CATECHOLAMINERGIC AXONAL REMODELING IN MOTOR CORTEX OF MICE FOLLOWING STROKE

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Abstract

Stroke is a leading cause of death and morbidity worldwide, and leaves stroke survivors with chronic disabilities. One of the key mechanisms that the brain triggers during stroke recovery is the sprouting of new axons and the formation of new neuronal connections. Meanwhile, studies have evidenced this phenomenon with methods using unspecific cell/axon markers.

The dopamine (DA) system is thought to be implicated in stroke recovery. However, the specific contribution and remodeling of this system to enhance stroke recovery, and whether D1-class receptors play a role in this process, remain unclear. Using a mouse photothrombosis stroke model, immunohistochemical methods, imaging analysis of axonal fiber density and branching in the motor cortex, we demonstrated a specific dopaminergic axon remodeling in the periinfarct region, with or without DA agonist administration. Axonal remodeling of noradrenergic fibers was subtle. In mice subjected to saline IP injection and physical rehabilitation (running wheels), we observed an increase of only DA fiber density in the periinfarct area as compared to the contralateral (intact) side. However, mice treated with DHX for 7 days followed by physical rehabilitation did not show difference between the two hemispheres. Our results suggest a modulatory effect of DHX on axonal remodeling mainly in the contralateral side. Interestingly, treatment of naïve mice with DHX had no effect of DA axon remodeling suggesting that D1-mediated axonal remodeling is stroke-dependent. We also established the temporal profile of post-stroke DA axon remodeling in the absence of DHX and physical rehabilitation. At 4 days post-stroke, there was a significant decrease in DA fiber density and a significant recovery was

measured after 28 days relative to the contralateral side. Altogether, our data highlight a major remodeling of DA axons in motor cortex following stroke, and a potential role for D1-class receptors in improving post-stroke recovery. Understanding adaptations of the DA system following stroke will have a great impact on stroke recovery research.

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List of Abbreviation

ACC: Anterior cingulate cortex

AIS: Axon initial segment

AMPH: Amphetamines

BDA: Biotinylated dextran amine

BDNF: Brain-derived neurotrophic factor

C domain: Catalytic domain

CA: Catecholamines

cAMP: Cyclic adenosine monophosphate

$CaCl_2$: Calcium Chloride

CaMKII: Ca2+/calmodulin-dependent protein kinase II

Cdk5: Cyclin-dependent kinase 5

CBF: cerebral blood flow

CIMT: Constraint-Induced Movement Therapy

CNS: Central nervous system

CREB: cAMP response element-binding protein

CSRP3: Cysteine and glycine-rich protein 32 Cardiac LIM protein (aka CLP, MLP, CRP3, LMO4, CMD1M, CMH12)

D-AMPH: Dextroamphetamine

D1R: D1 dopamine receptor

D2R: D2 dopamine receptor

D3R: D3 dopamine receptor

D4R: D4 dopamine receptor

D5R: D5 dopamine receptor

DA: Dopamine

DAPI: 4′,6-diamidino-2-phenylindole

DAT: Dopamine transporter

DBH: Dopamine β-Hydroxylase

DHX: Dihydrexidine

DOPAC: Dihydrophenylacetic acid

ET-1: Endothelin 1

ERK: Extracellular signal–regulated kinases

EPI: Epinephrine

FGF: Fibroblast growth factors

GAP-43: Growth Associated Protein 43

GDNF: Glial-Derived Neurotrophic Factor

GDF10: Growth differentiation factor 10

GFAP: Glial Fibrillary Acidic Protein

HCl: hydrochloric acid

HVA: Homovanilic acid

IGF: insulin-like growth factor

IHC: Immunohistochemistry

IP3: Inositol 1,4,5-triphosphate

IP: intraperitoneal

L-dopa: Levodopa

LC: locus coeruleus

M1: Primary motor cortex

MAOb: Monoamine Oxidase B

MAP2: Microtubule Associated Protein 2

MAPKAPK-2: MAP kinase-activated protein kinase 2

MCA: Middle cerebral artery

MCAO: Middle cerebral artery occlusion

MPH: Methylphenidate

NT-3: Neurotrophin-3

NT-4/5: neurotrophin-4/5

NGS: Normal goat serum

NE: Norepinephrine

NADH: Nicotinamide adenine dinucleotide

NET: Norepinephrine transporter

NFH: Neurofilament heavy

NGF: Nerve growth factor

OCT: Optimal Cutting Temperature

OGD: Oxygen-glucose deprivation

PAH: Phenylalanine hydroxylase

PBS: Phosphate-buffered saline

PDGF-AA: Platelet-derived growth factor

PFA: Paraformaldehyde

PNS: Peripheral nervous system

PI3K: Phosphoinositide 3-kinase

PRAK: p38-regulated/activated protein kinase

PKA: Protein kinase A

PT: Photothrombosis

R domain: Regulatory domain

ROS: Reactive oxygen species

SSC: Somatosensory cortex

TBS: Tris-buffered saline

TBS-T: TBS containing 0.5% Triton X-100

TBI: Traumatic brain injury

TH: Tyrosine Hydroxylase

TGF-β: Transforming growth factor-β

VEGF: Vascular endothelial-cell growth factor

VTA: Ventral tegmental area

VGF: Nerve Growth Factor Inducible

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Introduction

I. Overview of Stroke

Stroke incidents in Canada were more than 400,000 in 2013, and it is highly prevalent in elderly people (Krueger et al., 2015). The number of stroke related death has been decreasing; however, 82% of stroke patients still experience long-term motor deficits in the upper extremities (hands and arms) (Rathore et al., 2002; Livingston-Thomas et al., 2016). Stroke occurs when blood flow is lost to a specific area in the brain, either due to brain blood vessel blockage (ischemic stroke) that accounts for 80% of total stroke cases or due to rupture in the blood vessel (hemorrhagic stroke) accounting for the remaining 20% of stroke cases (Ojaghihaghighi et al., 2017). The initial clinical symptoms for the two types of stroke are distinct. Hemorrhagic stroke victims appear to suffer from agitated mood, acute onset headache, seizure, and mydriatic pupil, while ischemic strokes progress over many hours with victims experiencing gradual headaches (Ojaghihaghighi et al., 2017). Total cerebral blood flow (CBF) arrest in the stroke core leads to cell death within minutes, while the tissue around the stroke (penumbra) will survive, despite being subjected to reduced CBF (Xing et al., 2012). Cerebral ischemia triggers non-instantaneous ischemic cascade events (Xing et al., 2012). This begins with disruption in the energy supply to the tissue resulting in ionic balance disruption, decrease in glutamate reuptake, and increase in its release which is excitotoxic to the cells (Xing et al., 2012). This will ultimately cause free radical release and other several downstream signals and mechanisms that lead to irreversible neuronal damage (Xing et al., 2012).

II. Challenges in Stroke Recovery Research

Several studies have been conducted on different aspects of brain recovery after stroke to test drugs promoting neuroprotection, anti-inflammatory effects, reperfusion, and enhancement of recovery by a modulation of brain plasticity. Below I briefly review/discuss the therapeutic approaches that have been tested to promote recovery from stroke.

1. Neuroprotective Drugs

Drugs that target the ischemic cascades are considered to be neuroprotective drugs (Cheng et al., 2004). Research using Glutamate antagonists such as Selfotel (Boast et al., 1988), Eliprodil (Gotti et al., 1988; O'Neill et al., 1996; Patat et al., 1994) and Aptiganel (Aronowski et al., 1994; Cohen et al., 1994) block excitotoxicity caused by elevated extracellular concentration in preclinical stroke animal models but failed in clinical trials due mainly to their inefficacy and adverse side effects. Ischemic-triggered cascade also changes the ion channel permeability (ex: calcium channels). Increase in intracellular calcium concentration triggers many nucleases, proteases and phospholipases that lead to tissue damage (Kristián & Siesjö, 1998). Administration of nimodipine, a calcium channel blocker, was thought to be a neuroprotective following stroke (Audiologia et al., 2015). However, nimodipine was not clinically beneficial (The American Nimodipine Study Group, 1992). Free radical scavengers were also tested but they did not show neuroprotective effects clinically (Cheng et al., 2004).

2. Anti-inflammatory Drugs

Ischemic stroke induces an inflammatory response that recruit immune cells (neutrophils, monocytes, macrophages, T cells) and astrocytes to the site of the damage (Lindsberget al., 1996; Gerhard et al., 2000; Price et al., 2004; Buck et al., 2008). Activation and increased infiltration of

the inflammatory cells mediates the neurotoxic effect (ROS production and release of proinflammatory mediators such as chemokines and cytokines) of these cells and worsen stroke damage (Amantea et al., 2009; Kriz, 2006). To prevent the deteriorating effects of inflammatory cells and to promote neuroprotection after stroke damage, anti-inflammatory drugs that target *IL-1*-induced inflammation, microglial activation, leukocyte infiltration and other innate and adaptive immune responses were tested. Most of the tested drugs failed to successfully pass different phases of clinical trials either due to their side effects, not improving recovery or their futility (Cheng et al., 2004; Drieu et al., 2018).

3. Reperfusion Drugs

Alteplase (tissue plasminogen activator or TPA), a thrombolytic drug used to break up the blot clots occluding the cerebral artery and to facilitate the reperfusion of the ischemic brain tissue (Ogata et al., 2013; Picanço et al., 2014; Seitz & Donnan, 2015), is most efficient if administered hyperacutely after stroke.

The current pharmacological intervention for stroke has a very narrow therapeutic window that typically 4.5 hrs post-stroke (Malone et al., 2019). These interventions are only available in select cases and only in the hyperacute stage of stroke. At the chronic stage, neuroprotective and reperfusion drugs are less likely to show improvement on stroke ramifications.

4. Drugs Targeting Brain Plasticity

There is great interest in pharmacological interventions that are applicable for the chronic stage of stroke survivors to improve the long-term outcome after stroke. Improvement of behavioural outcome must be accompanied by enhancing recovery, which encompasses plasticity and growth following ischemic stroke. Recovery-based pharmacological interventions suggest that a wider time frame may help more stroke survivors to show recovery. After stroke,

there are endogenous neurorestorative mechanisms that the brain undergoes. Which typically mimics the reorganization observed in developmental stages (Zivin, 1998). The endogenous mechanisms that occur after stroke at the neuronal, glial, and vascular level are insufficient and suboptimal to promote neurological recovery. Drugs that target the monoaminergic system mainly serotonin (SSRIs) and dopamine (L-Dopa) have shown promise in enhancing the motor recovery outcome after stroke, however clinically results have been mixed (Rösser & Flöel, 2008; Viale et al., 2018). Therefore evidence suggests that restorative drugs that mainly target post-stroke recovery may improve behavioural outcome (Hermann & Chopp, 2012).

III. Brain Plasticity in Naïve Mice and After Stroke

1. Normal Brain Plasticity

Brain plasticity is characterized by any changes in neuronal wiring and function, and it is a continuous phenomenon throughout life (Schwartzkroin, 2001; Kolb et al., 2010). The brain when subjected to internal or external stimulus (sensorimotor input and training, natural rewards, stress, aging, etc…)(Kolb et al., 2010) can induce changes at the molecular, cellular, structural and functional levels (Shaw & McEachern, 2001). In the last two decades, it has been well established that the cortical areas (sensory, motor, visual and auditory) are in a continuously dynamic rather than fixed state (Bütefisch, 2004). There is a correlation between brain plasticity and behavioral outcome (Kolb & Whishaw, 1998). Plasticity is highly age-dependent, demonstrating variability in response to any stimulus when comparing young versus adult brains, more specifically in spine organization and density (Kolb et al., 2010; Kolb et al., 2003c).

The mechanisms neuron possesses to extend and elongate neurites, and ultimately reach their targets are distinct. The morphological alteration that a neuron must undergo to become a

functionally mature neuron can be summarized by 4 steps as follows: 1) initiation of a neurite extension, 2) growth cone guidance and navigation through complex environment, 3) elongation and growing, 4) the final target of the growing axon and formation of connections (Goldberg, 2003; Da Silva & Dotti, 2002).

- **Initiation step is divided into three sub-steps**: initial budding, neurite formation, and polarization. Those steps are highly dependent on actin cytoskeleton mobilization (Flynn, 2013).
- **The guidance and navigation** of the growth cone is greatly reliable on the guidance cues; repellant and attractant (Goldberg, 2003) (e.g. Draxin, Netrin-1, Slits Neurophilins, and Semaphorins). Early differentiation and guidance of the growing axon is influenced by several extracellular soluble molecules such as the fibroblast growth factors FGF2 and FGF7, transforming growth factor-β (TGF-β), vascular endothelial-cell growth factor (VEGF), insulin-like growth factor (IGF), the platelet-derived growth factor PDGF-AA and neurotrophins (Tucker et al., 2001; Labelle & Leclerc, 2000; Mendell et al, 2001).
- For an axon to **grow and elongate** there must be different factors then those required for its survival (anti-apoptotic proteins). In vitro neuronal cultures without any extracellular neurotrophin factors (e.g. nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5)), are not able to extend outgrowing axons from the cell body (Goldberg, 2003).

