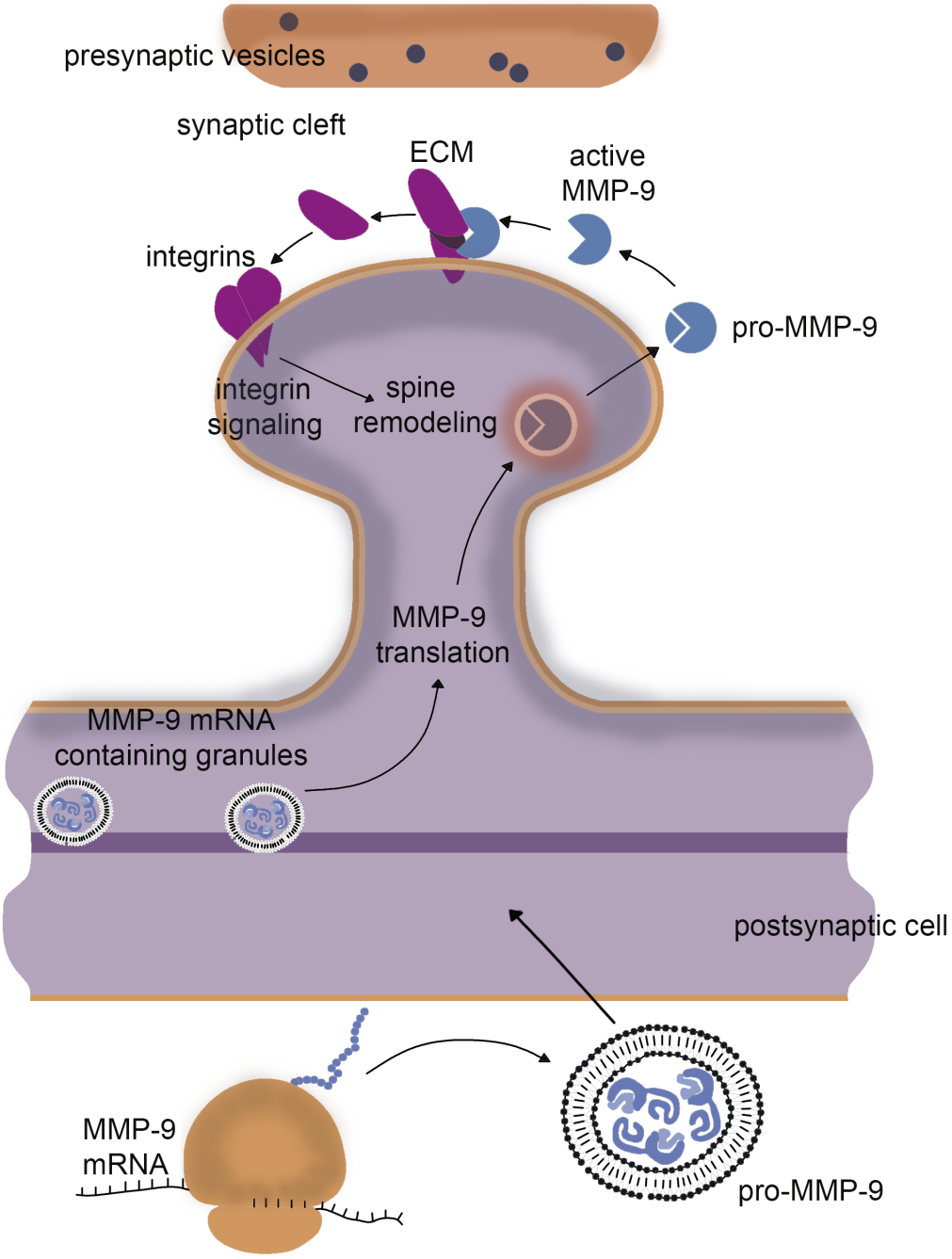

Figure 6. MMP-9 is locally released at the dendritic spines in a non-active form. The propeptide that it is blocking the enzymatic active site is cleaved off by a cascade of extracellular proteases. Once activated, MMP-9 cleaves ECM proteins, such as dystroglycan. The cleavage products act on integrin receptors to transduce the signals provoking actin cytoskeleton modifications underlying the growth of spines. (Adapted from Dziembowska et al., 2012 and Michaluk and Kaczmarek, 2007).

1.3.3 Beta-dystroglycan

Dystroglycan (DG) is a cytoskeleton-linked extracellular matrix receptor (Holt et al., 2000). It is formed by two sub-units, α and β, produced by proteolytic cleavage of a precursor, meaning that they are encoded by a single mRNA (Holt et al., 2000; Yamada et al., 2001; Zaccaria et al., 2001). This 160 kDa precursor propeptide is processed into α-DG form (120 kDa) and β-DG (43 kDa) (Holt et al., 2000).

α-DG is a highly glycosylated peripheral membrane protein. It binds to the extracellular matrix molecules laminin, agrin and perlecan with high affinity and in a calcium-dependent manner, and to the extracellular domain of β-DG (Zaccaria et al., 2001). β-DG spans the membrane, interacting intracellularly with the C-terminal cysteine-rich region of dystrophin or its homologue utrophin, or one of their truncated isoforms (Jung et al., 1995; Zaccaria et al., 2001) (Figure 7).

DG is expressed in a wide variety of tissues, including neural tissue. In the nervous system, given the presence of dystrophin, utrophin and their truncated forms, DG may participate in the formation of the dystrophin-associated glycoprotein complex (DGCs), which have an important functional role similar to that in the sarcolemma (Zaccaria et al., 2001). Even though there is not that much data showing evidence of DG presence in the brain, it has been shown that DG immunoreactivity for both subunits was widespread in the cerebral cortex, also there was β-DG-immunopositive hippocampal blood vessels; and α-DG present in postsynaptic specialization in the cortex (Zaccaria et al., 2001).

As referred above, there are some studies suggesting that β-DG can be degraded by MMP-9. First of all, a study showed that unidentified MMPs digest β-DG to reveal the 30 kDa product in the peripheral tissues (Yamada et al., 2001). Second, another study demonstrated that the appearance of the 30 kDa β-DG degradation product in the hippocampus coincides with increased levels of MMP-9 (Kaczmarek and Lapinska-dzwonek, 2002). Third, it was reported that β-DG expressed at the astrocyte endfeet can be specifically cleaved by macrophage-derived gelatinases (MMP-2 and MMP-9) in experimental autoimmune encephalomyelitis (Agrawal et al., 2006). Finally, Michaluk et al. provided evidence that β-DG is a target of neuronal MMP-9 in the brain *in vivo* as well as in neuronal cultures *in vitro*. This evidence was supported by the fact that MMP-9 was able to produce limited proteolytic cleavage of β-DG in brain neurons; this proteolysis occurred in response to synaptic activation and it was blocked by TIMP-1, a metalloproteinase inhibitor; the β-DG proteolysis into the 30 kDa form was absent in the MMP-9 knock-out mice; at last, the β-DG cleavage

correlates in time with increased MMP-9 activity and β-DG and MMP-9 co-localize in postsynaptic elements in the hippocampus (Michaluk et al., 2007).

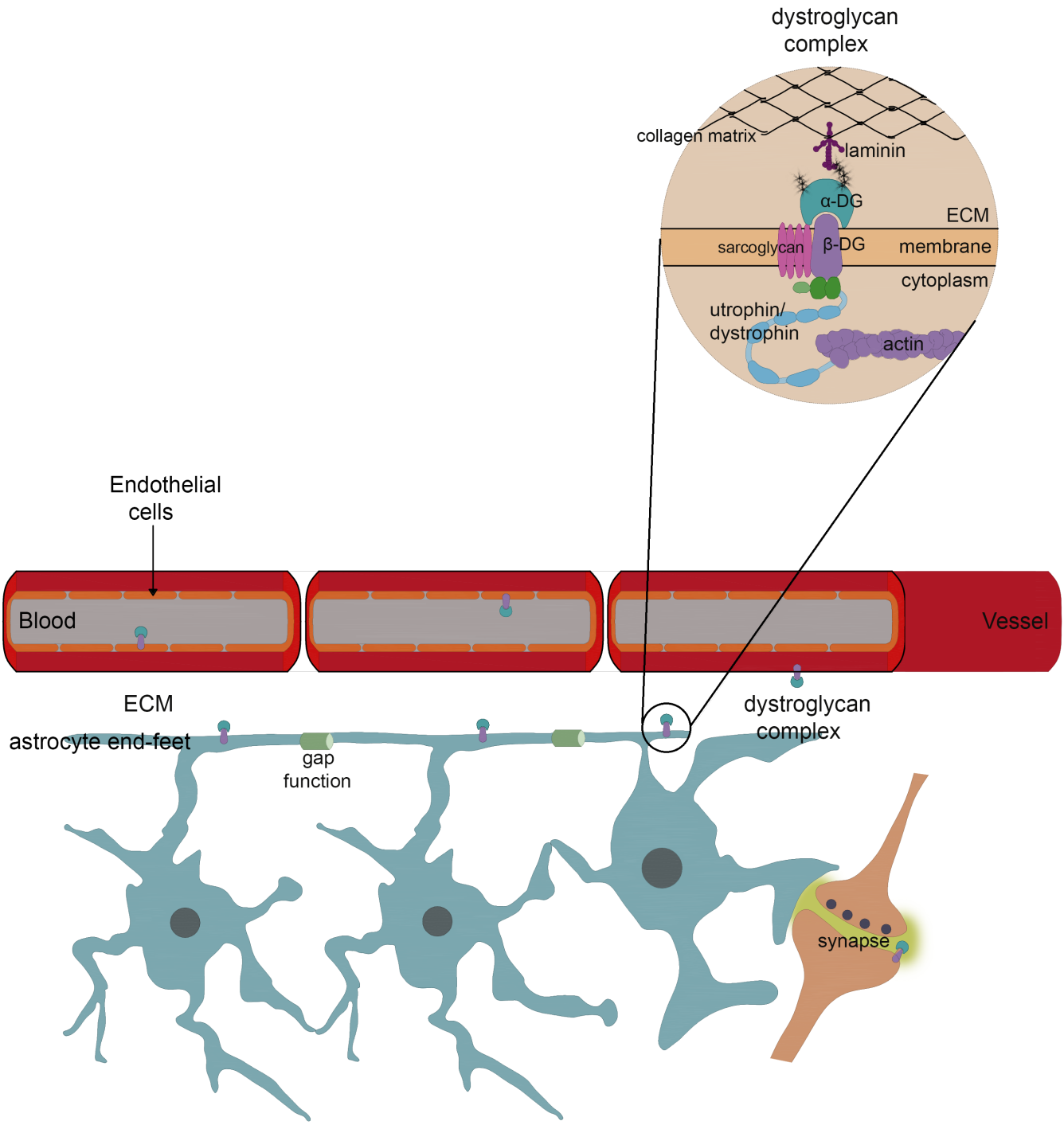

Figure 7. Distribution of dystroglycan complex in the brain. According with data presented previously, there are evidences that dystroglycan is widely spread in the brain. **Lower image**: It is connected to the endothelial cells in the brain vessels and on the astrocyte endfeet. Now, more recent data showed that beta-dystroglycan is also located in the synapses. **Upper image**: Dystroglycan complex is composed by two subunits: alfa and beta (α and β). α-DG binds to the extracellular matrix molecules such as laminin and to the extracellular domain of β-DG. β-DG is a transmembranal protein that binds to utrophin/dystrophin and it is connected that way to actin cytoskeleton protein. It is this unit that interacts with the ECM. (Adapted from Ross, 2002).

1.4 Experimental background

The next pages serve the purpose of explaining some of the techniques that can be employed in experimental stroke studies.

1.4.1 Experimental models for ischemic stroke

To study the underlying molecular and biophysical mechanisms in ischemic stroke, there are several *in vitro* and *in vivo* models available.

1.4.1.1 *In vitro* models:

Oxygen glucose deprivation (OGD) of cell cultures or brain slices is the most established and widely used *in vitro* model for ischemia (Chaturvedi and Kaczmarek, 2013; Mohr et al., 2011). The cells can be incubated in deoxygenated, glucose-free medium, lacking the supply of oxygen and nutrients (Chaturvedi and Kaczmarek, 2013).