In a normal adult brain, the most studied aspect of motor cortex plasticity is through synaptic plasticity (Holtmaat & Svoboda, 2009). In the adult brain of rodents, the dendritic arbors of excitatory neurons are stable at the trunk close to cell body; meaning it is not likely to further

elongate and form new branches (Petit et al., 1988; Brown et al., 2010; Trachtenberg et al., 2002). Typically, excitatory neurons in the cortical layers are connected by one presynaptic bouton and one postsynaptic spine to form one synapse. During motor skill learning (Yu & Zuo, 2011; Gipson & Olive, 2017), the spine density in the motor cortex increases within 1-48 hours lasting for months and it is thought to be related to the degree of learning acquisition (Xu et al., 2009; Yang et al., 2009). In vivo studies have demonstrated that axonal boutons are cell-specific and dynamic but axonal terminals are relatively stable in normal mice (Majewska et al., 2006; De Paola et al., 2006).

2. Brain plasticity after stroke.

Plasticity after stroke is altered at the molecular, cellular, network and structural levels, as I will discuss in the following sections below.

a) Molecular Changes

It has been reported that acutely (30-90 min) after cerebral ischemic stroke there is an induction of immediate early genes: c-fos, c-jun, and zinc finger genes (Akins et al., 1996). FGF and NGF trophic factors, which are mainly secreted by glial cells, also increase after stroke (2-5 days post-stroke, and after 1 month it was significantly increased concurrently with marked reactive astrocytosis) (Kogure & Kato, 1993). The gene and protein expression profiles of periinfarct neurons, which undergo growth of their extensions, is different from the molecular profile of the peri-infarct neurons not exhibiting morphological changes of their extensions (Li et al., 2010). Sprouting neurons trigger alteration of the expression of several genes related to growth factors, cytoskeletal modification, epigenetic modulators, and axonal sprouting and guidance molecules (e.g. GDF10) (Li et al., 2010).

b) Structural changes

Neuronal extensions (dendrites and axons) show high dynamic rate of structural changes following stroke (Carmichael et al., 2017). In the following sections, I summarized some of these changes occurring in different neuronal sub-compartments.

Axon Initial Segment: Immunohistochemical analysis performed within two weeks after stroke induction in the rodent forelimb motor cortex showed that the axon initial segment (AIS) is morphologically altered, becoming distally blunt and non-linear instead of being distally tapered off and linear (Hinman et al, 2013). The length of AIS decreases by approximately 15% from their original length in the peri-infarct regions (Hinman et al, 2013). Hinman et al. (2013) also showed the number of small AIS increases mainly in layers 2/3 in the peri-infarct zone, and there are arising new AIS from the proximal area of the existing AIS.

Dendrites: Longitudinal in vivo imaging of the pyramidal neurons in mice in the peri-infarct zone did not show an entire complete branch remodeled (grow or retract) (Brown et al, 2010). But the dendritic tips show significant remodelling by retracting mainly the tips close to the stroke area and preferential growth of the dendritic tips distal/away to the infarct zone mainly 2-6 weeks post-stroke (Brown et al, 2010). However, the total length of apical dendrites did not change (Brown et al, 2010). In agreement with these results, another study did not find a large-scale remodeling of layer 5 pyramidal neurons and robust remodeling of the dendritic branches (Mostany & Portera-Cailliau, 2011).

Axonal damage: In response to the loss of blood flow, neurons in the stroke core die which results in axonal degeneration and synapse loss, that ultimately damage the connections with the other adjacent or remote brain regions that are connected to the stroke area (Baron et al., 2014; Deller et al., 2007; Hinman, 2014). The middle cerebral artery occlusion (MCAO) model

in rodents showed significant decrease in myelinated axons in the periinfarct region one week post-stroke (Ueno et al., 2012). Moreover, distant areas can also be damaged and denervated due to their connections with the stroke area (Cramer & Bastings, 2000; Van Meer et al., 2010; Van Meer, Van Der Marel et al., 2010). The devastating brain damage arising from stroke also triggers growth-related events that allow surviving neurons near or away from the stroke to repair and form new connections, and eventually helping the brain to recover spontaneously (Ohab et al., 2006; Li & Carmichael, 2006; Wang et al., 2010).

Axons Outgrowth: Axonal sprouting is observed in rodent species, as well as in monkeys and humans (Carmichael et al., 2017). Studies have measured axonal outgrowth/regeneration in different stroke models, both in vivo and in vitro. In the rat MCAO model, Ueno et al., (2012) showed high myelinated axonal outgrowth in vivo in the periinfarct region 1-2 months post-stroke, consistent with elevated levels of phosphorylated neurofilament heavy (NFH) subunit, a marker of axonal growth (Bidmon et al., 1997; Sánchez et al., 2000). This observation was further confirmed in cortical neuron cultures using the oxygen-glucose deprivation (OGD) in vitro method to mimic ischemia (Ueno et al., 2012). This study also demonstrated that axonal growth was potentially mediated by phosphoinositide 3-kinase (PI3K)-induced activation of the serine/threonine kinase Akt and Akt-induced GSK-3β phosphorylation, leading to axonal regeneration (Ueno et al., 2012). Plasticity after axonal damage has been demonstrated in another in vitro setting (Abe et al., 1996; Bradke & Dotti, 2000). Indeed, axotomy of cultured differentiated hippocampal neurons induced these cells to regenerate axons from dendrites, which was confirmed both morphologically and molecularly (Bradke & Dotti, 2000).

In the cerebral cortex, sensory and motor areas display different pattern of neuronal connections within the peri-infarct areas after stroke. Stroke in the somatosensory cortex (SSC) induced an area of axonal sprouting 2-4 mm from the edge of the infarct to the motor and premotor areas (Carmichael et al., 2001; Li et al., 2010). In contrast, stroke caused a local increase in projections within the premotor and motor cortex (Overman et al., 2012). Axonal sprouting in the periinfarct areas detected by axonal tracing, using anterograde biotinylated dextran amine (BDA), can be seen after 2-3 weeks post-stroke (Carmichael et al., 2001), but more significantly after 1 month in rodents (Li et al., 2010; Li et al., 2015; Overman et al., 2012). In rodents, stroke in the forelimb motor cortex induces significant axonal sprouting from the motor cortex close to the infarcted site toward the somatosensory region (Li et al., 2015). Caracciolo et al. (2018) have shown that neurons anterior to the stroke site (forelimb motor cortex) are more specifically engaged in axonal sprouting and enhance functional recovery. Neurons located in the associative motor cortex and can be subjected to therapeutic manipulation for enhancing behavioral recovery. Stroke induces limited and suboptimal arrangement of axonal sprouting post stroke from the motor or sensory cortex to the surrounding areas that is insufficient for complete recovery (Dancause et al., 2005; Brown et al., 2009; Li et al., 2010; Overman et al., 2012; Ng et al., 1988; Abe et al., 1996).

Spontaneous biological recovery: Stroke patients show different degrees of recovery; however, rare cases have shown complete recovery where patients regain original brain function (Kwakkel et al., 2003). Photothrombotic stroke in the mouse forelimb motor cortex produces deficits in motor function, with a plateau in spontaneous recovery at day 42 (Clarkson et al., 2010). It has been suggested that one of the key components in brain plasticity to enhance motor

recovery after stroke is the newly developed neuronal circuits in the stroke ipsilateral side, an area associated with functional recovery (Clarkson et al., 2011; Clarkson et al., 2010). Blocking the effect of ischemia-induced genes that are responsible for axon growth inhibition (Ephrin-A5 signaling) and myelin-associated axonal growth inhibitors (Nogo-Nogo Receptor signaling), or delivering genes related to axonal outgrowth (GDF10) after stroke, causes significant increase in axonal sprouting that is correlated with behavioral recovery (Lee, Kim et al., 2004; Li et al., 2015).

3. Cortical reorganization

Stroke induces cortical map reorganization and form connections between motor, premotor, SSC and associated areas (Dancause et al., 2005; Brown et al., 2009; Li et al., 2010; Overman et al., 2012). This is principally due to the alteration in dendritic morphology (Kolb et al., 1997; Kolb & Gibb, 1991), elevated expression of growth-related proteins like GAP-43 (Stroemer et al., 1995), newly sprouting axons, and the newly formed intrahemispheric and interhemispheric connections (Overman et al., 2012; Carmichael et al., 2001) that are believed to be correlated with functional recovery. In the normal brain, the receptive fields of the SSC are clearly distinguished, so, one area in the brain responds to stimulation of one limb (Obi et al., 2018). After SSC stroke, a shift and reorganization/disorganization of the original contralateral topographic representation occur, so one area of the brain now responds to 3+4 limbs stimulation significantly after 4 weeks post-stroke (Obi et al., 2018). Stroke in the forelimb somatosensory cortex causes a shift in the sensory forelimb response toward the motor cortex that persists for 4 weeks post-stroke (Harrison et al., 2013; Caracciolo et al., 2018). Small strokes (~ 0.2 mm^3) in forelimb motor cortex did not affect the position or the excitability of the sensory forelimb area (Harrison et al., 2013). In contrast, the motor map is displaced from its usual

position to occupy the periinfarct regions, which culminates in the hyper-excitability of the periinfarct region within the motor cortex (Harrison et al., 2013; Caracciolo et al., 2018). Forced use of the affected limb through Constraint-Induced Movement Therapy (CIMT) or motor skill learning and extensive training have been shown to promote cortical remapping in periinfarct cortex (Nudo et al., 1996; Plautz et al., 2000; Könönen et al., 2005; Wolf et al., 2006). Evidence in human (Cramer, 2008) and animal studies (Caracciolo et al., 2018) suggest that sensorimotor recovery is greater if the remapping happens at its original place or closely adjacent to it. Collectively, these data show that after stroke there is cortical remapping.

4. Contralateral axonal sprouting

Axonal sprouting from the contralateral cortex to the ipsilateral hemisphere, brainstem, and spinal cord has also been seen following stroke (Wang et al., 2004; Chen et al., 2002; Seymour et al., 2005). It is consistently reported from functional magnetic resonance imaging (fMRI) that the contralesional side was activated while performing tasks (Calautti & Baron, 2003). There is a big debate on whether the activation of contralesional hemisphere has detrimental or supportive role for post-stroke functional recovery (Dancause et al., 2015; Buetefisch, 2015; Jones et al., 2013; Allred et al., 2010). In some patients, there is a beneficial role of contralesional motor areas on some aspects of recovery for complex motor behaviour (Lotze et al., 2006), but in other cases, it can interfere with the recovery of the affected limb (Mansur et al., 2005; Fregni et al., 2006). It is worth mentioning that in normal brain the two hemispheres are connected by interhemispheric connections that cross the corpus callosum such that one hemisphere inhibits the opposite one (Dancause et al., 2015). Reorganization and the increased activity in the contralesional hemisphere may be due to the loss of inhibition from the lesioned M1 on the

contralesional M1. Therefore, increases in contralesional M1 activity after stroke may result in an excessive inhibitory effect on the ipsilesional M1, which may interfere with recovery.

Meanwhile, other studies suggested that the contralesional hemisphere play a positive role in post-stroke recovery (Murase et al., 2004; Shimizu et al., 2002; Boroojerdi et al., 1996). Humans with chronic stroke subjected to theta burst stimulation for one session (Talelli et al., 2007) or multiple sessions (Talelli et al., 2012), to inhibit the contralateral M1 area did not show any benefits on grip force and skilled hand movement. In rodents with small infarcts in the somatosensory cortex, inhibition of the activity of the undamaged hemisphere after 3-4 weeks post-stroke produced modest impairments in the reaching task whereas animals with large infarcts subjected to inhibition of the contralateral region completely failed the reaching task (Biernaskie et al., 2005).

There are multiple plasticity events that have been shown in the contralateral hemisphere of rodents following MCAO or focal cortical injury (Jones & Schallert, 1992; Kawamata et al., 1997; Biernaskie & Corbett, 2001; Chen et al., 2002). Studies have notably shown an increased dendritic arborisation in layer V, which peaked after 18 days post-stroke (Jones & Schallert, 1992; Biernaskie & Corbett, 2001), axonal sprouting from the contralesional motor cortex to the ipsilesional striatum after 20-30 days (Napieralski et al., 1996; Carmichael & Chesselet, 2002; Cheng et al., 1998) and bilaterally to the red nucleus (Chen et al., 2002; Papadopoulos et al., 2002). It is reported that larger strokes in the primary motor cortex of monkeys showed more axonal sprouting from the contralateral hemisphere to the spinal cord after a long-term spontaneous recovery period between 6 to 12 months. Additionally, contralateral corticospinal projections have been seen in rats and mice (Benowitz & Carmichael, 2010; Lindau et al., 2014).

Rehabilitation and growth promoting protein manipulations such as fibroblast growth factor, inosine and anti-NogoA (Papadopoulos et al., 2002; Chen et al., 2002; Kawamata et al., 1997) enhance the functional recovery, which is associated with increased expression of axonal growth makers (GAP-43) in the contralesional side and increased axonal sprouting to the remote denervated areas from the intact side (Chen et al., 2002).

5. Rehabilitation promote axonal sprouting

Rehabilitation training is the most effective therapy to date for stroke survivors. It is thought that axonal sprouting is one of the key mechanisms for rehabilitation-induced functional recovery (Okabe et al., 2017; Okabe, Himi, et al., 2017; Jones & Adkins, 2015; Nudo et al., 1996). This has been demonstrated directly by retrograde and anterograde tracer experiments (Okabe, Himi, et al., 2017; Okabe et al., 2016), or indirectly by motor map reorganization (Jones & Adkins, 2015; Hamzei et al., 2006; Nudo et al, 1996; Taub et al., 2002; Plowet al., 2015; Frost et al., 2006; Fridman et al., 2004; Murata et al., 2015; Eisner-Janowicz et al., 2008). Nudo et al. (1996) showed that when animals were subjected to skilled hand movement training, the non-hand area (shoulder area) reorganized to represent the hand area after ischemic infarct of primary motor cortex. Axonal sprouting in post-rehabilitation training occurs in both hemispheres (Okabe et al., 2017). Approaches like: CIMT, skilled reach training, and rotarod training (Maier et al., 2008; Zhao et al., 2013; Starkey et al., 2011; Lee et al., 2013; Okabe et al., 2016; Okabe, Himi, et al., 2017) were used to study the axonal remodeling following rehabilitation training in rodents. It is well known that motor cortex directly controls the limb movements through corticospinal tracts (Ueno et al., 2018). Axonal sprouting in post-stroke with rehabilitation was mainly described in the projections from motor cortex (ipsilateral and contralateral side) to the spinal cord (Okabe et

al, 2017). Rehabilitation training following photothrombotic stroke in the caudal forelimb motor cortex of rats induces the formation of stem axons and collateral fibers in the dorsal column in the corticospinal tract projecting from the surviving ipsilateral forelimb motor cortex (rostral forelimb area) (Okabe et al., 2016; Okabe, Himi, et al., 2017).

IV.Catecholamine System

Catecholamines (CA) comprise 3 neurotransmitters, dopamine (DA), norepinephrine (NE), and epinephrine (EPI), that are present in the central and peripheral nervous systems (CNS and PNS) (Lot, 1993; Nagatsu, 2006). Notably, DA and NE are widely distributed in the cerebral cortex (Gaspar et al., 1989). Catecholamines are structurally similar in that they all possess a 3,4-dihydroxyphenyl (catechol) nucleus; however, despite their similar structure they exhibit different functions in CNS and PNS (Nagatsu, 2006). Catecholamines are synthesized from the amino acid phenylalanine, which is converted into to L-tyrosine by phenylalanine hydroxylase (PAH) primarily in the liver (Fernstrom & Fernstrom, 2018).

1. The Dopamine System

The major catecholamine neurotransmitter in the mammalian brain is DA. DA modulates many functions in the CNS including locomotor activity, cognition, emotion, positive reinforcement, food intake, and endocrine regulation (Missale et al., 1998).

a) Dopamine Synthesis

Dopaminergic neurons take up tyrosine (derived from phenylalanine metabolism and dietary proteins) from extracellular fluid in the brain by active transporters (Missale et al., 1998). L-tyrosine is converted to the DA precursor L-dopa by tyrosine hydroxylase (TH) using oxygen,

iron, and tetrahydrobiopterin as cofactors (Dunkley & Dickson, 2019; Daubner et al., 2011). L-dopa is rapidly metabolised to DA by the aromatic L-amino acid decarboxylase.

TH is the rate limiting enzyme in the CA synthesis pathway and is predominantly expressed in the cytoplasm (Pickel et al., 1975). However, there is evidence suggesting that TH can be trafficked to the axons and presynaptic nerve terminals where it can be translated locally and thereby facilitate downstream dopamine synthesis in the axons (Aschrafi et al., 2017; Gale et al., 2016).

TH consists of 3 domains: an N-terminal regulatory domain, a central catalytic domain, and a C-terminal tetramerization domain (Daubner et al., 2011). Regulation of TH activity occurs primarily by phosphorylation/dephosphorylation of the regulatory domain and via feedback inhibition exerted by CAs binding to the TH active site and promoting its inhibition (Dunkley et al., 2004) . The regulatory domain contains four regulatory serine residues, serine 8, 19, 31 and 40 (Daubner et al., 2011; Dunkley et al., 2004), that are subjected to phosphorylation (Daubner et al., 2011)(**Figure 1**). Phosphorylation of Ser8 has not been shown to have an effect on TH activity (Daubner et al., 2011). Phosphorylation at Ser40 has been shown to directly increase enzymatic activity in situ, in vitro and in vivo by promoting catecholamine dissociation from TH and thereby relieving the cathecholamine feedback inhibition (Dunkley et al., 2004; Dickson & Briggs, 2013). Phosphorylation at ser31 can moderately activate TH (Sutherland et al., 1993). Additionally, phosphorylation at Ser19 and at Ser31 increases the rate of phosphorylation at Ser40 by three- and ninefold respectively (Bevilaqua et al., 2001; Lehmann et al., 2006; Toska et al., 2002). Hierarchical phosphorylation at serine residues occurs in vitro, in situ and in vivo (Dunkley et al., 2004; Dunkley & Dickson, 2019). Once DA is synthesized, it will be released by the

presynaptic sites into synaptic cleft where it can interact with presynaptic and post-synaptic dopaminergic receptors (Nagy et al., 1978).

cInactive form

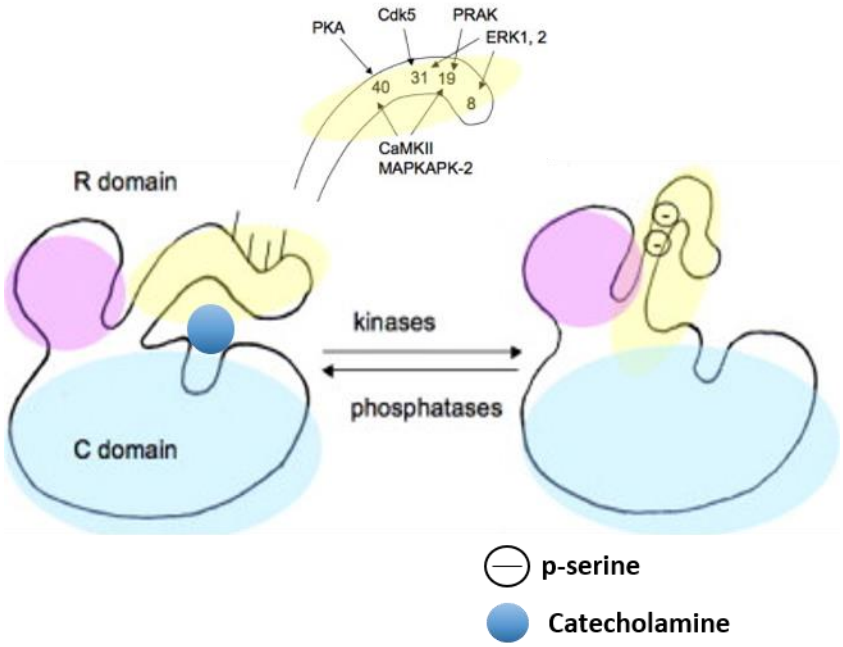


Figure 1: Structural Topology and Regulation of TH: The catalytic domain (C) light is in blue, The Regulatory (R) domain is in lavender with the mobile portion in yellow that contains serine residues phosphorylated by different kinases (PKA, CaMKII, MAPKAPK-2, ERK1 & 2 Cdk5 and PRAK). Upon phosphorylation of the R domain it moves out of its position obstructing access to the active site. Phosphatases then return the R domain to its inactivating position. Binding of catecholamines deactivate the enzymatic activity (shown in blue circle). Adapted and modified from Daubner et al., 2011 (figure 5 & 6).

b) Dopamine Receptors

Dopaminergic receptors belong to the GPCR family and are divided into 2 major classes: D1-class- (D1R and D5R) and D2-class- (short and long isoforms of D2R, D3R and D4R) subtypes. D1-class receptors are coupled to $G_{\alpha}s$/olf, while D2-class receptors are coupled to $G_{\alpha}i$/o, which respectively stimulate and inhibit adenylyl cyclase activity (Ayano, 2016). The D1 receptor is the most abundant subtype in the brain while the D2 receptors are the second most abundant subtypes (Romanelli et al., 2009). D1-class receptors are found mostly in the frontal and temporal

cortex, substantia nigra pars reticulata, olfactory bulb, nucleus accumbens, caudate, putamen, striatum, hypothalamus and hippocampus (Ayano, 2016; Grandy et al., 2016). D2-class receptors are found mainly in the VTA, nucleus accumbens, basal ganglia and frontal cerebral cortex (Malenka et al., 2009; Ayano, 2016).

c) The Dopaminergic Pathways

The dopaminergic system encompasses four major pathways (Björklund & Dunnett, 2007; Grattan, 2015; Brunelin et al., 2013; Ayano, 2016). The **nigrostiatal pathway** consists of dopaminergic neurons in substania nigra pars compacta that send projections to the basal ganglia and play an important role in the modulation of motor control and learning new motor skills. The **mesolimbic pathway** consists of dopaminergic neurons in the VTA that send projections to the amygdala, pyriform cortex, lateral septal nuclei and the nucleus accumbens, and plays a role in pleasure and reward seeking behaviors, addiction and emotion. The **mesocortical pathway** consists of VTA projections to the frontal cortex (including the motor cortex) and septohippocampal regions. The **tuberoinfundibular Pathway** consists of dopaminergic neurons in the arcuate nucleus that send projections to the pituitary gland, acting to regulate prolactin release**.**

d) Mesocortical Pathway Functions and Signaling through D1-Class Receptors

D1 receptors are expressed in high concentrations in prefrontal cortex. The primary motor cortex receives a high amount of dopaminergic projections mainly in layers V/VI from the neurons in the mesencephalic brainstem (mostly from the VTA) (Hosp & Luft, 2013; Berger et al., 1985; Hosp et al., 2011). The main functions of these projections are to mediate normal cognitive functions and contribute to the regulation of motivation and emotion. However, this pathway

has also been shown to be implicated in the acquisition of new motor skills and to promote motor plasticity (Hosp & Luft, 2013).

The destruction of these projections to M1 impedes motor skill- learning and long-lasting memory consolidation in rodents (Buitrago et al., 2004; Luft & Buitrago, 2005). Moreover, a role of mesocortical dopamine projections in experience-dependent changes of motor memory storage has been demonstrated at multiple biological levels, namely gene, network, physiology and synaptic transmission (Hosp & Luft, 2013; Monfils et al., 2005; Hikosaka et al., 2002). Roles of mesocortical projections have also been demonstrated in pathological conditions including Parkinson's disease and traumatic brain injury, which have been shown to have altered dopamine transmission to the motor cortex (Hosp & Luft, 2013).

D1 receptors are expressed in high concentrations in prefrontal cortex (mesocortical areas). For detailed review of signaling through D1-class receptor refer to Neve et al. 2004 review. Briefly, D1-class receptors are coupled to $G_{\alpha}s$/olf which activate adenylyl cyclase leading to increased intracellular cAMP. Subsequently, primarily through cAMP-mediated activation of PKA, D1R activation ultimately lead to the phosphorylation of sodium channels, calcium channels and ionotropic glutamate receptors that enhances the influx of cations through these channels and consequently increases neuronal excitability.

2. The Norepinephrine System

The NE system is responsible for modulating attention, arousal, and cognition during many behaviors. NE neurons express the enzyme dopamine-β-hydroxylase which converts DA into NE. The norepinephrine transporter protein (NET) is located on axonal terminals of noradrenergic neurons and is responsible for the reuptake of extracellular NE.

The locus coeruleus, located in the brain stem, is the primary source of NE projections to many different brain areas including: amygdala, cingulate gyrus, cingulum, hippocampus, hypothalamus, neocortex, spinal cord, striatum and thalamus. Two bundles of NE fibers pass rostrally through the brainstem, a dorsal bundle and a ventral bundle. The dorsal fibers project to the cerebral cortex, while the ventral fibers innervate other brain areas like the hypothalamus (Ungerstedt, 1971; Lindvall & Björklund, 1974). NE innervation is largely segregated in the cerebral cortex with projections being much more abundant in layer I than in layer V and VI (Chandler et al., 2014). The effect of NE is mediated by α- and β-adrenoreceptors that are widely expressed throughout all cortical layers (Vitrac & Benoit-marand, 2017). LC neurons extend projections to the motor cortex and it is believed that NE enhances some aspects of motor functions (Donaldson et al., 1976). Interestingly, physiological and pharmacological manipulations of adrenergic receptors in the motor cortex was found to change the excitability and firing rate of motor neurons (Vitrac & Benoit-marand, 2017; Plewnia et al., 2004). Additionally, it was demonstrated that NE enhances motor skill acquisition in humans.