1.4.1.2 *In vivo* models:

In vivo models have greater physiologic validity than *in vitro* models (Mohr et al., 2011). Models of cerebral ischemia can be separated into focal and global ischemia models (Bacigaluppi et al., 2010).

1.4.1.2.1 Models of global ischemia

These models are applied in rodents, pigs and dogs (Bacigaluppi et al., 2010) and can be induced by means of different approaches:

- Four vessel occlusion method (4VO): reversible common carotid artery (CCA) occlusion, which, combined with permanent interruption of the vertebral arteries via electro cauterization, results in a bilateral forebrain and brainstem ischemia (Pulsinelli and Brierley, 1979).
- Two vessel occlusion method (2VO): occlusion of the two common carotid arteries together with induction of hypotension for a limited time period This model was developed in our laboratory (Smith et al., 1984).
- Cardiac arrest and resuscitation: induction of ventricular fibrillation followed shortly

thereafter by defibrillation combined with chest compression and administration of epinephrine (Bacigaluppi et al., 2010).

1.4.1.2.2 Models of focal ischemia

Focal ischemia is the animal model that most closely mimic the changes that occur in cerebral infarction or stroke in humans (Braeuninger and Kleinschnitz, 2009; Sicard and Fisher, 2009) and it is typically performed in rodents such as rats or mice (Mohr et al., 2011).

- Occlusion of middle cerebral artery (MCAO) it is the most frequently used procedure and is induced through the insertion of a suture filament into the internal carotid artery, occluding the MCA at its origin. MCAO can be transient or permanent MCAO. In the transient MCAO (tMCAO), the suture filament is left transiently (times ranges between 30-120 min) before the suture is removed to enable the tissue reperfusion. In case of permanent MCAO (pMCAO) the suture is left in place and no reperfusion will occur (Bacigaluppi et al., 2010). After this procedure, the core is defined as the brain area which blood flow is reduced to less than 10%, affecting the lateral portion of the caudate putamen and the parietal cortex; by the other hand, the penumbra is the area which blood flow is reduced to less than 40%, affecting the entorhinal cortex (neocortex) and medial caudate-putamen (Mohr et al., 2011).
- Endothelin-1 model: this model achieves reversible occlusion of the middle cerebral artery (MCA) through direct or nearby application of the potent vasoconstrictor endothelin-1 (ET-1) (Canazza et al., 2014; Sharkey et al., 1993; Sicard and Fisher, 2009). When ET-1 is applied to the MCA there is a significant decrease of cerebral blood flow in its territory, resulting in an ischemic lesion pattern similar to that of direct surgical MCAO (Macrae et al., 1993; Sicard and Fisher, 2009). After approximately 20 minutes of severe cerebral blood flow reduction, there is a slow and progressive return of the blood flow to normal with the rate being dose-dependent (Macrae et al., 1993; Sicard and Fisher, 2009). In this model, there is an absence of lesions at injection site (Canazza et al., 2014).
- Thromboembolic model: it can be induced in animals through an injection of synthetic macro/microspheres (Bacigaluppi et al., 2010) or of a blood clot into the internal carotid artery (Bacigaluppi et al., 2010; Canazza et al., 2014; Sicard and Fisher, 2009). This model is of great interest to researchers because it is the closest in resemblance to human ischemic stroke and it can be used to evaluate thrombolytic therapies (Bacigaluppi et al., 2010; Braeuninger and Kleinschnitz, 2009; Canazza et al., 2014; Sicard and Fisher, 2009).
- Photothrombosis model (PT): In 1985, Watson et al. introduced PT as a technique to induce focal cerebral infarction in the cortical vasculature of rats (Watson et al.,

1985). In this model, a cortical brain lesion is induced by systemic injection of a photosensitive dye, such as Rose Bengal, and subsequent trans-cranial irradiation of the brain by a light beam, leading to the coagulation of the irradiated tissue (Bacigaluppi et al., 2010; Braeuninger and Kleinschnitz, 2009; Chaturvedi and Kaczmarek, 2013; Sicard and Fisher, 2009; Watson et al., 1985). Oxidative damage to the vascular endothelium caused by the altered dye leads to the platelet aggregation in that brain region and subsequent thrombotic vessel occlusion (Braeuninger and Kleinschnitz, 2009; Chaturvedi and Kaczmarek, 2013; Sicard and Fisher, 2009). This model does not require craniotomy because the light can penetrates the skull, so it can be less invasive (Braeuninger and Kleinschnitz, 2009). The main advantage of this procedure is the high reproducibility of lesion size and location, so the region of ischemia can be predefined and circumscribed being possible to selectively induce infarcts in cortical areas representing specific functions (Bacigaluppi et al., 2010; Braeuninger and Kleinschnitz, 2009; Sicard and Fisher, 2009). A disadvantage of this model is that vasogenic edema and blood-brain barrier breakdown occur within minutes which does not allow the formation of penumbra (Sicard and Fisher, 2009), creating a lesion with a small ischemic penumbra compared to other focal stroke models (Bacigaluppi et al., 2010).

1.4.2 Behavioral tests

Motor, sensory and cognitive deficits are common after stroke. Developing and using tests that have the ability to identify behavioral deficits is essential to measure the functional outcome in animal models and to give valuable insight of the biological changes in the brain following experimental stroke (Schaar et al., 2010). Since the stroke model employed in our experiments (PT) damages unilaterally the brain, the following behavioral tests can be considered relevant to assess the deficits.

1.4.2.1 Paw placement test

The paw placement (PP) test provides information on the tactile/proprioceptive response to limb stimulation (Madinier et al., 2014).

First described in 1995 by Garcia et al., the PP was part of the Neuroscore, an overall test done to evaluate neurological function and sensorimotor deficits after stroke (Garcia et al., 1995).

In this test, the animal is placed with the 4 paws in a platform top, hand-held and immobile, and the paws of the side to be tested along the edge. When the rat is moved over the edge, the paws loose the contact with the surface. Then the ability of the animal to replace the paw up back to the platform surface was assessed as described in 1992 by De Ryck et al. (De Ryck et al., 1992). For this assessment, it is also important to hold the head of the rat at 45º degrees angle avoiding the visual and whisker/snout stimulation (De Ryck et al., 1992; Madinier et al., 2014).

1.4.2.2 Corner test

The corner test is a sensorimotor functional assessment that has been shown to be reliable for identifying and quantifying sensorimotor and postural asymmetries; also to provide a simple way of detecting contralateral and ipsilateral turning biases (Schaar et al., 2010). In 1982, when this test was first described, it has been shown to be relevant to investigate the unilateral nigrostriatal damage in the rat (Schallert et al., 1982) and later, it was used to focal cerebral ischemia in the mouse (Zhang et al., 2002).

The apparatus consists of two boards placed closely together at a 30 degrees angle to form a narrow alley. An animal when placed facing the corner, both sides of the vibrissae are simultaneously stimulated which leads the animal to rear and turn 180 degrees. Intact animals will usually turn around to the right or left randomly while animals with unilateral brain damage will preferentially turn around in the ipsilateral direction, leading with the non-impaired limb and displaying and asymmetry in corner turning (Chen et al., 2010; Hua et al., 2002; Schaar et al., 2010; Zhang et al., 2002).

Baseline or pre-training is recommended for the reduction of variability and identification of preferential side. In addition to identifying sensorimotor deficits, the corner test has been shown to be an objective assessment of long-term functional outcome (up to 90 days) after stroke in both the rat and the mouse (Schaar et al., 2010; Schallert, 2006).

1.4.2.3 Corridor test

The corridor test is a test of sensorimotor integration that depends on a rat's ability to retrieve food from either side of its body (Dowd et al., 2005; Fitzsimmons et al., 2006).

It has been shown that rats with unilateral dopamine depletion and with unilateral lateral hypothalamic lesions neglect the pellets on the contralateral side of their bodies, and preferentially retrieve pellets from the ipsilateral side (Dowd et al., 2005; Fitzsimmons et al., 2006; Marshall and Teitelbaum, 1974)

In this test, restricted fed (hungry) rats are placed into a long, narrow corridor in which pots of sugar pellets are placed on the left and right sides, they are then free to retrieve pellets from either side of their bodies (Dowd et al., 2005; Fitzsimmons et al., 2006).

Baseline performance is required for the same reasons of the corner test.

1.4.3 Biomolecular techniques

Quantitative analysis of molecular proteins, such as Western blot and zymography, define the amount of protein in a sample or how active that protein is. Since qualitative analyses just show the presence or absence of the protein of interest, it is important to perform additional analyses in a quantitative way. Qualitative and quantitative analyses are made in same way, but in quantitative analyses imaging techniques are then used to assess the quantification of either protein amounts or enzymatic activity.

Western blot analysis is a powerful tool for protein detection where we can know the exact protein amount in the sample. Zymography is used for detection of enzymatic activity of matrix metalloproteinases.

1.4.3.1 Western blotting

The Western blot is an analytical technique used for specific protein detection in a sample of tissue extract.

In this technique, the proteins are separated by gel electrophoresis. The proteins run through a polyacrylamide gel under denaturing sodium dodecyl sulfate (SDS) conditions and they are separated by the molecular weight.

The proteins in the gel are transferred to a membrane of polyvinylidene fluoride (PVDF) by electric current, then they are incubated with specific antibodies for the target protein (Renart et al., 1979; Towbin et al., 1992).

The blocking step is also very important. This step prevents the non-specific binding of the antibody to the membrane and other proteins. For that it can be used bovine serum albumin (BSA) or non-fat dry milk in a tris-buffered saline (TBS) with a detergent such as Tween 20.

This reduces extra bands in the final product of the Western blot (Renart et al., 1979; Towbin et al., 1992).

After incubation with the specific primary antibody, there is the detection step. During the detection step, the membrane is "probed" for the protein of interest with a secondary antibody linked to a reporter enzyme, normally horseradish peroxidase. When this enzyme is exposed to an appropriate substrate (chemiluminescent agent), the substrate is cleaved and the reaction produces luminescence in proportion to the amount of protein. It is then exposed to the light then showing an image of the bands having the molecular size of the protein of interest. The bands are quantified by densitometry.

1.4.3.2 Zymography

A currently used technique to analyze the expression of MMPs is zymography. In this technique, it is possible to identify MMPs by the degradation of their preferential substrate and by their molecular weight. One can also determine whether the MMP is in an active or pro form (Snoek-van Beurden and Von den Hoff, 2005).

In zymography, the proteins are separated by electrophoresis under SDS conditions, such as in Western blot. The separation occurs in a polyacrylamide gel containing a suitable substrate that is co-polymerized with the acrylamide (called zymogram) (Heussen and Dowdle, 1980).

Since SDS causes reversible denaturation of MMPs, the gel must be rinsed, so that SDS is removed and the MMPs can renature and recover their enzymatic activity (Heussen and Dowdle, 1980; Snoek-van Beurden and Von den Hoff, 2005). Then, during the incubation of the gel in an activation buffer, the renatured MMPs in the gel will digest the substrate included in the gel (Snoek-van Beurden and Von den Hoff, 2005).

Subsequently the gels are exposed and the void will appear as bands quantified by densitometry.

Gelatin Zymography is the zymography used for detection of gelatinases, MMP-2 and MMP-9 and it is based on some specific principals such as: during electrophoresis, the MMP substrate gelatin is integrated in the polyacrylamide gel; the separation of MMP-TIMP by SDS enables independent detection; both pro- and the active forms of MMPs can be distinguished by their molecular weight (Snoek-van Beurden and Von den Hoff, 2005).

Chapter 2
Aims

The study underlying this master thesis was conducted to investigate changes of some components of the ECM in the brain after experimental stroke, and how housing animals in an enriched environment after stroke affects these components. First of all, we were interested to know if EE affects the expression of β-DG after experimental stroke.

Also, because there is evidence showing β-DG as a target of MMP-9, we decided to see how this matrix metalloproteinase is affected by stroke and consequently after recovery. Since MMP-9 is a gelatinase, we analyzed as well the activity of gelatinase MMP-2.

In addition, we wanted to know how was the behavioral outcome after one week of recovery after experimental stroke when animals were either housed in an enriched or standard environment.

Chapter 3
Methodology

3.1 Animal studies

This work was done with 31 animals, which were divided into two groups: Group A (n= 20) and Group B (n = 11). Group A was used to study infarct volume measurement, immunohistochemistry and immunofluorescence. Group B was used to study behavioral assessment and to perform Western blotting and zymography. The experiments were carried out on male Sprague Dawley rats (8 weeks, Charles River).

3.2 Experimental design

We performed two studies. Both studies had the same duration of recovery: 7 days after experimental stroke. The day when PT was performed was considered day 0. After 2 days, there was a selective sorting and randomization into either EE or STD cages during 5 days. At day 7, the rats were sacrificed.

In the first study, group A of animals (n=20), served the purpose of assessing the infarct volume and in the same brains immunohistochemistry analyses were performed (Figure 8).

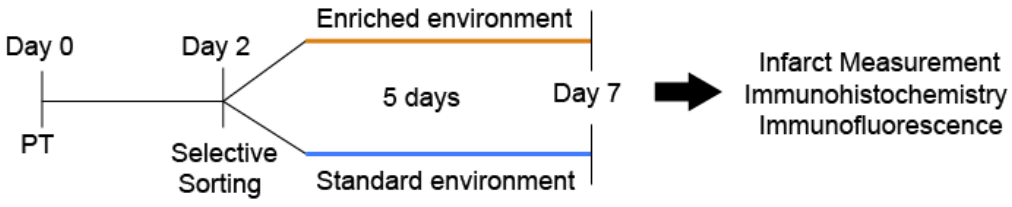

Figure 8. Study group A: n=20. Sham EE: n=5; Sham STD: n=5; EE: n=5; STD: n=5. These animals were used for infarct volume measurement, immunohistochemistry and immunofluorescence.

In the second study, group B of animals (n=11), the animals were used for behavioral assessment after experimental stroke, as well as Western blotting and zymographic analyses (Figure 9).

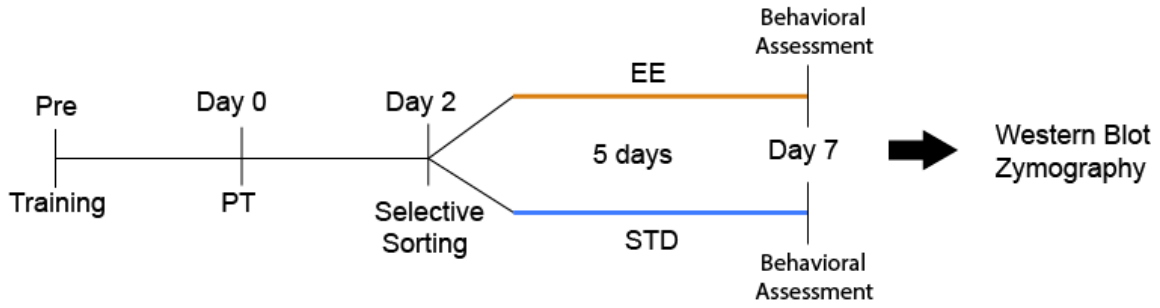

Figure 9. Study group B: n=11. Sham n=4; EE n=4; STD n=3. These animals were used to perform behavioral assessment and techniques such as Western blotting and zymography.

3.3 Permanent focal ischemia by PT

Rats of both groups A and B were housed under reverse light conditions, with the testing performed during the dark period when the rats are active.

The animals were anesthetized by isoflurane (approximately 2% in O_2 under spontaneous ventilation) and placed into a stereotaxic frame with sensorimotor cortex coordinates. Corporal temperature was monitored during surgery using a rectal temperature probe and animals were kept at 36.8–37.6 ± 0.3 by means of a heating pad with feedback control (Supplementary Table 1). The skin above the skull was incised. Thereafter, the dye Rose Bengal (3.2 ml at 10 mg/ml) was injected in the tail vein. Two minutes after injection, the skull was illuminated with cold light (Schott, KL 1500 LCD) on an area of 8 to 4.5 mm for 20 min (from +4 to -4 mm antero-posterior and from 0.5 to 5 mm on the left from bregma) (Figure 10). The tail and the scalp incisions were sutured and the rats transferred to their home cage with free access to food pellets and water. The functional deficit was assessed 2 days after the onset of the stroke using the paw placement test and then after one week. Control rats (sham), after the incision of the skull, were subjected to Rose Bengal injection without subsequent illumination (Group A = 5; Group B = 4).

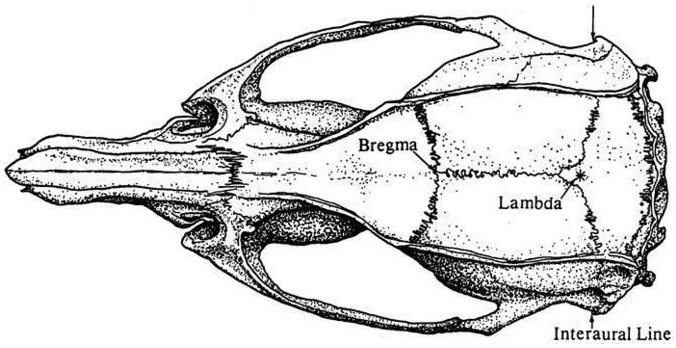

Figure 10. Location of bregma. The PT was perfomed from +4 to -4 mm antero-posterior and from 0.5 to 5 mm on the left from bregma (from Paxinos and Watson, 2013: The Rat Brain in Stereotaxic Coordinates).

3.4 Selective sorting, randomization and EE

On day 2 after PT, a selective sorting was performed, where only rats with total paretic limbs were included in both studies (score in paw placement test = 0, see below). This was done in order to obtain groups with a similar functional deficit. After this selective sorting, animals were randomized and kept either in pairs in standard laboratory cages (STD) (Group A = 5, Group B= 3) or all together (Group A n=5, Group B n=4) in a EE cage with 8 rats (animals excluded from the study were used to fill up the cages) for 1 week as described in both Figures 8 and 9. No animal died during either surgeries or recovery.

3.5 Behavioral tests

3.5.1 Paw placement

The paw placement (PP) test provides information on the tactile/proprioceptive response to limb stimulation. Animals (Group B) were placed with all 4 paws on the table top, and the paws to be tested along the edge. Each rat was moved over the edge with the paws to be tested in loose contact with the table surface. The ability of the animals to place the limb back onto the table surface when the rat was moved toward the edge was evaluated. Importantly, the head was held at 45º angle, so that visual and whisker/snout stimulation was prevented. The placement of each paw was recorded when the rat was moved towards the edge. Sensorimotor dysfunction was assessed by using a score of 1, 0.5 and 0: (1) – the paw is immediately placed on the table surface; (0.5) – the limb is extended, but some

movements and attempts to place the paw on the surface of the table; (0) – the paw is totally immobilized, hanging down, with no movement. To obtain the assessment during recovery, each rat was tested at 2 and 7 days after PT stroke.

3.5.2 Corner and corridor tests

The corner and corridor tests were performed in the same platform. The corridor had a length of 150 cm, and the pots with 10 sugar pellets were placed 10 cm of distance between each other; one of the pot on the right side, 10 cm after the other on the left side. In the end of each side of the corridor, there was a corner forming ~45º degrees. The animals of group B performed 3 baselines before PT to assure they recognized the environment. The assessment of both tests was done 7 days after experimental stroke. Each head turn was counted as 1 point. After that, the percentage of the turning biases for each side for both, corridor and corner test were calculated.

3.6 Tissue collection

3.6.1 Group A

At 7 days after PT, rats of group A were anesthetized with isoflurane and perfusion-fixed with 4% paraformaldehyde (PFA) solution. Brain were collected and immersed in 4% PFA solution for 4 h, then transferred into 25% sucrose phosphate buffer solution (0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4) and stored at 4ºC. Brains were then cut in 30 µm slices using a microtome and the slices were kept for infarct size measurement, immunohistochemistry and immunofluorescence. All the slices were stored in anti-freeze solution (30% ethylene glycol, 30% glycerol, 0.01 M NaH_2PO_4, 0.03 M Na_2HPO_4) at -20ºC.

3.6.2 Group B

At 7 days after PT, rats of group B were deeply anesthetized with isoflurane and decapitated. Brains were removed, freed from meninges and immediately frozen in isopentane (-40ºC) and further cooled down to -70ºC on dry ice and -80ºC for storage. Tissue samples were collected from 2 to 3 mm thick sections (from the beginning of the infarct to posterior areas), of the SS (Figure 11) and further homogenized in cell lysis buffer (see Western blot and Real-time gel zymography section).

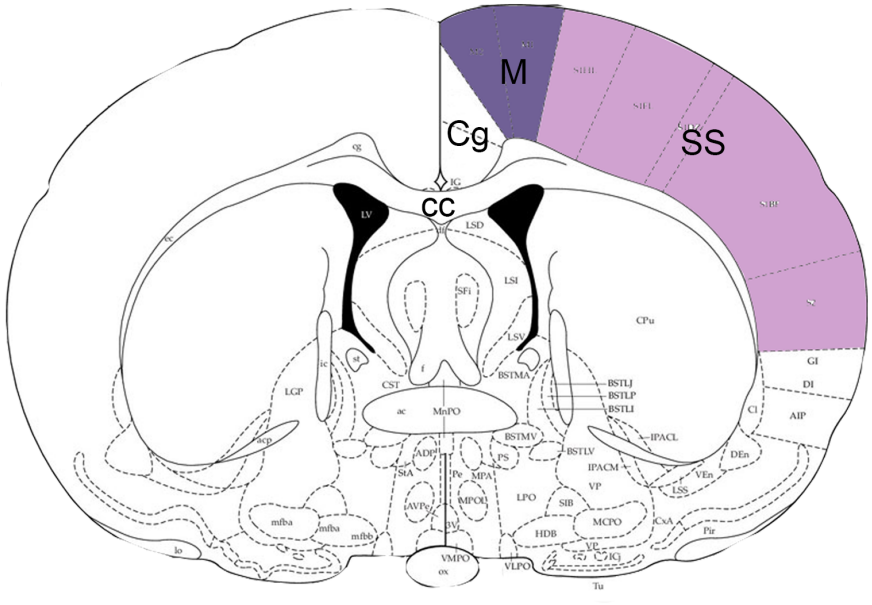

Figure 11. Brain areas. After PT, the motor cortex was injured (M). The somatosensory cortex (SS) was left intact and further homogenized. (Cg: cingulate cortex; cc: corpus callosum) (Adapted from Paxinos and Watson, 2013: The Rat Brain in Stereotaxic Coordinates).

3.7 Infarct volume measurement

For each animal of group A in EE and STD cages, the brain slices were immunostained with the monoclonal NeuN antibody (Neuronal Nuclei, Millipore, Hampshire, UK; dilution 1:1500). All slices were scanned, the non-lesioned area of the infarcted hemisphere and the non-lesioned contralateral hemisphere were outlined and measured using ImageJ software (National Institute of Health, USA). The infarct volume (mm^3) was then calculated by integration of the different areas (Swanson et al., 1990).

3.8 Western blotting

Whole cellular protein extractions from the dissected SS region were performed by mechanical homogenization and incubation in lysis buffer: 20 mM Tris (pH 7.5), 150 mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton-X100, 2.5 mM sodium pyrophosphate, 1mM β-

glycerolphosphate, 1mM orthovanadate, 1mM PMSF, supplemented with a protease inhibitor cocktail (P2714, Sigma). Following 15 min incubation, samples were centrifuged at 14000 rpm, for 20 min. Total protein concentration was determined by the Bradford assay, using bovin albumin (Sigma) as standard.