V. Catecholaminergic System and Stroke.

Amphetamines induce the release of monoamines (mainly dopamine and norepinephrine) and promote excitatory neurotransmission in the CNS (Heal et al., 2013), and have been the subject of stroke recovery research. Despite basic research studies suggesting benefits of amphetamine administration in the functional recovery of motor function following stroke (Bradley & Damiano, 2019; Sprigg & Bath, 2009), clinical trials using amphetamines have led to conflicting results with respect to an improvement of post-stroke motor recovery (Walker-

Batson, 2013). Consequently, studies have focused efforts on the potential benefits of specifically activating a single catecholamine system or receptor, which will be discussed in the following sections.

1. Noradrenergic System and Stroke

The role of the noradrenergic system in stroke is not well understood. During transient global stroke in rats an 18-fold increase of NE in the hippocampus was reported. Following the increase, NE levels were found to gradually return back to the baseline after 40 min (Globus et al., 1989). Windle and colleagues (Windle et al., 2007) used the neurotoxin N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) to selectively destroy norepinephrine projections from the locus coeruleus in rats after ET-1 stroke model. NE depletion was found to facilitate recovery for animals that did not undergo any physical rehabilitation and not house in enriched environment. Moreover, NE depletion did not impede recovery facilitated by intensive rehabilitation using the staircase reaching test

Additionally, it was found that blocking the adrenergic receptors, (alpha-1, alpha-2, and beta subtypes) has an acute effect on decreasing the stroke volume in rodents (Monai et al., 2019) and facilitates functional motor recovery when administered 30 min before stroke or up to 2 hours post-stroke. This neuroprotective effect is thought to occur via regulation of extracellular potassium ions that were shown to be abnormally elevated extracellularly due to the stroke (Monai et al., 2019). In contrast, increasing the signaling specifically through beta-2 receptors reduces the pro- and anti-inflammatory responses of microglia and macrophages 3 hours after stroke onset and increases the stroke volume (Lechtenberg et al., 2019).

In humans, a single dose of the noradrenaline reuptake inhibitor reboxetine promoted recovery for the affected hand on some behavioral tests (increased maximum grip power and index-tapping frequency) around 2 hours after its administration (Wang et al., 2011). This recovery was associated with normalized neural and network activity (reduction of abnormal increase in cortical activity in both hemispheres) in motor and sensory motor cortex during behavioural tests (Wang et al., 2011). However, it is not known if reboxetine enhances recovery over the long term or if it results in any side effects. Additionally, reboxetine was more effective at promoting recovery in patients with lesions occurring closer to the time of examination (Wang et al., 2011). Overall, our current understanding of the role of the noradrenergic system in stroke is incomplete.

2. Dopaminergic System and Stroke

a) Stroke-Associated Alterations in Dopaminergic System Post-Stroke

Experimental studies have shown fluctuations in dopaminergic system components during and after stroke in rodents and non-human primates (Gower & Tiberi, 2018).

For in vivo and in vitro stroke models that impact the striatum, a significant increase of **DA levels** in the striatum has been reported following stroke onset (Gower & Tiberi, 2018). This increased DA level eventually returns back to a normal level regardless of stroke model or ischemic time periods. However, studies that used different stroke models have found different timing for the return of DA levels back baseline, with reports of anywhere from 30 min to several hours post-stoke (Gower & Tiberi, 2018). Interestingly, the level of **DA metabolites** (DOPAC and HVA) were nonreciprocal with DA levels. They were found to significantly decrease during stroke, then rise after stroke offset (Globus et al., 1988; Kawano et al., 1988; Slivka et al., 1988).

Cerebral ischemia in rodents also causes a massive increase in DA levels that goes back to normal levels in the ipsilateral hemisphere after 2 to 24 hours of stroke onset (Zervas et al., 1974; Harrison et al, 1979; Ahagon et al., 1980). In non-human primates, 1 hour after stroke there is a significant increase in DA levels in cortical areas, however 3 hours after stroke DA levels were significantly decreased in the ipsilateral hemisphere in comparison with the contralateral hemisphere (Zervas et al., 1974; Ishihara et al., 1979; Gower & Tiberi, 2018). Additionally, the DA level (but not DA metabolite levels) in the contralateral side SSC was also increased 1 week post-stroke, while after 2 weeks it was reduced in comparison to sham operated animals (Obi et al., 2018). Finally, DA synthesis enzymes, TH and DOPA decarboxylase, and the **dopamine transporter (DAT)** were found to be downregulated in the ipsilateral hemisphere SSC in comparison to the contralateral hemisphere at 2 and 24 hours in rats post-stroke (Uzdensky et al., 2017).

No clear picture of the expression of dopaminergic receptors after stroke has been established. However three studies have reported an increase in D2R expression 2-14 days after stroke in the subcortical areas, on either the ipsilateral or contralateral side (Dawson et al., 1994; Martín et al., 2013; Sieber et al., 2014), while two studies have reported a decrease in D1R expression on the ipsilateral side in an MCAO stroke model (Abe et al., 2004; Sieber et al., 2014). Interestingly, a study using D1R- and D2R-antagonists before 30 min of stroke onset showed that the potential toxic effect of the increased level of DA during ischemia is likely due mainly to the actions of D2R, while D1R could be involved in mechanisms that cause neuronal inhibition and neuroprotective effects (Hashimoto et al., 1994). Another study also reported that D2R

antagonists exert neuroprotective effects if they are applied directly after stroke (Yulug et al., 2006 a,b).

b) Effects of Dopaminergic System on Post-Stroke Recovery: A Current Dilemma

Research testing the involvement of the DA system in post-stroke recovery has focused on enhancing DA neurotransmission through the use of direct DA receptor agonists and indirect dopaminergic agonists such as the DA precursor L-dopa, or amphetamine derivatives, which less specifically affect DA transmission but instead more broadly activate catecholaminergic transmission in general. A recent review by Gower and Tiberi (Gower & Tiberi, 2018) that discussed the therapeutic targeting of the dopaminergic system in the facilitation of recovery after stroke can be consulted for a detailed overview of this topic. Here, I will summarize some important facts about the conflicting findings.

Several pre-clinical and clinical studies were conducted using DA-stimulating drugs including amphetamines (AMPH), dextroamphetamine (D-AMPH) and methylphenidate (MPH). However, these studies were inconclusive as their therapeutic benefits on cortical stroke recovery were limited (Gower & Tiberi, 2018; Kumar & Kitago, 2019; Bradley & Damiano, 2019). Behavioral tests to assess recovery after AMPH administration in pre-clinical research such as beam walk test, tactile placing, cylinder, horizontal ladder, foot fault test, skilled reaching test were used. The majority of these pre-clinical studies have reported the beneficial effect of AMPH on motor and sensorimotor recovery when repeated administration was used. Moreover, an immediate effect following AMPH administration was observed in some behavioral tests while in others the beneficial effects were recorded after a delay following the drug administration (Papadopoulos et al., 2009; Schmanke & Barth, 1997; Stroemer et al., 1998; Wolf et al., 2014; Gower & Tiberi,

2018). On the contrary, other preclinical studies have reported that AMPH has no positive effect on recovery (Rasmussen et al., 2006; Alaverdashvili et al., 2007; Auriat & Colbourne, 2008; Brown et al., 2004; Gower & Tiberi, 2018); either due to factors such as drug administration timing, location of the lesion, methods for performing the lesion and/or different methodology for performing the behavioral tests.

In Clinical studies, some found it beneficial and others showed a trend in recovery especially when combined with physical therapy (Crisostomo et al., 1988; Gladstone et al., 2005; Martinsson & Wahlgren, 2003; Schuster et al., 2011; Gower & Tiberi, 2018). Conversely, other studies did not report any beneficial effect of AMPH administration (Treig et al., 2003; Sonde & Lökk, 2007; Gower & Tiberi, 2018). A more recent clinical study using an administration of 10 mg of D-AMPH 10-30 days post-stroke followed by 1 hour physical training for a total of 6 sessions did not show evidence of motor functional improvements compared to placebo-control patients (Goldstein et al., 2018). These mixed results could be due to many factors such as proper dosage, time and period of drug administration, dissimilarity in experimental design and the control for variabilities (e.g. the age, co-morbidities, within the experiment, location and size of stroke lesion location, age of stroke onset, genetic polymorphisms, and the large spectrum of action of DA-enhancing drugs (Gower & Tiberi, 2018)).

The studies of AMPH effect in stroke recovery led to studies investigating the effect of selectively stimulating the dopamine system either by L-dopa or specific dopamine receptor agonists. One study investigated the effect of L-DOPA on rats. Three different doses (1, 5 or 20 mg/kg) were administered for 5 days, and it was found that the 20 mg/kg group was significantly improved relative to the control animals at 7 days post-infarct (Ruscher et al., 2012). Clinical

studies using L-dopa to improve motor recovery from stroke showed some promises, but overall results from clinical studies are still mixed and inconclusive (Stinear, 2019). Potential reasons for this are that length of intervention and time since injury varied greatly across studies with treatments ranging from a single dose to a 7-week trial, and started as early as 5 days- to as late as 20 years after injury (Gower & Tiberi, 2018; Bradley & Damiano, 2019). Longer administration, often combined with physical therapy, appears to have a more significant impact on motor outcomes, and may do so by modulating motor cortex excitability and the ability to learn and encode motor memories (Gower & Tiberi, 2018; Bradley & Damiano, 2019).

Limited studies have been done on direct dopamine receptor-specific agonists. Three studies to date, two in humans and one in mice, have assessed the effect of D2R agonists on motor functions post-stroke. In a rat study, the partial D2R agonist aripiprazole was administered at day 14 post–stroke for 4 days and led to significant improvement in recovery by facilitating proper reorganization of the receptive fields in the SSC (Obi et al., 2018). However, the two human studies using ropinirole and rotigotine did not find significant improvement on various tests of motor function for patients given the D2R antagonist compared with placebo patients (Cramer et al., 2009; Gorgoraptis et al., 2012).

Collectively, these results show that the dopamine system plays an important role in stroke recovery outcomes, and that optimizing timing, dose and duration of treatments targeting the dopamine system should be an important goal moving forward in stroke recovery research.

VI. Role of D1-class Receptors in the Enhancement of Post-Stroke Motor Recovery: Findings from the Tiberi Lab

Previous work in our lab has used a D1-class agonist, dihydrexidine (DHX), to investigate the role of D1-class receptors (Mottola et al., 1992) in promoting motor recovery following stroke. Timeline of the proof-of-concept study is depicted in (**Figure 6**). Six-week old mice were acclimated in reverse/light dark cycle for two-weeks, which was followed by training and assessment of pre-stroke motor functions on three validated behavioural tests: the adhesive removal test, the horizontal ladder test, and the cylinder test (Schönfeld et al., 2017). Photothrombosis (PT) was used to induce stroke in the left motor cortex. After stroke, the three behavioral tests were performed again to determine post-stroke motor deficits, and mice were randomized in two experimental groups to receive single daily i.p. injection of 4 mg/kg of DHX or saline for 7 consecutive days. At the end of drug treatment, behavioural tests were performed to assess motor function. Animals were then placed in cages with 24-hour free access to a running wheel for 9 days as a form of physical exercise. Finally, animals were sacrificed at 31 days post-stroke.

Our results showed a functional recovery on the adhesive test after the 7 days of drug administration (**Figure 2**), while on the ladder test animals showed recovery after physical training (running wheels) (**Figure 3**). Saline and DHX-treated mice did not show any motor recovery on the cylinder test (**Figure 4**). To shed further light on the potential underlying mechanisms of the DHX treatment combined with physical exercise in promoting faster motor

recovery on the adhesive removal test and horizontal ladder test, I aimed to investigate the impact of DHX treatment on the modulation of axonal sprouting.

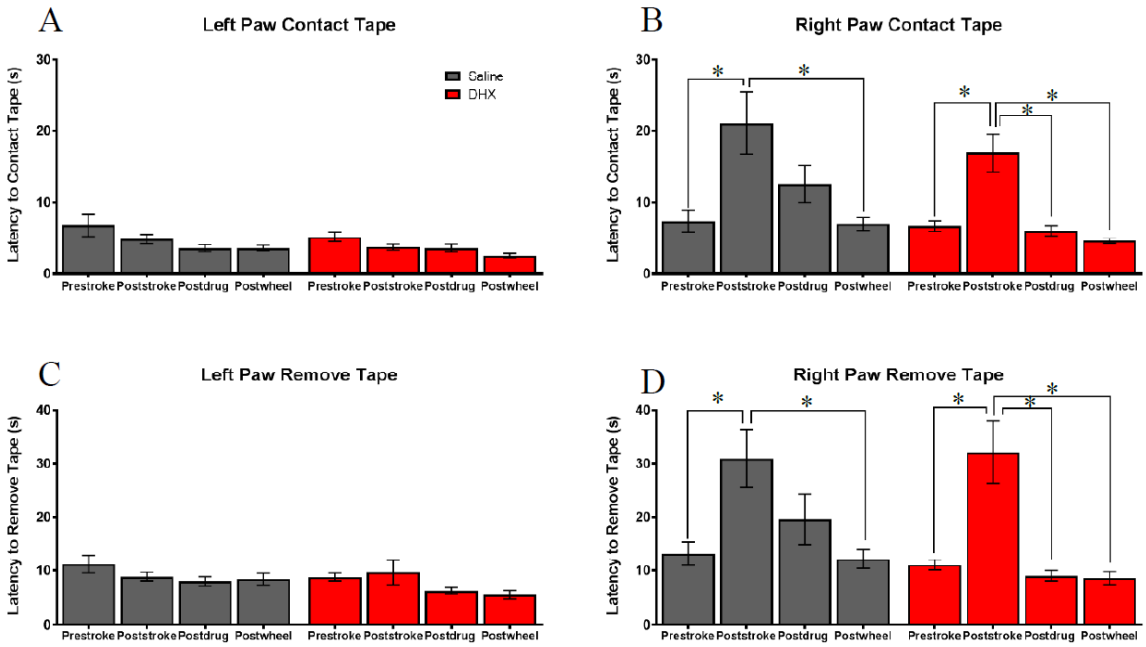


Figure 2: Adhesive Removal Test Results. Time to contact and remove the tape on the left paw are shown in panels A and C respectively. B and D, Saline-treated mice recovery was detectable after running wheel intervention for contacting and removing tape. DHX-treated mice recovery was detectable after drug treatment for contacting and removing tape (Gower A., M. Sc. Thesis, University of Ottawa, 2018).