Twenty micrograms of protein of brain samples of both ipsilateral and contralateral sides of the SS belonging to the rats of group B were separated on a 10% SDS polyacrylamide gel. Blocking was performed onto polyvinyldifluoride (PVDF) membranes using blocking buffer (20 mM Tris, 136 mM NaCl, pH 7.6, 0.1% Tween 20, 5% nonfat dry milk), and detected using a monoclonal antibody against Beta-dystroglycan (Abcam, England, UK; dilution 1:500). After incubation overnight at 4ºC, signals were obtained by the binding of a secondary anti-mouse-HRP antibody (Sigma, Germany; dilution: 1:10000) and visualized by exposing the membrane under a CCD camera (LAS1000, Fujifilm, Japan) using a chemiluminiscence kit (Immobilon Western Chemiluminescent HRP Substrate, Millipore, Hampshire, UK). Membranes were stripped and reprobed for β-actin (Sigma, Germany; dilution: 1:75000). After densitometric analysis using the ImageJ software (National Institute of Health, USA), beta-dystroglycan expression was calculated as percentage of β-actin expression.

3.9 Real-time gel zymography

The extraction of MMPs from brain tissue followed the procedure by Szklarczyk (Szklarczyk et al., 2002) with some modifications. Tissue samples were weighed and then homogenized in a ice-cold buffer containing 10 mM $CaCl_2$ and 0.25% Triton X-100 in water (20 µl of buffer/1 mg of wet tissue). The homogenates were centrifuged at 9100 rpm for 30 min at 4°C.

The entire supernatant containing soluble proteins (Triton X-100-soluble) was quantitatively recovered. Proteins from the supernatant were precipitated in 60% ethanol for 5 min at 4°C and then centrifuged at 14000 rpm for 5 min. The precipitate was solubilized in 100 µl of non-reducing SDS-sample buffer for 15 min at 37°C.

The pellet (Triton X-100-insoluble) was resuspended in a buffer containing 50 mM Tris, pH 7.4, and 0.1 M $CaCl_2$ in water, heated for 15 min at 60°C, and then centrifuged at 12500 rpm for 30 min at 4°C. This treatment results in releasing ECM-bound MMPs into the solution. The final supernatant was considered a Triton X-100-insoluble fraction. After centrifugation, the entire supernatant was quantitatively recovered. The proteins from the supernatant were precipitated and finally solubilized in 50 µl of sample buffer.

Protein concentration was measured by the Bradford method, using 20 µl of samples removed immediately before precipitation. Samples were stored at -80°C.
The samples prepared from the Triton X-100 insoluble fraction were separated on 8% SDS-PAGE gel co-polymerized with 0.2% FITC-labeled gelatin. FITC labeling of the gelatin (Sigma) followed the protocol of Hattori (Hattori et al., 2002). Gels were washed twice for 30 min in 2.5% Triton X-100 to remove SDS and incubated for 2 d in 50 mM Tris, pH 7.4, 10 mM $CaCl_2$, 1µM $ZnCl_2$, 1% Triton X-100, and 0.02% sodium azide at 37°C. Gels were digitally photographed in UV light (ChemiDoc XRS+ System, Bio-Rad). Enzymatic activity is detected as dark bands against a bright fluorescent background and it was quantified by densitometry of gelatinolytic bands using the ImageJ software (National Institute of Health, USA).

3.10 Immunohistochemistry

Free-floating brain slices of group A were rinsed three times in PBS (phosphate buffer saline) and quenched in 3% H_2O_2 (Hydrogen Peroxide) and 10% MetOH (Methanol) for 12 minutes. After washing in PBS, the sections were blocked with blocking solution (5% normal donkey serum, Jackson ImmunoResearch, UK and 0.25% Triton X-100) in PBS) for 1 h at room temperature. The sections were incubated overnight at 4ºC with primary antibody (mouse monoclonal anti-beta-dystroglycan, Abcam, England, UK; dilution: 1:200) diluted in blocking solution. Following three rinses with 1% normal serum and 0.25% Triton X-100 in PBS, the sections were incubated with appropriate secondary biotinylated antibodies (donkey anti-mouse-biotin; Sigma-Aldrich, USA; dilution 1:500) in blocking solution for 90 min at room temperature (RT). Visualization was achieved via the Vectorstain ABC kit (vector) using 3,3-diaminobendizine-tetrahydroklorid (DabSafe, Saveen Werner, Sweden), 8% NiCl and 3% H_2O_2. Bright field pictures were acquired using an Olympus BX60 microscope (Solna, Sweden).

3.11 Immunofluorescence

Free-floating brain slices of group A were rinsed three times in PBS and blocked with blocking solution (5% normal donkey serum, Jackson ImmunoResearch, UK and 0.25% Triton X-100 in PBS) for 1 h at RT. The section were incubated overnight at 4ºC with primary antibody (mouse monoclonal anti-beta-dystroglycan, Abcam, England, UK; dilution: 1:200;

rabbit anti-PSD-95, Synaptic Systems, Germany; dilution 1:500; rabbit anti-SYP-Ab4, Thermo Fisher Scientific, USA; dilution 1:200) diluted in blocking solution. Following thee rinses with 2% normal serum and 0.25% Triton X-100 in PBS, the sections were incubated with appropriated secondary antibodies (donkey anti-mouse-biotin, Sigma-Aldrich, USA); dilution 1:500) conjugated with either fluorescent dyes Cy3/Cy5 (Jackson ImmunoResearch, UK) and Streptavidin Alexa Fluor 488 conjugate (Molecular Probes, Invitrogen, USA) at a dilution of 1:100 in blocking solution for 90 min at RT. Fluorescent dyes were imaged using a confocal laser-scanning microscope (Zeiss LSM 510, Jena, Germany).

3.12 Statistical analysis

All data are presented as mean ± SD (standard deviation), unless otherwise stated. Statistical analysis was performed with the two-tailed unpaired Student's t-test for the infarct size comparison, Kruskal-Wallis for the behavioral tests, one-way ANOVA for the Western blot analyses and Real-time gel zymography. $P<0.05$ was considered statistically significant. Data handling and statistical processing were performed using Microsoft Excel and GraphPad Prism Software.

Chapter 4
Results & Comments

4.1 Enriched environment does not affect infarct size after PT

We started with the analysis of the infarct volume size. Figures 12A and B show the location of the cortical infarct one week after PT as displayed in the NeuN stained coronal section of a rat brain. The lesion is localized approximately 8 mm caudally from the frontal cortex, affecting fore and hind paw motor areas of the motor cortex. The cingulate (cg) cortex was left intact such as large parts of the SS (Figure 11). The lesion extended from the cortical surface down to the corpus callosum (cc), but did not transect the white matter.

The lesion localization in the EE (Figure 12B) and STD (Figure 12C) groups was the same. The mean size of the infarct in the EE at 1 week after PT was 36±15 mm^3, while in the STD housed animals the mean infarct volume was 33±13 mm^3 (Figure 10D).

Thus, EE does not affect the volume of the infarct. Animals housed either in EE or STD cages, at 2 days after PT and staying there 5 days, had the same infarct size.

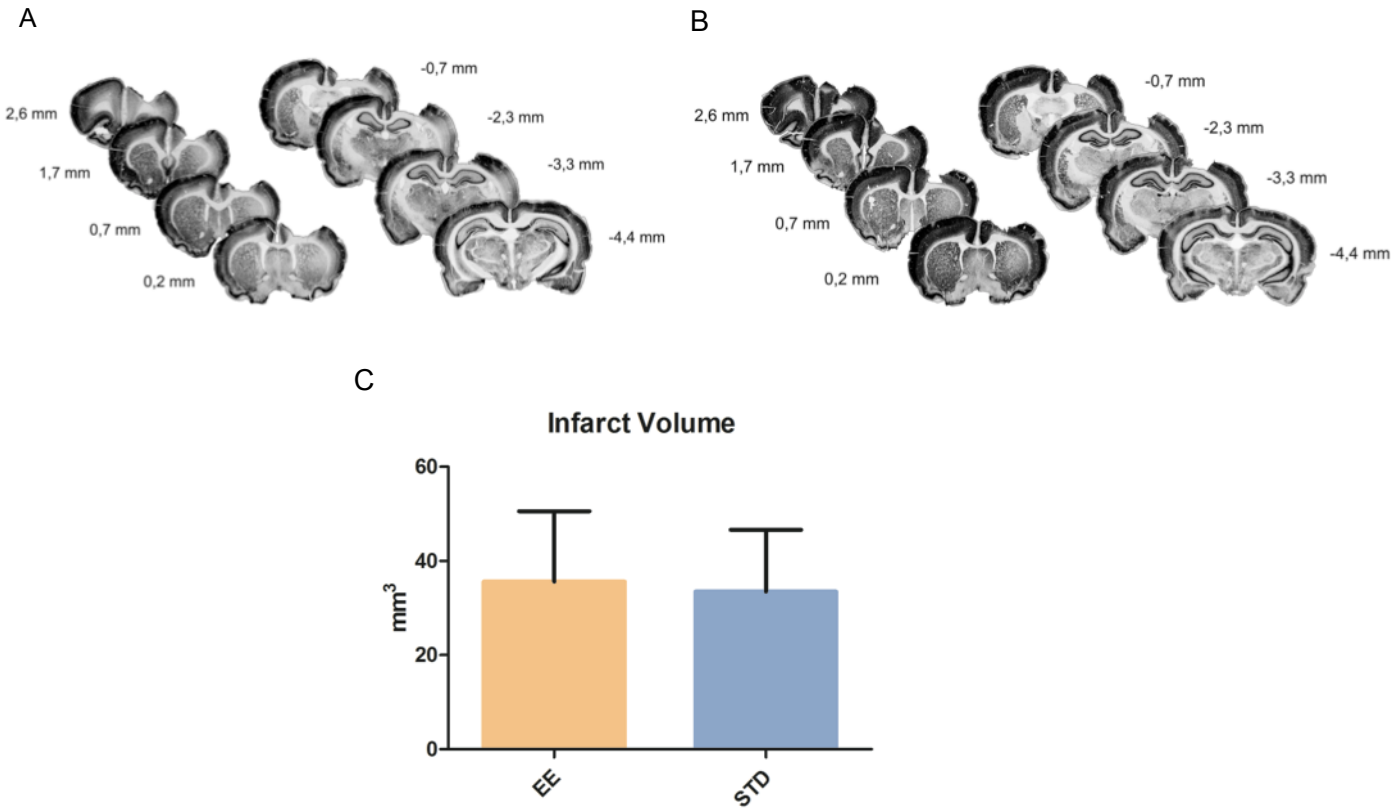

Figure 12. Infarct volume measurement. Infarct volume was measured at 1 week after PT and estimated by integration of the lesioned area on brain slices taken the distance from bregma showed in the figures (A) and (B). NeuN staining staining was performed to distinguish between intact and death tissue. (A) Coronal brain slices of a representative brain infarct with the respective distances from bregma for the EE housed animals and (B) STD housed animals. (C) Infarct volume expressed in mm^3. Values are presented as mean ± SD and tested with two-tailed unpaired Student´s *t*-test (n=5 for each housing condition; EE: enriched environment; STD: standard environment).

4.2 Enriched environment enhances sensorimotor function after lesions in the motor cortex

4.2.1 Paw placement test

Early this year, a study performed in the same lab where I have accomplished my thesis showed that the animals housed in EE after experimental stroke recovered the limb placement ability better than STD animals and this was assessed by the PP test (Madinier et al., 2014). In that study, a difference was found in behavioral recovery between the two different housing groups, EE and STD, in the PP test.

As described in the methodology, in this test, visual and whisker/snout stimulation was prevented, so the behavioral response can be only be due to tactile/proprioceptive response to the limb stimulation.

In both groups, EE and STD, the fore and hind paws on the side of the lesion were unaffected. Whereas the contralateral paws were both affected as it is shown in Figure 13.

At 7 days of stroke recovery, the EE housed animals recovered quickly. They achieved scores close to the ones of the sham animals. There was no significant difference between the two groups (EE and sham), both fore (Figure 13A) and hind paws (Figure 13B) recovered. Even when both paws were analyzed together, there was no significant difference (Figure 13C).

In contrast, the STD housed animals did not recover any function of both fore and hind paws 7 days after stroke, receiving a score of 0 (Figure 13D). There was a significant difference in the score obtained by the STD animals, comparing to the sham animals, where both of their paws have a score of 1 (Figure 13F).

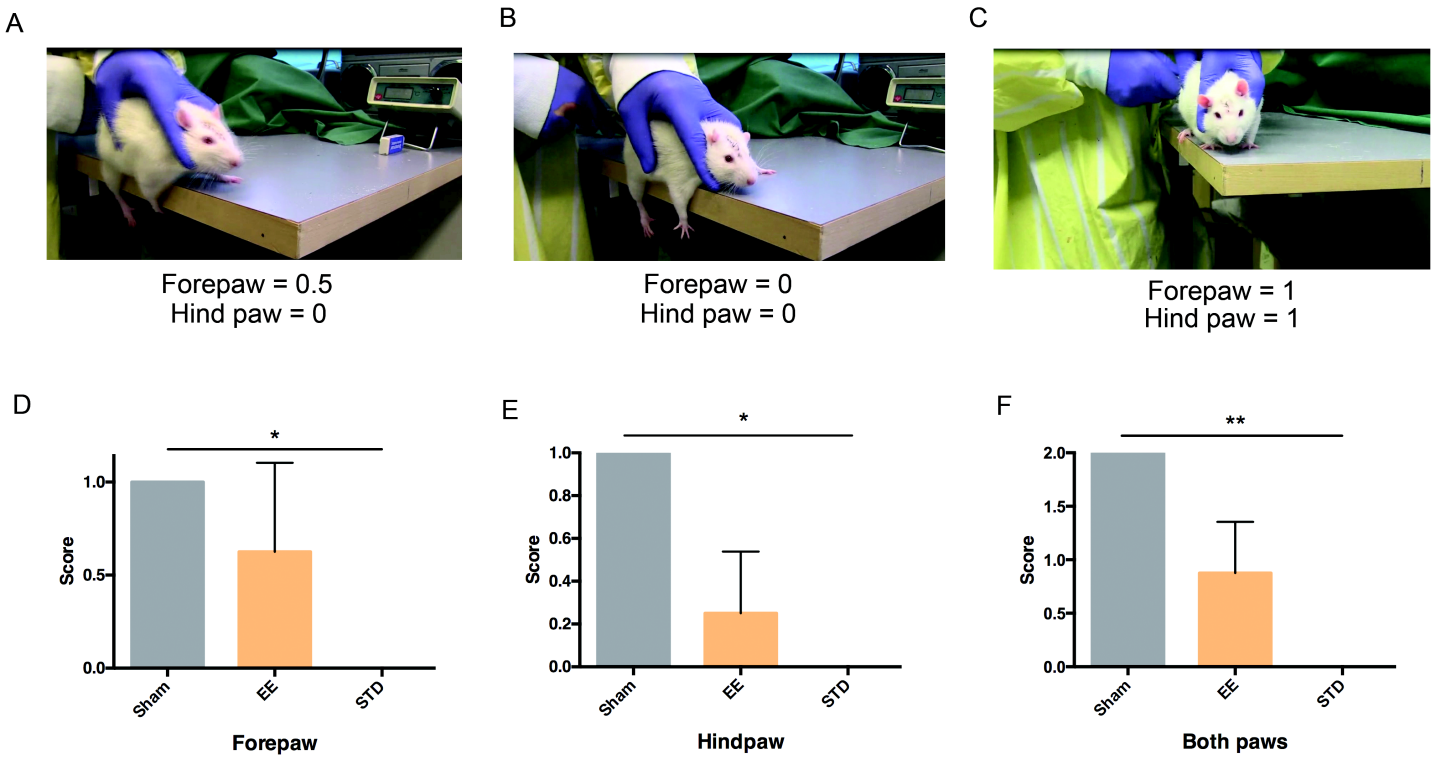

Figure 13. Paw placement test. Limb placing ability of rats subjected to PT under conditions that prevent visual and whisker/snout stimulation during testing. (A) The hind paw is hanging, totally paretic. This behavior is scored with 0. The forepaw, where the rat is grabbing the edge of the table but it is not able to put it up, it is scored with 0.5. (B) Both paws, hanged and paretic, are scored with 0. (C) The rat does not hang in any point the both paws, putting automatically the paw back to the edge of the table. This behavior is scored with 1. This is the normal score before PT for rats. (D) Fore paw placement, (E) hind paw placement and (F) both paws placement. The scores are the mean value of the score for the group with SD, tested with Kruskal-Wallis (Group B: Sham n=4, EE n=4, STD=3; *: $p<0.005$) when Sham compared to STD; EE: enriched environment; STD: standard environment).

4.3 The corner and corridor tests

The lesion caused to the motor cortex impairs paw functions in the contralateral side of the lesion. As mentioned above, the corner and corridor tests are used to assess sensorimotor of postural asymmetries and turn biases. They have been used in unilateral lesions, for instance in experimental models of Parkinson's disease, where the contralateral body side is affected.

4.3.1 Corner test

In this test, it was counted how many 180º degrees turns and which side has been chosen by the rat during 10 minutes of the test, after passing through all the corridor way. With the total of turns performed, it was calculated the percentage of turns done to each side.

There was no difference either on turning to the right or turning to the left between sham animals and PT animals, or even between EE and STD housed animals (Figure 14).

Hence, it looks like PT does not affect the turn biases at 7 days of recovery after stroke.

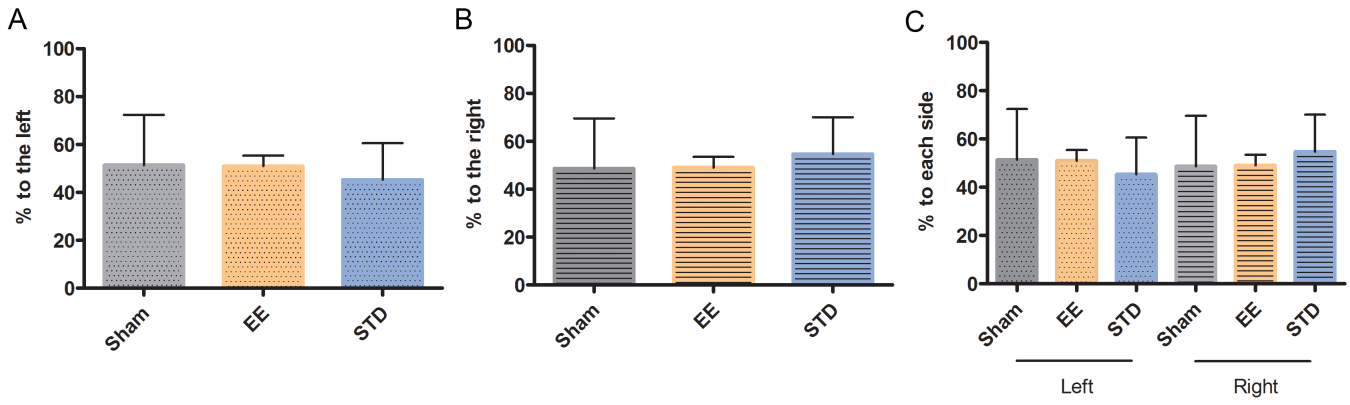

Figure 14. Corner test. Turn of 180º biases of rats subjected to PT during 10 min testing. (A) Percentage of body turns to the left and (B) to the right. (C) Compares both sides percentage. Each body 180º degrees turn of the rat after facing a 45º corner is counted as 1. Then it was calculated the total of turns and also the percentage of the number of turns to each side. No significant changes between groups. The percentage is the mean value of the score for the group with SD, tested with Kruskal-Wallis (Group B: Sham n=4; EE n=4; STD n=3; EE: enriched environment; STD: standard environment).

4.3.2 Corridor test

In the corridor test, each side of the food retrieval was counted during the 10 minutes of the test along the corridor way. Likewise the corner test, where all the food retrievals were counted and the percentage of head turns to the right and left was calculated.

In the same way as in the corner test, in the corridor test there was no differences in the preferable side for food retrieval between sham animals and PT animals, and between EE and STD housed animals (Figure 15).

Thus, PT does not affect the side of food retrieval in the affected animals at 7 days of recovery after stroke.

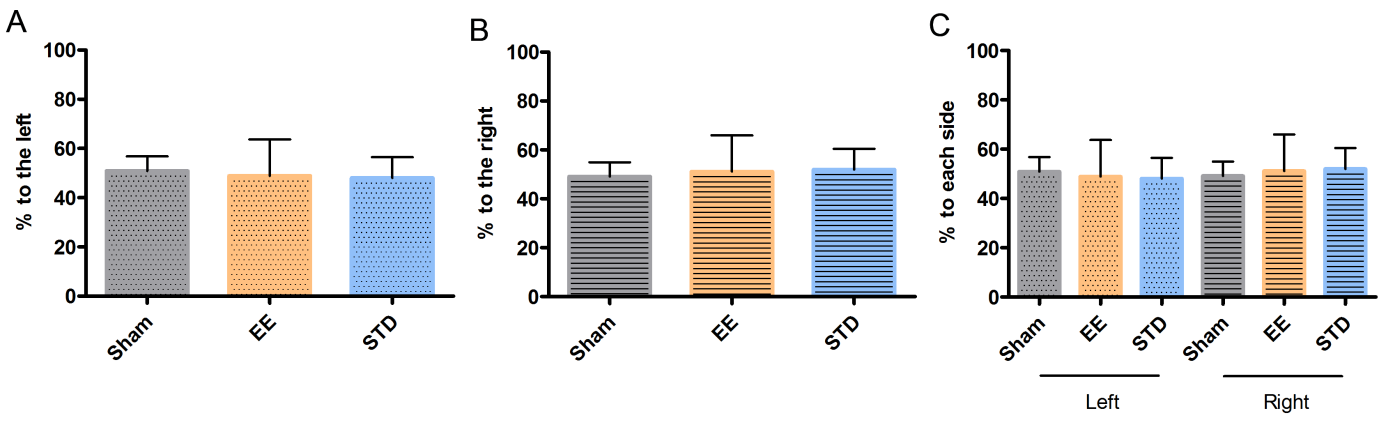

Figure 15. Corridor test. Turn biases for food retrieval of rats subjected to PT during 10 min testing. (A) Percentage of head turns to the left and (B) to the right. (C) Compares both sides percentage. Each head turns towards the pot with sugar pellets of the rat across the corridor is counted as 1. Then it was calculated the total of turns and also the percentage of the number of turns to each side. No significant changes between groups. The percentage is the mean value of the score for the group with SD, tested with Kruskal-Wallis (Group B: Sham n=4; EE n=4; STD n=3; EE: enriched environment; STD: standard environment).

4.4 PT affects degradation of β-DG in the SS

As mentioned above, β-DG is mainly found linked to the endothelial cells of vessels in the brain (Zaccaria et al., 2001). We confirmed this location (Figure 16A). Basically, in most of brain areas, β-DG was found in vessels when stained with a specific antibody (Supplementary Figure 1).

To assess the levels of β-DG in the somatosensory cortex, we employed the Western blot technique. Sham, EE and STD animals either in the ipsilateral (Figure 16B) or contralateral side (Figure 16C), showed the upper band (43 kDa) basically with the same density. Regarding the lower band, the 30 kDa form, a difference between sham and the injured animals was noted. The 30 kDa band is essentially not seen in sham group, while in the most of the EE and STD animals, we can see the product of the degradation.

Analyzing the levels of degradation of β-DG, we saw that the levels of the 43 kDa band were affected by PT, with no differences between EE and STD housed animals, either in ipsilateral or contralateral side (Figures 16D and 16E).

In the SS of the ipsilateral side, the levels of the 30 kDa product of degradation of β-DG are higher in both EE and STD housed animals compared to sham animals (Figure 16F). Hence, experimental stroke enhances the degradation of β-DG. Also, in the EE group more degradation is seen, though this difference did not reach statistical significance. Thus, EE may stimulate an increased degradation at 7 days of recovery after PT comparing to STD in the ipsilateral side.

Interestingly, in the contralateral side, significant degradation of β-DG is only seen in EE housed animals comparing with sham animals (Figure 16G). STD animals showed some tendency for that degradation as well, even if it was not statistically significant.

When we performed immunofluorescence with an antibody against β-DG (Figure 17), we found that not only vessels were stained (Figure 17C).