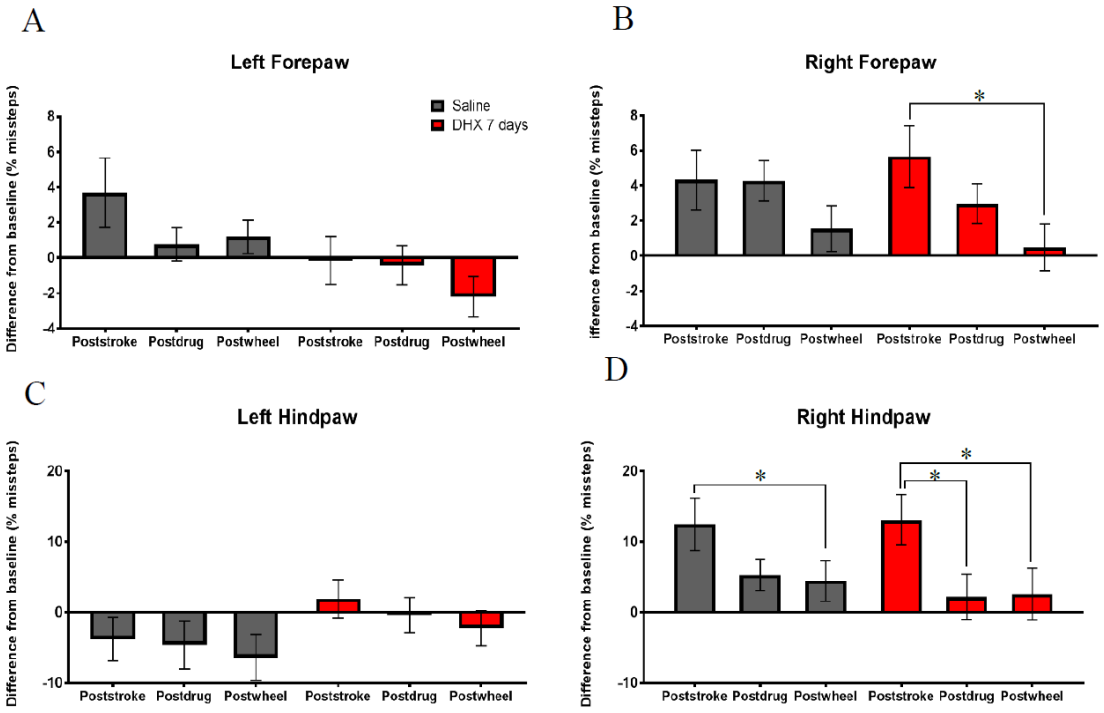


Figure 3: Horizontal Ladder Test Results. Performance on left forepaw and hindpaw are shown in panels A and C respectively. Panel B represent the results of the right forepaw. DHX group showed significant changes over the recovery period. Panel D shows the results of the right hindpaw. In saline-treated mice a statistically difference was reached after running wheels. In DHX-treated mice a statistically different detectable difference was seen after drug treatment. All data is expressed as the difference from the prestroke baseline of the percentage of missteps (Gower A., M. Sc. Thesis, Universitv of Ottawa. 2018).

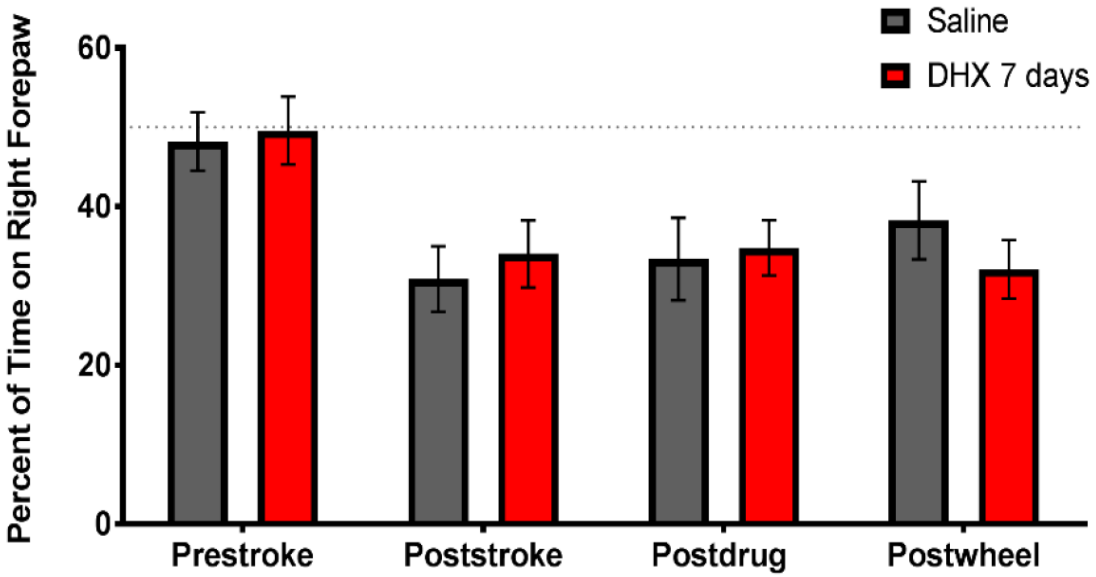


Figure 4: Cylinder Test Results. This graph represents the se of the Right Forepaw during the cylinder test. Data is reported as the percentage of time spent on the right forepaw (Gower A., M. Sc. Thesis, University of Ottawa, 2018).

VII. Rationale, Objectives and Hypothesis of My Study

1. Rationale:

Identification of optimal treatments to improve stroke outcome is limited by incomplete understanding of the neurobiological underpinnings of functional recovery in different brain systems. It is crucial to better understand the endogenous/spontaneous mechanisms that CNS undergoes to assist in the development of new therapies designed to enhance motor recovery after stroke. It has been shown that DA plays an important role in stroke recovery. However, how DA exerts its effect during stroke recovery is poorly understood. Hence, elucidating the alterations of the DA system after stroke may be a first step toward the development of transformative therapies. Such advancement can be achieved by understanding and characterizing the post-stroke changes of the DA system, in order to identify new therapeutic targets. As discussed above, axonal sprouting is one of the main factors to promote recovery. Activation of the dopaminergic D1R promotes motor cortex plasticity, exerts an effect on acquisition of motor skills, and enhances long-term potentiation (Hosp et al., 2011; Molina-Luna et al., 2009; Pekanovic & Atiemo, 2015). DHX increase the rate of cAMP synthesis that activate downstream cAMP response element-binding protein (CREB) which stimulates expression of multiple genes (BDNF, c-Fos, VGF, NGF and TH) responsible for axonal growth, neuronal functional, dopamine synthesis and synaptic plasticity (Delghandi et al, 2005; Lei et al., 2009; Moore & Goldberg, 2011; Caracciolo et al., 2018). Therefore, DHX-induced activation of D1-class receptors may play a role in formation of new connections and enhancing axonal sprouting in stroke animals.

2. Objectives of my thesis are three-fold:

a) Assess the effect of DHX on DA fiber remodeling (i.e. describe anatomically the changes in catecholaminergic fibers occurring around the stroke and in the contralateral side) and characterize these fibers (i.e. phenotyping fibers that undergo remodeling).

b) Assess the effect of DHX on DA fiber remodeling in naïve mice.

c) Determine the timing of monoaminergic fiber remodeling after stroke (i.e. follow fiber remodeling in the short- and long-term after stroke);

3. Hypothesis:

Dopamine, and notably D1-class receptors, modulate fiber remodeling after stroke, supporting a role of DA in post-stroke recovery.

Methods

I. Study Design

The goal of the present study was to assess the potential remodelling of monoaminergic fibers following ischemic stroke and if DHX treatment modulates it. The approach of the first part of my objectives was done using IHC on brain slices from stroke mice used in our previous study (herein referred to **Cohort 1**). The brains were collected from mice that underwent various manipulations, including PT-induced stroke, 7-day drug treatment (DHX or saline), 9-day free-access to running wheels and a battery of behavioural tests performed at different steps of the study as depicted **Figure 6**. For Cohort 1, IHC was performed on brain sections from saline and DHX-treated mice (30-day post-stroke) to image catecholaminergic fibers. For the second part of my study, we used naïve mice in order to test the effect of a 7-day treatment DHX in absence of running wheels exercise (**Cohort 2**). For the third part of my study, mice underwent PT-induced stroke to determine the timing of stroke-induced TH-positive fiber remodeling (**Cohort 3**). An apotome optical sectioning and fluorescent microscopy was used for acquiring 3D images done in collaboration with Dr. Baptiste Lacoste (OHRI). Computational reconstructions and quantifications of 3D images were done by our collaborator Dr. Cesar Comin (University of São Paulo, Brazil) in a blinded and unbiased way. Finally, it is worth mentioning the experiments have been also performed to optimize the tissue quality and antibodies, which have been used throughout the project for different purposes (see **Table 1**and **Table 2** for more details).

II. Animals

All mice used were male C57BL6 mice ordered from Charles River (Kingston, NY, U.S.A.). Animals were single housed upon arrival in Greenline cages (Techniplast, Italy) with standard

mouse cage bedding, a nesting square and a cardboard house. Food (Teklad 2018 Rodent Diet, Envigo) and water were available ad libitum. All mice were housed in reverse light-dark housing, with lights off at 7:00 and on at 19:00. Cohort 2 animals (8-week old) received drug treatment for 7 days and then sacrificed at 5-day post-drug treatment to be in line with our original timetable (**Figure 6**) Cohort 3 mice (6-week old) were handled for 2 weeks before stroke induction and then sacrificed by cardiac perfusion at 4, 10 and 28 post-stroke days. All animal procedures were approved by the University of Ottawa Animal Care Committee.

Mouse outcomes during my study:

- Cohort 2: originally, 12 mice were ordered, 6 mice per each group (saline and DHX). One animal from DHX group died on the 3rd day of drug injection.
- 3 batches of animals were ordered for cohort 3. This mouse cohort were used for our timepoint study (**Figure 5**)

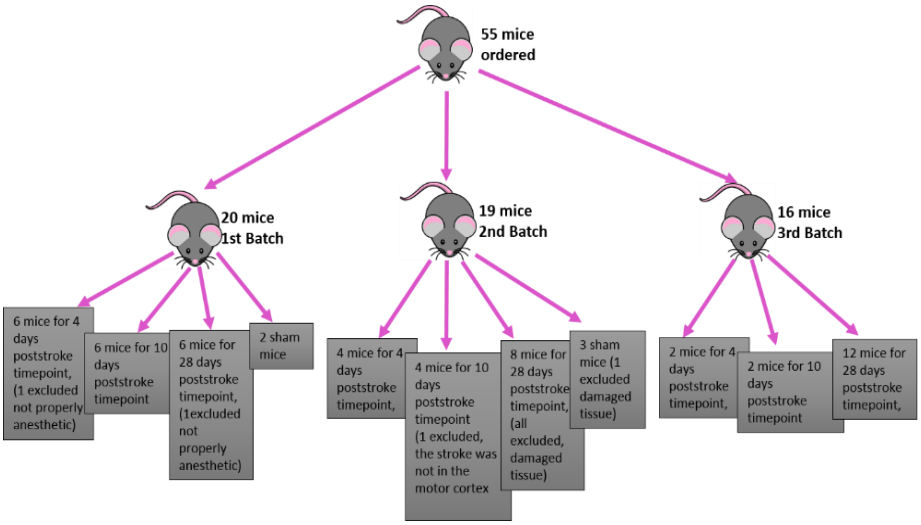


Figure 5. Mouse Outcome in the Time Course Study. For the time course study, 3 separate batches of animals were used. For mice assigned to the 4-day poststroke time point, 11 mice out of 12 were used for brain analysis (1 mouse was excluded because of an experimental issue during PT procedure). For animals assigned to the 10-day poststroke time point, 11 mice were included in the study and one mouse was excluded as the stroke lesion located outside the motor cortex coordinates. For animals assigned to the 28-daypoststroke, 17 out 26 mice completed the study. Indeed, one animal was excluded because it did not display any sign of stroke and 8 brains could be used because of bad tissue quality (i.e cracked sections that were not optimal for IHC). Sham mice group (n=4) that completed the study, one brain had to be excluded for bad tissue quality.

III. Timeline of the Studies.

- Cohort 1: IHC was performed on sections from brains extracted at 1-month post-stroke (**Figure 6**).

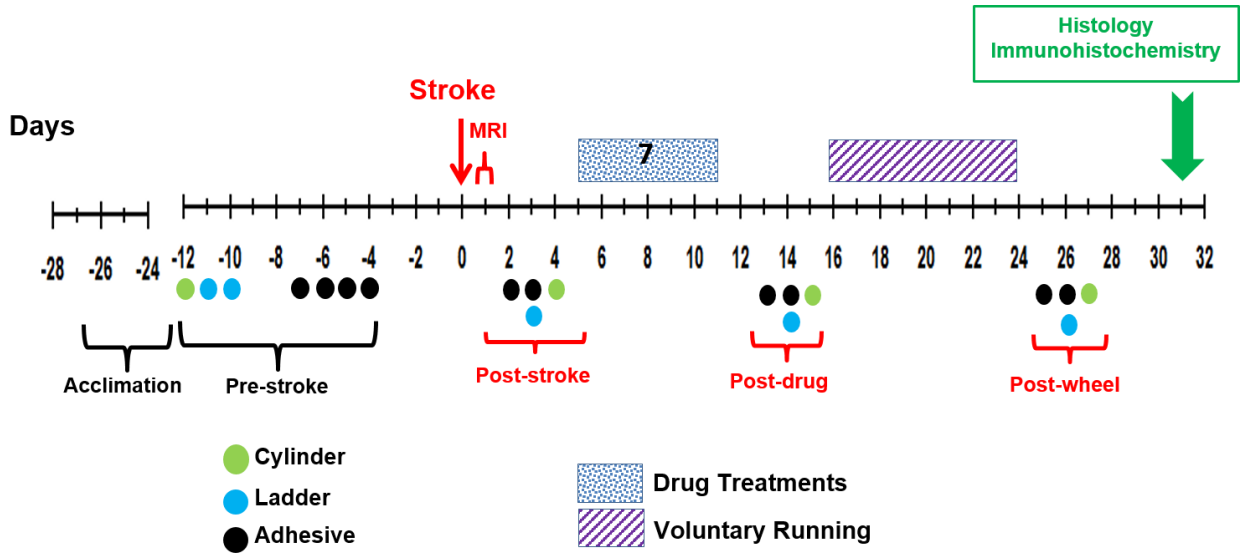


Figure 6: Timetable for cohort 1 tissue that was used to test the effect of DHX on post-stroke motor recovery in a photothrombosis mouse model.

- Cohort 2: IHC was performed on sections from brains extracted 5 days after drug treatment (**Figure 7**).

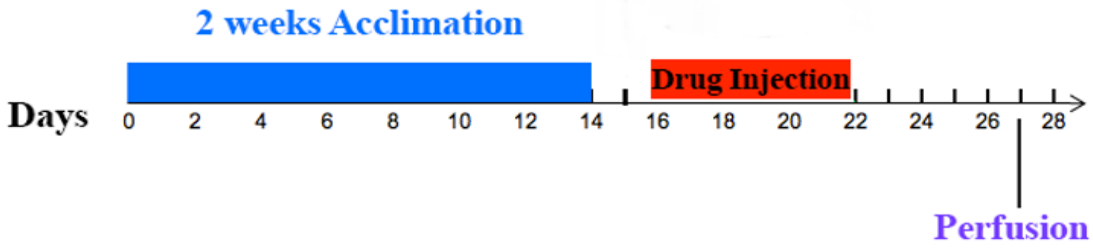


Figure 7: Timetable for the study of the effect of a 7-day treatment with DHX (4 mg/kg) in naive (non-stroke) mice.

- Cohort 3: IHC was performed on sections from brains extracted 4, 10 and 28 days post-stroke (**Figure 8**).

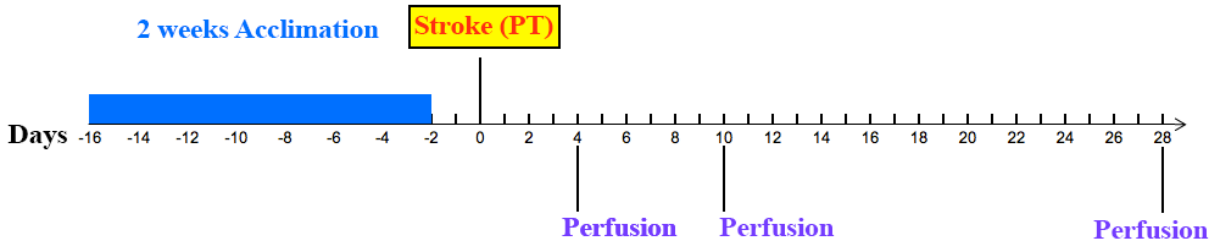


Figure 8: Outline of time-course study.

IV. Photothrombosis Stroke Induction

Photothrombosis was used to induce focal ischemia using the photosensitive dye Bengal Rose administered through intraperitoneal (IP) injections (Watson et al., 1984). Strokes were induced at 0.7 mm anterior and 1.5 mm lateral to bregma, to target the left side forelimb representation area of the primary motor cortex (Paxinos & Franklin, 2013). The day before photothrombosis surgery, animals were weighed. Rose bengal (Tocris, Bristol, U.K.) was prepared the day of surgery by mixing rose bengal powder with 1× phosphate-buffered saline (PBS) to a concentration of 10 mg/ml. The solution was vortexed thoroughly and then sterilized using a 0.22-µm syringe filter. Filtered rose bengal was stored in amber tubes and every effort was made to prevent exposure to light during the preparation process.

The day of surgery, mice were kept covered from light as much as possible. Anesthesia was administered with induction at 5% isoflurane in about 2 L/min oxygen, until the animal was unresponsive to touch. The animal was then transferred from the induction box to a facemask

with the same isoflurane concentration. The animal's heads were shaved and cleaned with chlorohexidane. The animals were given 1 ml of saline subcutaneously on the back, as well as applications of xylocaine jelly (2% lidocaine hydrochloride, AstraZeneca) in the ears and Tear Gel (Alcori, Mississauga, ON, Canada) over the eyes. Animals were then moved to the surgical table, with anesthesia maintained at the induction level. The animals were fixed in a stereotactic frame, the skull exposed through a midline incision, cleared of connective tissue and dried. Rose bengal was administered intraperitoneally at a dose of 10 ul/g of mouse, such that mice received 1 mg of rose bengal per g of body weight. At this time a 5-minute timer was started. The laser, mounted on a stereotaxic frame was centred such that the laser was above bregma on the exposed skull, and at a height of 3cm above the skull. Then laser was moved according to the coordinates described above. At the end of the five-minute period the laser (power ~20mW; wavelength: 532 nm (Beta instruments)) was turned on and left on for 10 minutes. After the ten minutes the laser was turned off and the incision was closed using Vetbond glue (3M Company). Throughout the surgery efforts were made to keep body temperature around 37°C. Sham-operated animals underwent the entire surgical process but the laser was kept off.

Mice were moved to recover in an incubator at 37°C until conscious and then were moved to clean standard cages, with the addition of a soft foam sheet covering the cage filling for increased comfort and 8-10 Cheerios (General Mills) to promote feeding and a bit of their old nest. Mice were returned to their housing room and were wellness-checked in the evening and the following day. Mice were given bupivicaine to reduce pain 3 times, upon wound closure, 6 and 24 hours postop.

V. Pharmacological treatment

Dihydrexidine (DHX) hydrochloride: ($C_{17}H_{17}NO_2$, Tocris, Bristol, UK, CAT #: 158704-02-0) a prototypical full and selective D1 receptor-class agonist. This drug tested in small clinical trials showed a therapeutic potential for improving the cognitive and working memory in schizophrenia and schizotypal disorder (George et al., 2007; Rosell et al., 2015).

Drug administration: DHX was administered by IP injections. The drug was dissolved in sterile 0.9% saline and vortexed well to ensure complete mixing. Then, the drug was sterilized using a 0.22 µm syringe filter and aliquoted into sterilized Eppendorf tubes. With the exception of weighing out the drug and vortexing, preparation was performed in the biological safety cabinet to keep the process as aseptic as possible. DHX was given intraperitoneally at a volume of 10 µl/g of mouse (4 mg/kg). In my experiments, the side of injection was alternated each day during the 7-day treatment period.

VI.Tissue preparation

Animals were deeply anaesthetized by intraperitoneal injection of 0.1 ml of sodium pentobarbitol (65 mg/ml, euthanyl). Cardiac perfusion was performed once the animals were deeply anesthetized through incision to open the chest cavity and expose the heart. A needle connected to a pump (Dynamin Peristaltic Pump, Model RP-1) was inserted into the left ventricle, and a nick was created in the right atrium. The pump was then turned on, at a rate of 7 ml/min, to pump 30 ml of 1× PBS. Then pump was turned off, the intake was switched to perfuse with

100 ml of 4% (w/v) paraformaldehyde (PFA; Sigma) prepared in 1× PBS, and the pump was turned back on.

At the end of perfusion of animals, brains were collected and soaked in 4% (w/v) PFA for 30 min. Brains were then transferred to 15% (w/v) sucrose prepared in 1× PBS and gently shook overnight at 4°C. On the next day, brains were transferred to 30% (w/v) sucrose for at least 24 hours and ensure brains had sunk to the bottom of the falcon tube to warrant maximum cryoprotection to the tissue.

- **For Cohort 2,** brains were cut on microtome (Leica SM 2000R, SM 2010R). Briefly, brains were trimmed using a 1 mm brain matrix (Zivic Instruments, 5325) and mounted on a dry-ice cooled stage. Mounted brains were then covered in dry ice, and cut. Serial coronal sections were collected into 9 consecutive vials filled with 1× PBS, (VWR, BDH1125-4LG). Brain sections were stored at -20°C. This procedure is consistent with the procedure done to process cohort 1 brain tissue.
- **For Cohort 3**, brains were embedded in an embedding mold filled with Optimal Cutting Temperature (OCT) compound placed on dry ice to quickly freeze the brains. Then brains were transferred to -20°C overnight and stored to -80°C until used for cutting by cryostat (Leica CM 1850, Leica Biosystems). On the cutting day, brain was mounted on a chuck using OCT and kept for 1 hr on the cryostat chamber before starting to cut sections at 30-µm thickness. Serial sections were mounted on 9 slides (each slide is a representative of sections throughout the brain), dried and stored at -80°C for IHC.

N.B. Sham animals for cohort 3 were perfused at 28 days post-surgery.

VII. Immunohistochemistry (IHC)

IHC for Cohort 1 and 2: Brains with stroke volume between 0.35 mm^3 and 0.75 mm^3 were selected for the study. Stroke volume bigger than 0.75 mm^3 was excluded to avoid using sections showing damaged corpus callosum. Sample size was 9 for all groups. Brains were cut in series of 9 vials, and 2 slices were taken from one vial for IHC. Stroke tissue was visually seen in 2-4 slices per each vile. Before starting IHC, 3 steps were done (antigen unmasking, permeabilization, and denaturation). Between each of the 3 steps, three times rinses with 0.5%TritonX in 1X Tris-buffered saline (TBS) were performed. 1) For the antigen unmasking step, the slides were submerged in hot 0.01M citric acid solution (pH 6) for 15 min. 2) For the permeabilization step, slides were submerged in 0.1% trypsin in 0.1M Tris and 0.1% $CaCl_2$ solution for 10 min. 3) For the denaturation steps, sides were placed in 2N HCl prepared in (Tris-Buffered Saline) TBS for 30 min.

Next, a blocking solution, consisting of 10% (v/v) normal goat serum (NDS) (Cedarlane, 005-000-121) and 0.5% gelatin (w/v) (M319-500ML, VWR, USA) made TBS containing 0.5% (v/v) Triton X-100 (TBS-T), was applied for 90 min. The blocking solution was then removed and without washing, the primary antibody solution was applied. Primary antibodies were diluted in the blocking solution and kept overnight at 4°C. The next day, the primary antibody was removes and slides washed 4 times for 5 min with 0.5% TBS-T. The concentrations of primary antibodies were as follows: guinea pig anti-TH (1:500, Synaptic Systems), rabbit anti-NET (1:300, Synaptic Systems), rabbit anti-MAP2 (1:500, Abcam), rabbit anti-TH phosphorylated at ser31 (1:500, Cell Signaling Technologies), and rabbit anti-TH phosphorylated at ser40 (1:500, PhosphoSolutions).