In summary, β-DG is localized in brain vessels and its degradation into the 30 kDa form product in the somatosensory area of both hemispheres is significantly increased after experimental stroke, particularly in animals housed in an EE.

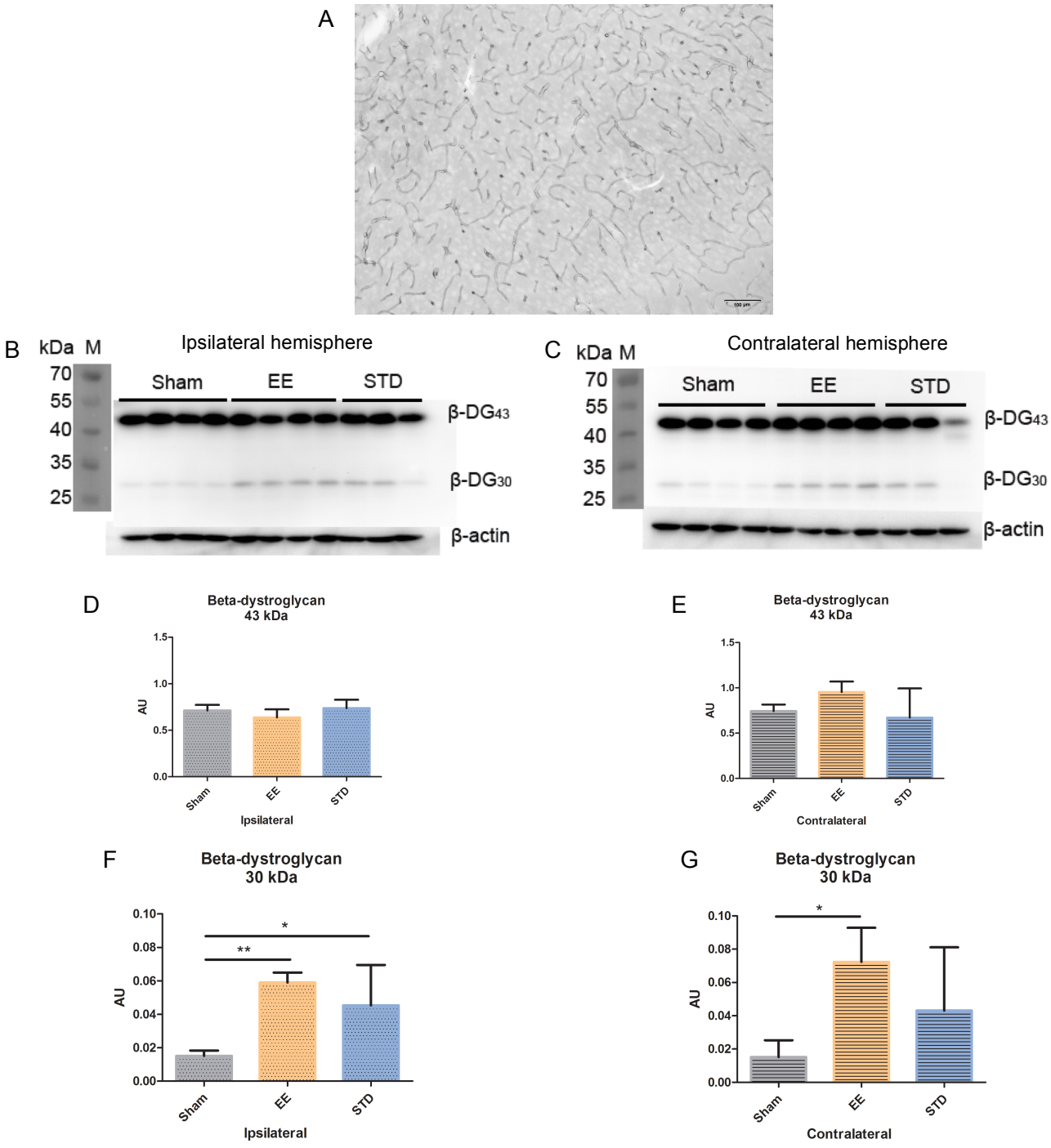

Figure 16. Expression of beta-dystroglycan in the somatosensory cortex. (A) Beta-dystroglycan immunostaining of the cerebral cortex shows the presence of β-DG mainly in vessels. Scale: 50 µm. (B) Expression levels of β-DG 43 kDa and its degradation product 30 kDa in the somatosensory cortex of the ipsilateral and (C) contralateral hemisphere of Sham (n=4), EE (n=4) and STD (n=3) at 7 days of recovery after PT. Final quantification of both forms of β-DG in the total of 11 animals expressed in arbitrary units (AU). (D) Quantification of the levels of 43 kDa form of β-DG in ipsilateral side and (E) contralateral side. No statistically significant differences between groups, Sham, EE and STD. (F) Quantification of the levels of 30 kDa form of β-DG in ipsilateral side and (E) contralateral side. Data displayed as means ± SD and tested with one-way ANOVA followed by Bonferroni correction (*: $p<0.05$). (EE: enriched environment; STD: standard environment; M: marker; AU: arbitrary units; β-DG43: beta-dystroglycan 43 kDa; β-DG30: beta-dystroglycan 30 kDa; β-actin: beta actin.

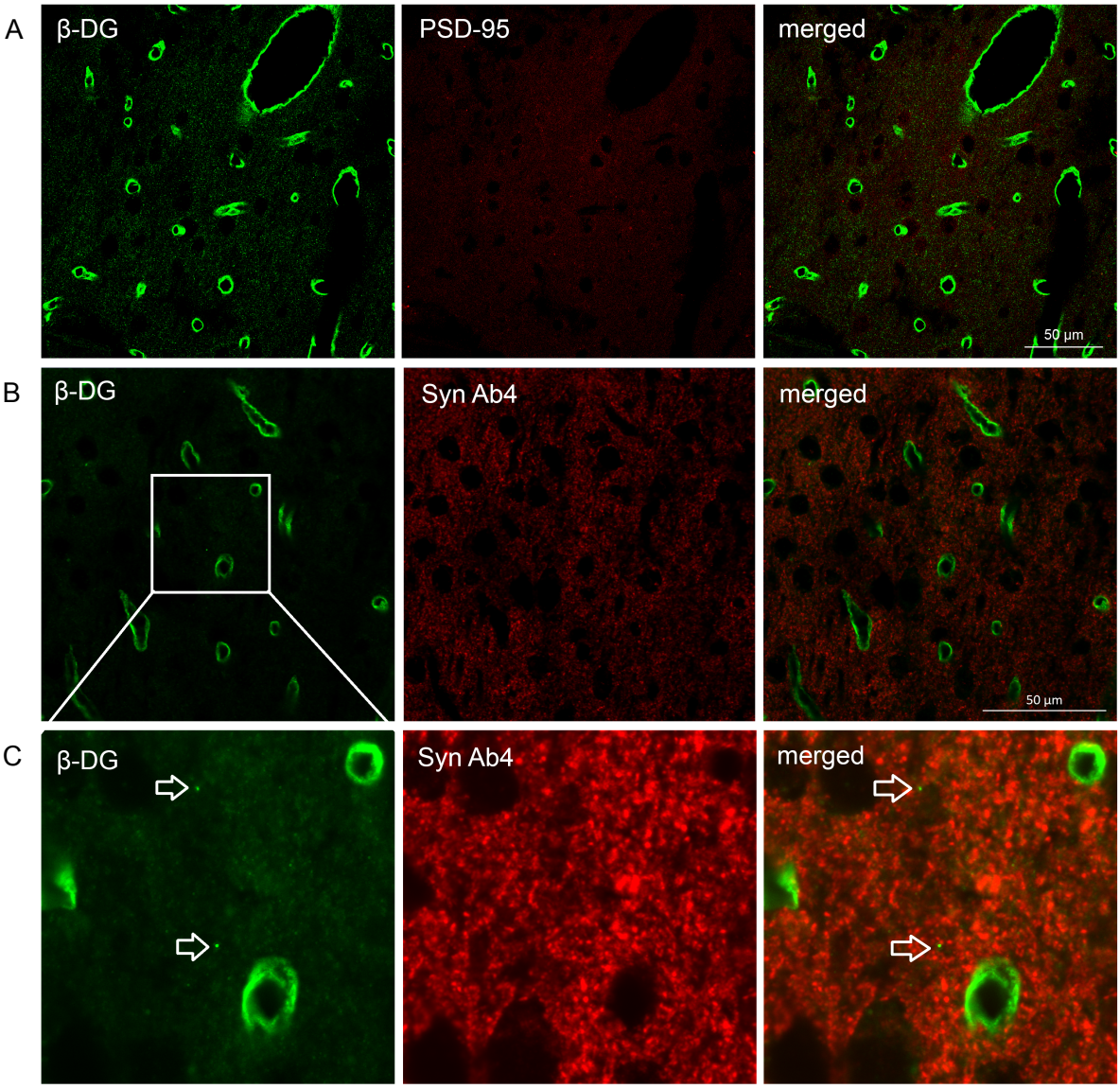

Figure 17. Expression of beta-dystroglycan surrounding vessels in the somatosensory cortex. Confocal images of β-DG (green) with two synaptic proteins (red): PSD-95 (A) and Synaptophysin Ab4 (B). (A) Overall view of β-DG co-stained with PSD-95, showing that besides the vessels, there is specific signal surrounding the vessels. (B) Higher magnification of β-DG co-stained with Synaptophysin, showing specific dots surrounding the vessels. (C) Crop of the upper image to zoom in the image. Arrows: beta-dystroglycan specific dots. Scale: 50 µm.

4.5 PT affects the enzymatic activity of gelatinases MMP-9 and MMP-2 in the SS cortex

As mentioned above, MMPs are known as being brain matrix modulators at delayed stages after stroke (Zhao et al., 2006). Therefore, MMPs may have a role in brain plasticity and ECM remodeling (Zhao et al., 2006) and that EE may aid in functional recovery after stroke by these mechanisms (Madinier et al., 2014). Since the gelatinases MMP-9 and MMP-2 have been implicated in cerebral ischemia (Lo et al., 2002) and β-DG was indicated as an enzymatic target of MMP-9 (Michaluk et al., 2007), we decided to analyze MMP activity in the somatosensory cortex at 7 days of recovery after PT.

To assess the enzymatic activities of MMP-9 and MMP-2, we employed the Real-time gel zymography. Sham, EE and STD animals were analyzed both in the ipsilateral (Figure 18A) and contralateral side (Figure 18E). Three bands representing gelatinolytic activity were analyzed: the upper band, pro-MMP-9 (92 kDa), the mid band, active-MMP-9 (82 kDa) and lower band, MMP-2 (62 kDa). The difference between sham and the injured animals was noted in basically all bands in both hemispheres.

Analyzing the levels of enzymatic activity of pro-MMP-9, in the ipsilateral side, STD animals presented a significant enhanced MMP-activity when compared to sham animals (Figure 18B). However, EE animals did not show a statistical significant difference in the enzymatic activity. In the contralateral side, neither EE nor STD showed difference in the pro-MMP-9 activity when compared to sham, even though the injured animals appeared to have a tendency for a higher gelatinolytic activity (Figure 18F).

Regarding the enzymatic activity of active-MMP-9 in the ipsilateral side of PT, both EE and STD animals had a significant higher MMP-9 activity when compared with non-injured animals (Figure 18C). When analyzing the contralateral side, only EE animals showed a significant enhanced gelatinolytic activity (Figure 18G).

Finally, MMP-2 enzymatic activity was enhanced in both groups of injured animals compared with sham. This result was observed in both hemispheres, ipsi- (Figure 18D) and contralateral (Figure 18H).

It is evident that PT affects the enzymatic activity of gelatinases MMP-9 and MMP-2.