The secondary antibodies, goat anti-guinea pig alexa fluor 647 (1:300, Thermofisher), goat anti-rabbit alexa fluor 488 and alexa fluor 594 (1:300, Thermofisher), goat anti-mouse alexa flour 488 and alexa fluor 594 (1:300, Thermofisher) were diluted in the blocking solution and applied for 2 hrs at room temperature. Then, slides were subjected to 4 washes of 5 min with TBS-T. Lastly, DAPI (4',6-diamidino-2-phenylindole;1:5000, Sigma-Aldrich) a blue-fluorescent DNA stain was applied for 5 min. At the end of incubation period, slides were coverslipped and kept to dry before imaging.

For co-localization IHC studies, 3 brains from saline group were used for double staining experiments. Free floating sections were incubated with TH and phospho-THser31 or TH and phospho-THser40. Prior to incubation with antibodies, sections were immediately incubated with blocking solution without pre-treatment steps. Through all the steps, the sections were kept on a shaker and mix gently.

IHC for Cohort 3: there was no 3-step pretreatment prior to applying blocking solution. Brain tissue from this cohort was used to perform TH staining, and images were collected for the assessment of fiber density and branch point quantification.

In all experiments, no-primary and no-secondary antibody controls were run in parallel. There was no specific staining with these controls.

VIII. Fluorescence microscopy and image acquisition

Immunostained sections were examined under a Zeiss Axio Imager M2 microscope equipped with a digital camera (Axiocam 506 mono) as well as the Zeiss ApoTome.2 module for optical

sectioning also equipped with 10×, 20× and 40× objectives. Images were processed using Zen 2 software. For each slice 20× images for the cortex were taken. Higher magnification images 40× were taken of the ipsilateral and its corresponding area in the contralateral side. To sample the area adjacent to the stroke that visually showed high staining, most of the slices, two 40× images were taken around the stroke toward the midline and 2 images on the contralateral side. The images where positioned to be at the edge of the stroke, the second image was positioned right above the corpus callosum and close to edge of the stroke as depicted in **Figure 10**A and **Figure 14**A For time course study one 40X image on each brain side was taken, same position to "ROI 2" (**Figure 10**A and **Figure 14**A). 18 µm and 13.20 µm deep z-stacks, 0.25 µm each plane in the z-stack, were acquired with 40× images to visualize TH and NET staining, respectively.

IX. Infarct Size

Tissue from a single slide was taking from each brain. The slides were dried for 30 min at room temperature. Neurotrace 640/660-far red fluorescent Nissl Stain (1:200 N21483, Thermofisher) was applied on slides, incubated for 2 hours, washed with 0.5% (v/v) TBS-T and counterstained with DAPI. Then, slides were coverslipped and kept to dry. Images were taken for ipsi- and contralateral sides showing a stroke when observed with 10× objective. The area of the stroke was measured using Fiji software. To calculate the stroke volume, the sum of infarct areas was multiplied by 9 (to account for the 9 representative slides taken) and by 30 (for slices cut at 30 µm) to account for the thickness of each slice.

X. 3D images reconstructions.

The quantification of the 3D images was done by our collaborator as stated above. The 3D images were smoothed using a median filter with a size of 0.681 microns. Then, an adaptive thresholding operation was applied. Voxels having intensity larger than the average intensity of a circular region of radius 23 microns around the voxel were classified as a fiber. Otherwise, the voxels were associated with background. The resulting image contains voxels having only two colors: black for the background and white for voxels associated with a fiber. Connected white components with a volume smaller than 1-micron^3 were removed from the image. A thinning procedure (Palagyi & Kuba, 1998) was then used for obtaining the medial axes of the segmented fibers. Such a procedure allows the representation of each fiber as a set of 1D curves.

The medial axes were then represented using a graph structure. Each fiber termination and bifurcation were represented as a node and fiber segments were represented as connected pairs of nodes. The length of each fiber segment was then calculated. Fiber segments having a length smaller than 1.5 microns and at least one termination point was iteratively removed from the image. This is so because small segments are usually caused by spurious intensity variations. The process needs to be iterative because the removal of a fiber segment can reveal a new small segment that also needs to be removed.

The fiber density was then calculated as the total length of the fibers contained in the stack divided by the stack volume. Branching points were identified as graph nodes being associated with at least three fiber segments. The branching point density of the stack was calculated as the number of branching points divided by the stack volume.

The reconstructions were generated using the Visualization Toolkit (VTK) library. The radii of the fibers shown in the reconstructions were obtained using the values of the distance transform of the binary fiber images at the positions of the medial axes. **Figure *9*** summarizes the different steps of image processing.

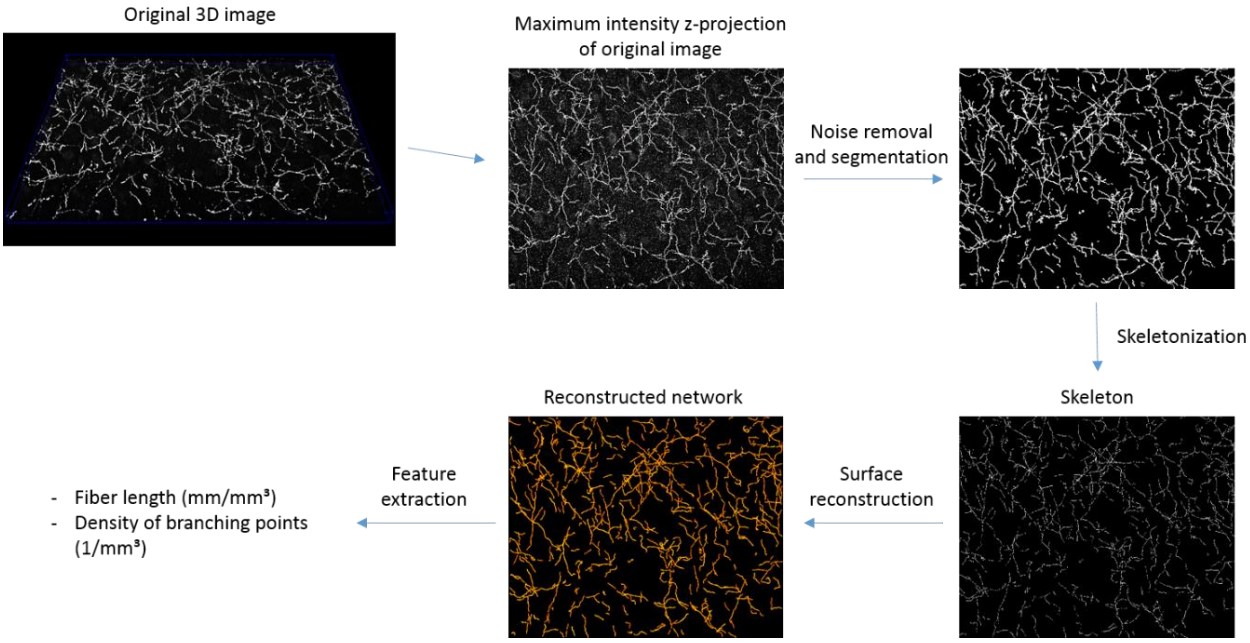


Figure 9: Summary of steps used for processing 3D images.

XI. Statistical analysis.

Statistical Analysis was performed by GraphPad Prism (version 8.1). Values are reported as mean ± standard error of the mean (SEM). A Two-way ANOVA (e.g. 'treatment and brain side' and 'post-stroke days and brain side") followed by post-hoc test (Sidak's multiple comparison) was used for monoaminergic fiber remodeling after stroke readouts (density and branch points).

A Mann-Whitney U test (appropriate for small sample size) was used for two group comparison for healthy mice receiving saline or DHX. One-way ANOVA followed by post-hoc test (Tukey's multiple comparison) was performed to compare the infarct sizes between the 3 groups (4 days poststroke, 10 days poststroke and 28 days poststroke). $p< 0.05$ was considered significant.

Perfusion/Postfixation Procedures	Tissue Preservation Methods (pre- and post-cutting)	Brain Cutting Method	Thickness of Brain Slices
#1 • PBS perfusion for 6 min (40 ml) • 4% (w/v) PFA perfusion 10-15 min (70-100 ml) • 4% PFA (w/v) overnight	**Brains:** 30% (w/v) sucrose 0.1% (w/v) sodium azide in 1× PBS at 4°C **Slices:** 0.1% (w/v) sodium azide in 1× PBS at 4°C	Microtome	30 µm
#2 • PBS perfusion (30 ml) • 4% (w/v) PFA overnight	**Brains:** Preserved in OCT-filled molds at -80°C **Slices:** (1) Kept on slide at -80°C (2) Free floating sections kept in antifreezing solution at -20°C	Cryostat	30 µm 50 µm
#3 • PBS perfusion (30 ml) • 50 ml 4% (w/v) PFA • 30 min 4% (w/v) PFA post-fixation	**Brains:** Preserved in OCT- filled molds at -80°C **Slices:** (1) Kept on slide at -80°C (2) Free floating sections in antifreezing solution at -20°C	Cryostat	30 µm 50 µm
#4 • PBS perfusion (30 ml) • 100 ml 4% (w/v) PFA perfusion • 30 min 4% (w/v) PFA post-fixation	**Brains:** Preserved in OCT- filled molds at -80°C **Slices:** (1) Kept on slide at -80°C (2) Free floating sections in antifreezing solution at -20°C	Cryostat	30 µm 50 µm

Table 1 Perfusion and Post-Fixation Methods. Summary of different perfusion and post-fixation protocols tested during Masters Studies to optimize brain tissue fixation for IHC.

Antibody	Catalog #	Source	Perfusion Methods Tested	Brain Markers	Outcome and Results
Chicken anti-TH	ab76442	Abcam	Perfusion 1 (**see Table 1**)	Catecholaminergic Systems	No signal with any condition notably in cortical areas
Mouse anti-TH	22941	ImmunoStar	Perfusion 1, 2, 3 and 4 (**see Table 1**)	Catecholaminergic Systems	Cortical areas stained using perfusion 3 and 4 without antigen retrieval (AR). Staining was not sharp using other perfusion methods.
Guinea pig anti-TH	213 104	Synaptic Systems	Perfusion 1, 2, 3 and 4	Catecholaminergic Systems	Cortical areas appeared stained following perfusion 1 with AR, but also following perfusion 2, 3, and 4 without AR. However, perfusion 3 gave optimal results.
Rabbit anti-TH phosphorylated at Ser40	p1580-40	Phospho Solutions	Perfusion 1 and 3	Activated TH	Showed staining in cortical areas in IHC-free floating section method after perfusion 1, and after perfusion 3 without AR.
Rabbit anti-TH phosphorylated at Ser31	13041S	Cell Signaling Technology	Perfusion 1 and 3	Activated TH	Showed staining in cortical areas in IHC-free floating section method after perfusion 1, and after perfusion 3 without AR.
Rabbit anti-DAT	284003	Synaptic Systems	Perfusion 1, 2, 3 and 4	Dopaminergic Axons	IHC with free floating method or mounted slides, with or without AR, after all perfusions. Quality of cortical staining was low and not in line with what literature suggests.
Rat anti-DAT	SC-32258	Santa Cruz	Perfusion 1, 2, 3 and 4	Dopaminergic Axons	IHC with free floating method or mounted slides, with or without AR, after all perfusions. Quality of cortical staining was low and not in line with what literature suggests.
Rat anti-DAT	MAB369	Millipore Sigma	Perfusion 1, 2, 3 and 4	Dopaminergic Axons	IHC with free floating method or mounted slides, with or without AR, after all perfusions. Quality of cortical staining was low and not in line with what literature suggests.

Mouse anti-DAT	AMAB91125	Millipore Sigma	Perfusion 1, 2, 3 and 4	Dopaminergic Axons	IHC with free floating method or mounted slides, with or without AR, after all perfusions. Quality of cortical staining was low and not in line with what literature suggests.
Rabbit anti-DA	IS 1005	ImmuSmol	Perfusion 1, 2, 3 and 4	Dopaminergic Systems	No signal with any condition
Rabbit anti-D1R	GR309340-1	Abcam	Perfusion 1	D1R-expressing Neurons	No signal with any condition
Rabbit anti-D1R	GR3176670-1	Abcam	Perfusion 1	D1R-expressing Neurons	No signal with any condition
Rabbit anti-D1R	GR120585-5	Abcam	Perfusion 1	D1R-expressing Neurons	No signal with any condition
Rabbit anti-NE	IS 1042	ImmuSmol	Perfusion 1, 2, 3 and 4	Noradrenergic System	No signal with any condition
Rabbit anti-DBH	22806	Immunostar	Perfusion 1, 2, 3 and 4	Noradrenergic System	No signal with any condition
Rabbit anti-NET	260 003	Synaptic Systems	Perfusion 1, 2, 3 and 4	Noradrenergic Axons	Good quality of staining in all perfusions (AR needed for perfusion 1)
Rabbit anti-GFAP	GTX78212	GeneTex (Cederlane)	Perfusion 1, 2, 3 and 4	Reactive Astrocytes, Glial Scar	Good quality of staining in all perfusions (AR needed for perfusion 1)
Goat anti-Human CSRP3	C19020856	US Biological Life Science	Perfusion 1, 2, 3 and 4	Axonal Regeneration	No signal with any condition
Rabbit anti-GAP-43	ab16053	Abcam	Perfusion 1, 2, 3 and 4	Axonal Regeneration	Staining was dim and not sharp, hard to detect axons
Rabbit anti-MAP2	ab183830	Abcam	Perfusion 1, 2, 3 and 4	Somatodendritic Compartment	Good quality of staining in all perfusions (AR needed for perfusion 1/free floating sections for IHC)

Rabbit anti-Tau	314002	Synaptic Systems	Perfusion 1, 2, 3 and 4	Axons	Staining was dim and not sharp, difficult to detect axons

Table 2: List of antibodies used. Summary of all antibodies tested for different purposes during Masters Studies. CSRP3, Cysteine and glycine-rich protein 32 Cardiac LIM protein (aka CLP, MLP, CRP3, LMO4, CMD1M, CMH12); DA, dopamine; DAT, Dopamine Transporter; D1R, Dopamine D1 Receptor; DBH, Dopamine β-Hydroxylase; GAP-43, Growth Associated Protein 43; GFAP, Glial Fibrillary Acidic Protein; NE, Norepinephrine; NET, Norepinephrine Transporter; TH, Tyrosine Hydroxylase.