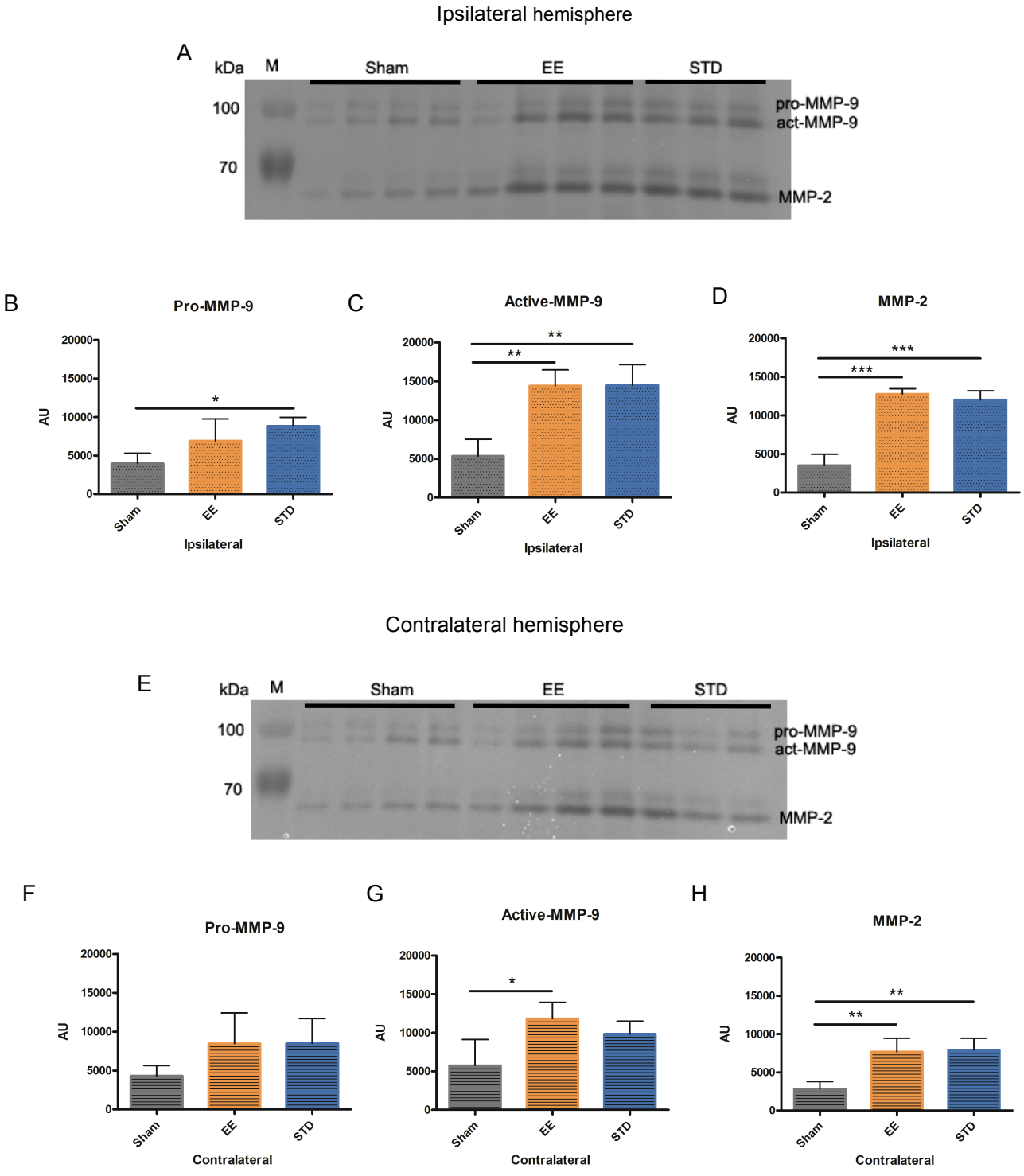

Figure 18. Enzymatic activity of gelatinases MMP-9 and MMP-2 in the somatosensory cortex. (A) Enzymatic activity levels of pro-MMP-9, active-MMP-9 and MMP-2 in the somatosensory cortex of the ipsilateral and (E) contralateral hemisphere of Sham (n=4), EE (n=4) and STD (n=3) at 7 days of recovery after PT. Final quantification of MMPs enzymatic activity in the total of 11 animals expressed in arbitrary units (AU). In the ipsilateral side, quantification of the enzymatic activity of pro-MMP-9 (B), active-MMP-9 (C) and MMP-2 (D). Statistically significant enhanced expression in the injured groups EE and STD when compared with sham. In the contralateral side, quantification of the enzymatic activity of pro-MMP-9 (F), active-MMP-9 (G) and MMP-2 (H). Only MMP-2 showed enhanced enzymatic activity of both injured groups. Data displayed as means ± SD and tested with one-way ANOVA with post hoc multiple comparison test (Tukey test) (*: $p<0.05$). (EE: enriched environment; STD: standard environment; M: marker; AU: arbitrary units; matrix metalloproteinase (MMP)-9: pro-MMP-9: 92 kDa; act (active)-MMP-9: 82 kDa); MMP-2 (62 kDa).

Chapter 5
Discussion

In this study, we show that rats housed in an EE after PT, an ischemic stroke model that affects the fore and hind limb motor areas, have an improved neurological recovery comparing to the STD housed ones, seen by a tactile/proprioceptive limb placing test. The effects of EE in the functional recovery are not due to a decrease in infarct size but correlate with degradation of the brain extracellular matrix molecule β-DG, possibly by MMPs.

The following discussion will be focused on the enhancement of somatosensory functions by EE and the possible underlying biological mechanisms.

5.1 PT model: somatosensory neglect

The PT model of stroke is advantageous in studying recovery mechanisms after experimental stroke, since the localization and size of the lesion can be controlled very well, meaning that it will have little variability among animals (Madinier et al., 2014).

With this model, plasticity processes affecting various brain regions can be studied – from small infarcts induced by single vessel PT occlusions (Sigler et al., 2008) to large brain lesions involving entire functional entities such as the SS (Moon et al., 2009; Starkey et al., 2012). Depending on the size and localization of the lesion, also the recovery is affected and the degree of neglect (inability to process and perceive stimuli on one side of the body).

PT affects the brain leading to deficits in laterality, sensory, motor function and also learning (Madinier et al., 2014).

When we induced the PT lesion, the fore and hind limb motor areas were affected, leaving the somatosensory cortex essentially intact (De Ryck et al., 1989; Madinier et al., 2014) (Figure 12). The ischemic lesion by PT caused a deficit in a sensorimotor test, the paw placement test. As reporter earlier, this test measures the deficit in tactile/proprioceptive response when visual stimuli and whisker/snout contact is prevented (De Ryck et al., 1992, 1989). At 2 days after PT, we saw that the behavioral outcome had a considerable deficit when it was tested with PP showing that the tactile/proprioceptive response was affected.

Then, the extensive lesion to primary motor cortex caused deficits in fine motor skills due to damage of the cortico-spinal tract (CST) and loss of somatosensory functions (Madinier et al., 2014). Hence, PT appears to induce a specific somatosensory neglect (De Ryck et al., 1992, 1989; Madinier et al., 2014).

5.2 EE reverses sensorimotor deficits and dysfunction

Enriched environment induces biological effects in the brain that could account for the positive effect in recovery (Wieloch and Nikolich, 2006). We initiated enriched housing at 2 days after performing experimental stroke, when the infarct can be considered fully matured (Madinier et al., 2014). The rats were kept in EE during 5 days. This time did not affect infarct volume size when compared with rats housed in STD cages. Therefore, improved performance after EE must be associated with recovery of neuronal functions, connectivity and brain plasticity after stroke.

At the end of the 7 days period studied, the animals housed in the EE cages improved from the sensorimotor deficit, assessed by tactile/proprioceptive response that is analyzed by the paw placement test. The whisker and visual contact were prevented, so the improvement of the behavioral performance is because of the tactile/proprioceptive response. While the group of animals housed in EE recovered some of the limb placement response, we observed that the changes in the scores between sham animals and EE were not significant; in the STD animal group, not even a single rat recovered the limb placement response at 7 days of recovery after PT. Although the EE housed animal group did not fully recover the fore paw placement response, data published earlier this year by our group showed that at 12 days of recovery in EE, the animals totally recovered the ability of placing the fore paw back on the edge of the table (Madinier et al., 2014).

Regarding the corner and corridor test, animals subjected to PT comparing with sham animals at the end of the 7 days study period did not show any difference. Even when compared to the baseline percentages, there was no difference before or after experimental stroke for all the groups: sham, EE and STD animals. As shown before, these tests are used for sensorimotor functional assessment, which has been shown to be reliable for identifying and quantifying sensorimotor and postural asymmetries, when there is a unilateral brain lesion (Schaar et al., 2010). Even if the corner test has been shown as suitable for assessment after experimental stroke, in that study the model used was MCAO, not PT (Zhang et al., 2002). There is evidence that the corner and corridor tests take advantage of

multiple partial sensory and motor asymmetries associated with nigro/striatal dysfunction (Fitzsimmons et al., 2006; Schallert et al., 1982; Zhang et al., 2002). The experimental PT stroke model used in our work does not affect either the substantia nigra or the striatum, but only the primary motor cortex, unlike the stroke model MCAO. Hence, our results suggest that corner and corridor tests are not suitable for the behavioral assessment after ischemic stroke lesion that affects specifically the primary motor cortex.

Therefore, we observed a beneficial effect of EE when using a test of sensory ability and proprioception. This suggests that the STD housed animals fail to integrate sensory stimuli to trigger a motor response, particularly when visual and whisker/snout stimuli are absent. EE, stimulating the sensorimotor network, involved in the sensorimotor response, leads to enhancement of cellular and network plasticity. The recovery of tactile sensation and proprioception could be envisaged to involve sensorimotor cortex also remote from the lesion (Madinier et al., 2014).

From earlier studies performed in our lab, we know that EE improves cortical plasticity by reducing the density of PNNs surrounding PV/GABA neurons in the ECM (Madinier et al., 2014). In the same line of that study, we aimed to assess which matrix metalloproteinase could be involved in those changes of brain extracellular matrix and, in particular, if beta-dystroglycan, indicated as target for MMP-9, is part of these ECM changes.

5.3 EE enhances degradation of β-DG into a 30 kDa product

Here, we confirm the presence of β-DG mainly in vessels, as shown before (Zaccaria et al., 2001). Some data show that β-DG is expressed by endothelial cells and astrocytes, both *in vivo* and *in vitro*. Besides, β-DG is expressed on astrocyte endfeet in all cerebral microvessels. These data suggests that a fraction of dystroglycan complex could be the principal adhesion receptor system for anchoring astrocytes to the vascular basal lamina (Milner et al., 2008).

We also show that the levels of β-DG 43 kDa form in the SS stay regular between all groups. However, we show that PT enhances the degradation of β-DG (into a 30 kDa product). In animals of the EE group, the degradation of β-DG is more extensive than the STD group, and when compared with no injured animals as well. The degree of β-DG degradation in sham animals is very low. This suggests that at 7 days of recovery after stroke, the beta-dystroglycan enhanced degradation may be involved in the functional recovery of the EE housed animals comparing to STD animals, which present a lower β-DG degradation level is

lower and worse behavioral outcome. The more extensive degradation of β-DG is seen in both brain hemispheres, indicating that not only the ipsilateral hemisphere is involved in the functional recovery, but also the contralateral hemisphere. This is suggested by a study that showed that functional recovery after one-sided stroke depends in part on the intact contralateral cortex. The recovery previously observed after the right-sided motor cortex stroke was largely abolished by a second left-sided PT stroke (second stroke) (Bachmann et al., 2014). Those results suggest that contralateral cortex contributes to functional recovery probably via direct corticospinal fibers to the denervated hemicord as well as via polysynaptic routes (Bachmann et al., 2014; Benecke et al., 1991; Jankowska and Edgley, 2006). The authors also saw that neurons in the contralateral sensorimotor cortex contralateral to the stroke increased their projections into the left hemicord over time. Hence, the study suggested that cortical fibres project ipsilaterally as well as contralaterally to the spinal cord, and plastic changes occurs on both sides (Bachmann et al., 2014). Another study demonstrated an increased size of cutaneous receptive fields in both hemispheres, in response to tactile stimulation. Their results show no differences between the sizes of receptive fields in the two hemispheres (ipsi and contralateral) in lesioned animals, demonstrating that both hemispheres are affected by brain ischemia (Reinecke et al., 2003).

It is clear from our results and others, that the β-DG expression is manly in the brain vessels, however when analyzing confocal images, we saw that there was a specific signal of this protein outside the vessels and outside the cells. Surrounding the neurons, where the ECM is present, we can see that the protein is present, evident as dots. Indeed, earlier studies have shown that β-DG is also present in the post-synaptic elements (Michaluk et al., 2007; Zaccaria et al., 2001), suggesting that these dots are related with their previous findings. The limited degradation of β-DG after stroke as seen in our Western blots was not depicted in the immunostaining.

Regarding the widely presence of β-DG in brain vessels, it has been shown earlier that animals that exercise after brain injury show an increase in the expression of neurotrophic factors, which regulate neuronal survival and differentiation, synaptic plasticity, as well as angiogenesis in the brain (Endres et al., 2003). A recent study showed that the up regulation of Fibroblast Growth Factor-2 (FGF-2), a neurotrophic factor, induced by EE promote functional recovery through enhanced angiogenesis after brain injury (Seo et al., 2013). Another study showed that with the increased number of brain vessels in a model of rat cerebral hypoperfusion, there are changes in the DGC, more specifically in laminin and β-DG that are associated with the process of vascular remodeling. Their main finding is that the alterations of expressions of these proteins, including β-DG, seem to correlate with angiogenesis rather than BBB disruption, supporting the functional recovery (Wappler et al.,

2011).

Thus, β-DG is a very widely spread protein in the brain, more evident in the vessels but also surrounding the neurons. At 7 days of recovery after stroke, EE leads to an enhancement of the degradation of β-DG in the SS in both ipsi- and contralateral hemispheres, which could facilitate brain plasticity after experimental stroke and enhance functional recovery.

5.4 PT enhances gelatinolytic activity in the SS cortex

In this work, we show that PT enhances the enzymatic activity of both gelatinases, MMP-9 and MMP-2. Since there are two types of fractions from the zymography samples, triton-insoluble fraction (where MMPs are bound to the ECM) and the triton-soluble fraction (MMPs in the cytosol), we decided to analyze the triton-insoluble fraction because we are interested in studying changes that occur in the ECM at 7 days of recovery after experimental stroke.

As mentioned before, MMPs have been implicated in different stages of stroke, including acute and recovery phases (Chaturvedi and Kaczmarek, 2013; Clark et al., 1997; Planas et al., 2001; Rosell and Lo, 2008; Zhao et al., 2006). In the acute phase (the first hours after stroke) the MMPs seem to have a deleterious role (Chaturvedi and Kaczmarek, 2013; Rosell and Lo, 2008), while in the delayed phases they appear beneficial, mediating dendritic plasticity and ECM remodeling of the ischemic brain (Rosell and Lo, 2008; Zhao et al., 2006).

There are many studies showing the particular expression of MMP-9 and MMP-2 in recovery after brain ischemia (Copin and Gasche, 2007; Kurzepa et al., 2014; Planas et al., 2001; Zhao et al., 2006). After the discovery of β-DG being a synaptic target of MMP-9 in response to enhanced neuronal activity (Michaluk et al., 2007), we aimed to assess if MMP-9 and MMP-2 activities change during recovery after PT.

Here, we show that injured animals, either in EE or STD groups, have a statistically significant enhanced enzymatic activity of MMP-9 and MMP-2 when compared with non-injured sham animals. These results support our data of increased degradation of β-DG at 7 days of recovery after PT.

Pro-MMP-9 has a lower expression compared with active-MMP-9 in both hemispheres, showing that most of the MMP-9 is actually in the active form in the ECM, which can contribute to ECM remodeling and brain plasticity. Since brain plasticity is crucial for recovery after stroke, enhanced active-MMP-9 might have a critical role in this functional and behavioral recovery.

MMP-9 and MMP-2 activity is higher in the ipsilateral side compared to the contralateral side. However, analyzing specifically the contralateral active-MMP-9 graph, we saw that only EE animals had a significant difference when compared with non-injured animals. The results are in line with an increased degradation of β-DG in the contralateral side (Figure 16G). Although in this work, we do not have any substantial proof that β-DG is a MMP-9 target, our results suggest a good correlation between these proteins.

Hence, MMPs enzymatic activity is affected by PT and might have a key role for functional recovery. The differences in the gelatinolytic activity between EE and STD groups are not evident at 7 days of recovery, even if some studies showed the main recovery results due to MMPs changes between 7 and 14 days after stroke (Zhao et al., 2006). Additional experiments are then needed to establish the role of MMPs at more delayed time of recovery.

Chapter 6
Conclusion

The PT stroke lesion, which affects the primary motor cortex, the fore and hind paw motor areas, is a model to study the mechanisms of somatosensory dysfunction and recovery after stroke.

We show that EE environment does not affect the infarct size of the injured animals. Hence, the recovery observed in the behavior is due to neurological functional recovery and not to differences in the infarct size.

The EE housed animals recovered the tactile/proprioceptive paw placement response after being 5 days there. Animals housed in the STD cages did not recover any limb placement response. Thus, EE has a beneficial effect in the sensorimotor abilities, which was seen by the paw placement test.

When analyzed the corner and corridor tests, we found that because PT does not make a lesion in either the substantia nigra or striatum, so those behavioral tests are not suitable to analyze any recovery due to the enriched housing.

From earlier studies, we know that the EE improves cortical plasticity and sensorial information processing, by changing the brain ECM. Since β-DG is a target of a matrix metalloproteinase, we decided to study the effects of enriched housing on this protein.

Hence, we confirmed that β-DG is mainly localized in the vessels of the brain but it is also expressed surrounding the cells. We suggest that the β-DG localized outside of the vessels it is expressed in the ECM, more specifically in the post-synaptic elements.

We also propose that the β-DG degradation, which leads to a formation of a 30 kDa β-DG product, it is enhanced in the EE housed animals more than in the STD ones in both SS ipsi and contralateral side, and this may promote cortical plasticity and functional recovery. This degradation can be due to the increased activation of the matrix metalloproteinases, seen before in other studies after experimental stroke.

Finally, we saw that MMP-9 and MMP-2 enzymatic activity are enhanced after PT in both hemispheres, ipsi- and contralateral.

Regarding our future work, the role of β-DG in stroke recovery is still unknown, so further investigation is needed. Our work was done only at one week of recovery after stroke, so it

could be interesting to investigate the levels of degradation of β-DG and gelatinases enzymatic activity (MMP-9 and MMP-2) at different time points of recovery after experimental stroke. Another possible objective for the future is the analysis of the triton-soluble fraction of the zymographic samples, and so being able to study the expression of MMPs in the cytosol.

Chapter 7
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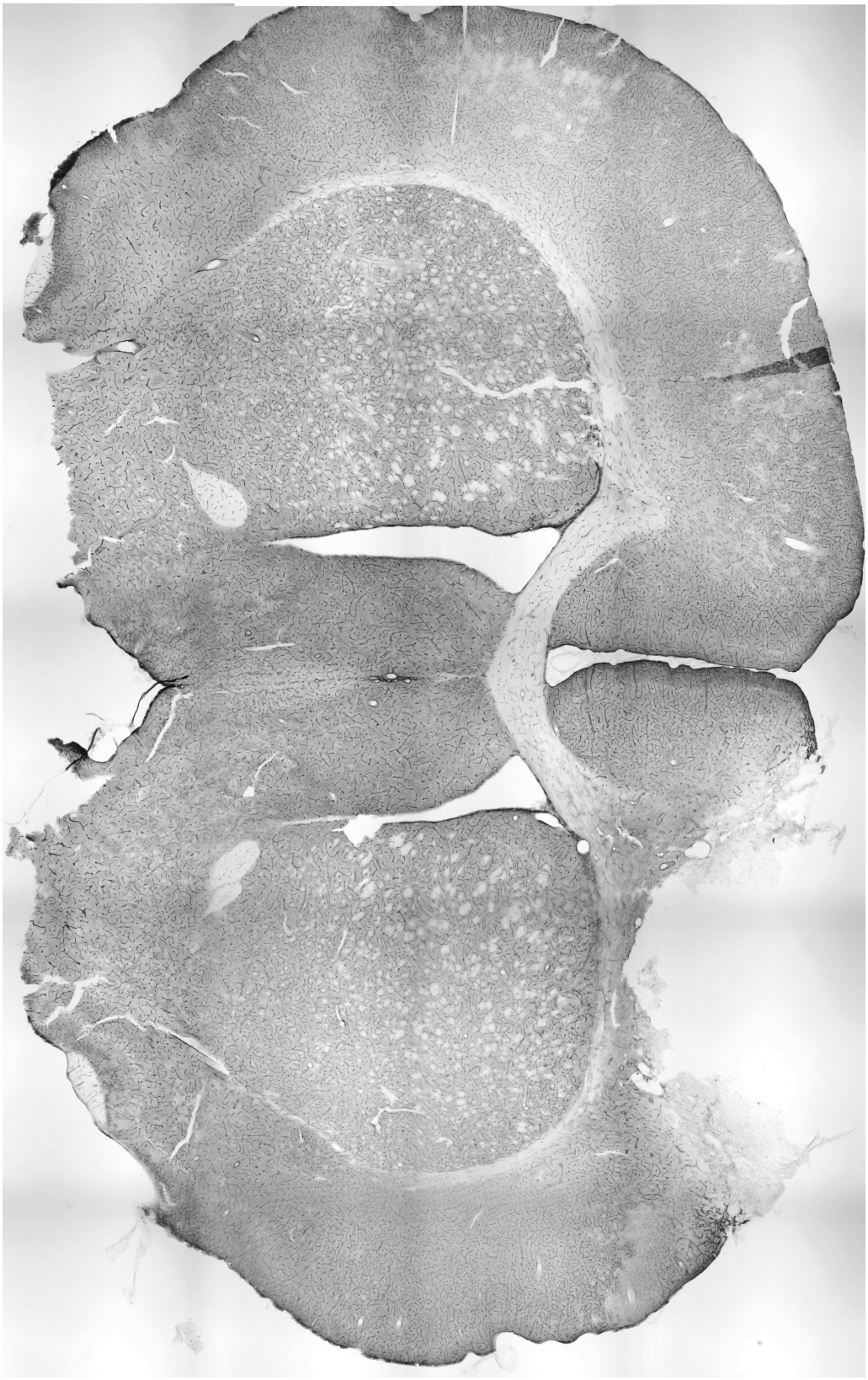
Chapter 8
Supplementary Data

	Weight OP	Anesthesia	Hemisphere	KL 1500 LCD Settings		Ilumination (min)	OP temp °C Rectal	Rose Bengal Administration 15mg RB/kg rat and 10 mg RB/1 ml salin	Place of Infarct/Lamp	Infarct Size
Sham EE	386	10,08	Left	x	x	x	37,3	26 mg in 1.7 ml saline		x
	450	10,49	Left	x	x	x	37,4			x
Sham STD	349	13,19	Left	x	x	x	37,6	34 mg in 2.2 ml saline		x
	413	13,42	Left	x	x	x	37,4			x
EE	358	11,08	Left	4.2C	3000K	20	37,2		0.5mm relativ from bregma, +4mm bregma,-4mm bregma	OK++
	318	13,23	Left	4C	3000K	20	36,8			OK++
	343	10,57	Left	4C	3000K	20	37,2			OK++
	358	9,12	Left	4C	3000K	20	37,4	48 mg RB in 3.2 ml saline		OK++
STD	374	13,10	Left	4C	3000K	20	37,5		0.5mm relativ from bregma, +4mm bregma,-4mm bregma	OK++
	334	14,07	Left	4C	3000K	20	37,5			OK++
	352	11,23	Left	4C	3000K	20	36,8	48 mg RB in 3.2 ml saline		OK++

Supplementary Table 1. Study group B: n=11. Sham n=4; EE n=4; STD n=3. Animal settings to perform photothrombosis.



Supplementary Figure 1. Coronal section stained with beta-dystroglycan. Basically all brain section is stained with beta-dystroglycan, showing that it is widely expressed in the brain in the vessels.