Results

I. Objective 1: Assessment of DHX Effect on Catecholamine Fiber Remodeling in Mice after Stroke.

For this objective, brains from cohort 1 were used for IHC and image analysis to check if the fibers of catecholaminergic system underwent any remodeling. To test this, 5 markers were used: TH (marker for dopaminergic and noradrenergic systems), NET (specific marker for the noradrenergic system), and MAP-2 (somato-dendritic marker) and phophoTH at serine 31 or serine 40 (Markers for the phosphorylated activated forms of TH).

1. TH-Positive Fiber Remodeling in Motor Cortex of Saline and DHX-treated Mice

In order to test if a catecholaminergic axonal remodeling happens in DHX-treated mice following stroke recovery period, 3D image reconstruction and quantification for density and branch point of TH-positive fibers in selected region of interests (ROIs) were performed. As shown in **Figure 10** A and B, two ROIs at higher magnification (40×) were taken around the stroke and in corresponding regions of the contralateral side. In saline group, the ipsilateral side significantly displayed high TH-positive fiber density and branch point compared to contralateral side ($p=0.001$, $n=9$ for both groups) in both boxes and on the averages of those two boxes (***Figure 10***C and **Supplementary Figure 1**). However, in DHX-treated mice there were no detectable statistical difference in fiber density and branch point between ipsilateral side and contralateral side ($p=0.447$ and $p=0.466$, respectively) (**Figure 10**C and **Figure S1**). In both graphs (**Figure 10**C), all animals in saline-treated mice showed increase in fiber density and branch points from the contralateral side, however, in DHX treated mice almost half of the animals showed less fiber density and branch points compared to the contralateral side. There was no statistical difference between saline contralateral side with either side of DHX-treated mice in fiber density and branch

points (p=0.43 and p=0.09 for the density of TH-positive fibers between contralateral side of saline-treated mice and contralateral side of DHX-treated mice and between contralateral side of saline-treated mice and Ipsilateral side of DHX-treated mice respectively. p=0.80 and p=0.24 for the number of branch points of TH-positive fibers between contralateral side of saline-treated mice and contralateral side of DHX-treated mice and between contralateral side of saline-treated mice and Ipsilateral side of DHX-treated mice respectively). These data strongly suggest that D1-class receptors can modulate poststroke TH-positive fiber remodeling.

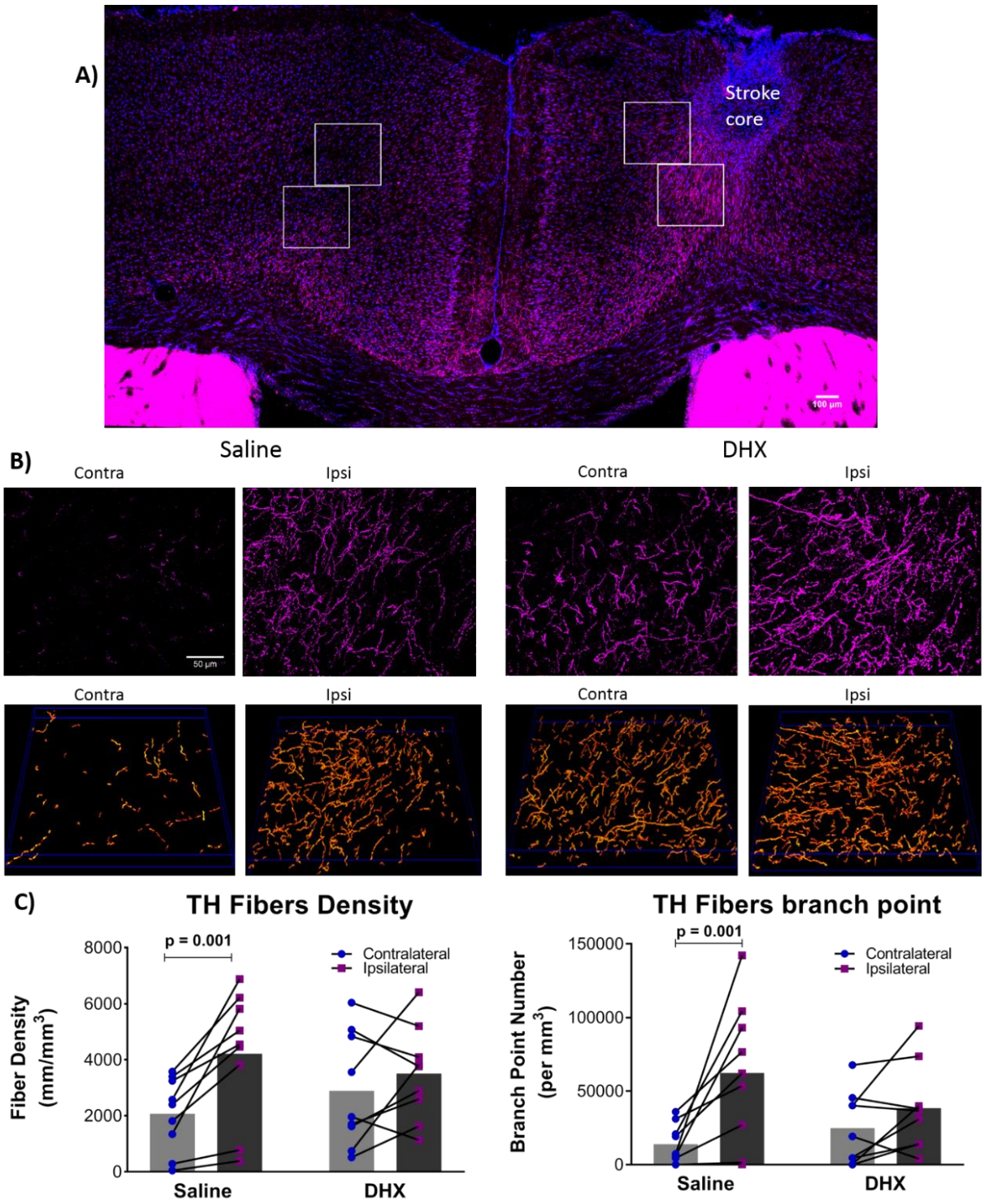


Figure 10 Remodeling of TH-positive fibers at 28-day poststroke following saline and DHX treatment. A, Representative example of a 10× image magnification for TH immunostaining of saline-treated brain. The location of ROI1 and ROI2 on 10× image was taken at 40× magnification for the 3D reconstruction and quantification. **B**, Representative example of 40× images (upper panels) and their 3D reconstruction (lower panels) is shown. Images of contralateral (contra) and ipsilateral (IPSI) sides are shown in left (Saline group) and right (DHX group) panels**. C,** Graphs represent the averages of the two ROIs for the quantification of the density and number of branch point of TH-positive fibers. Significant p value is shown on the graphs. Values are reported as mean ± S.E.M, with n = 9 for both groups. There was no significant interaction between the treatment and the brain hemisphere, however, p value was 0.054 for both fiber density and number of branch points. A two-way ANOVA using Sidak's multiple comparison test was performed with a significance level (α value) set at 0.05

2. TH-Positive Fibers are not Dendrites

To confirm the axonal nature of TH-positive fibers, a somatodendritic marker, MAP2, was used in IHC. No co-localization between TH and MAP2 was observed (**Figure 11**) which is indicative of the axonal nature of TH-positive fibers.

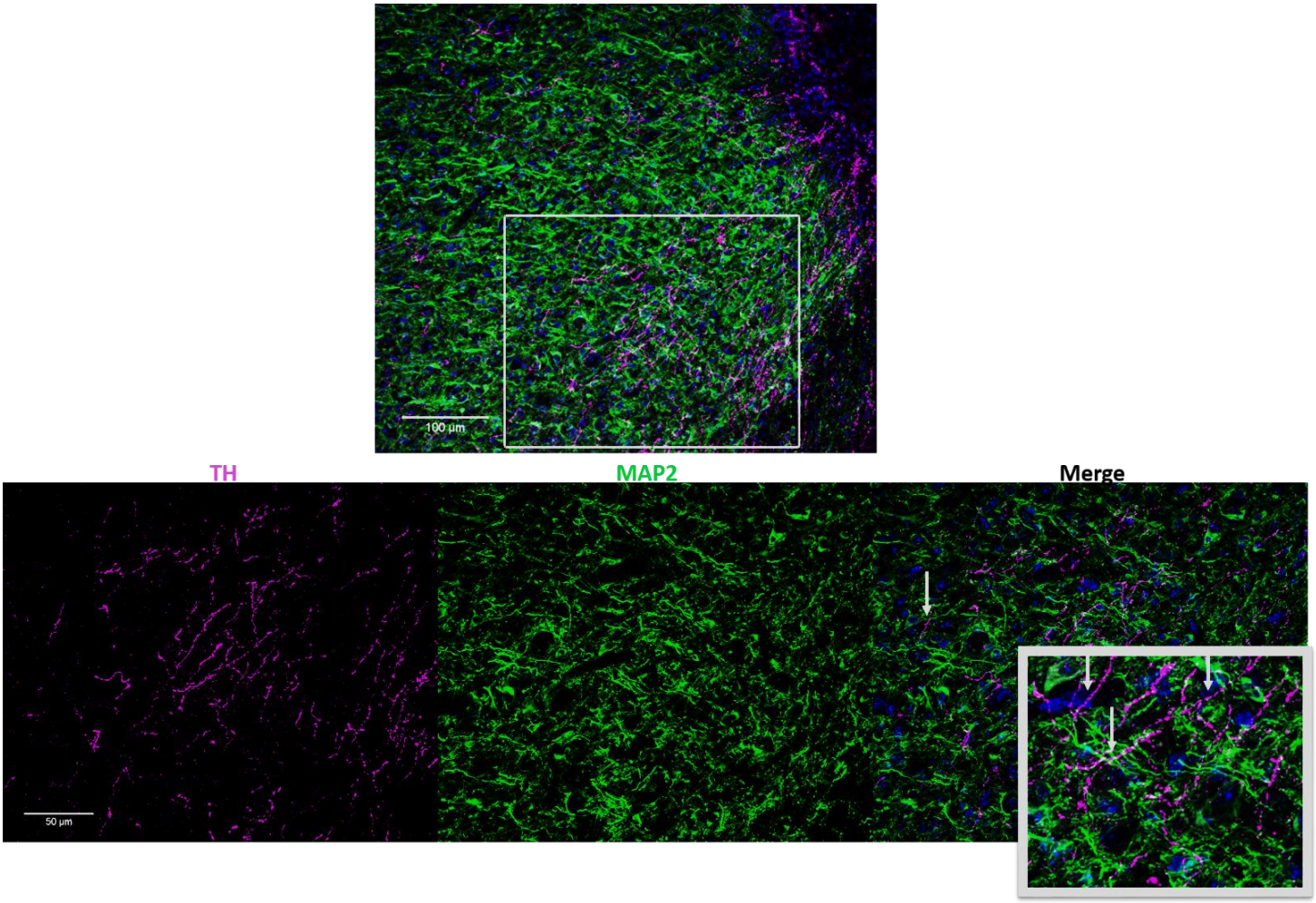


Figure 11 TH-positive fibers are not dendrites: Upper panel is 20× image for TH and MAP2 co-staining. Lower panels are 40× images for the box indicated in the 20× image. Merged images did not show co-localization between TH and MAP2. Arrows indicate representative fibers showing no obvius co-localization. The white box is the magnification of the merged image.

3. TH are Not Noradrenergic Axons.

In order to test if TH-positive fibers were of dopaminergic or noradrenergic origin, co-staining for TH and NET, or TH and DAT. Due to technical issues with commercially available DAT antibodies we tested, no definitive conclusion could be drawn from IHC in cortical areas. In contrast to DAT, no technical difficulties with NET staining were encountered. Analysis of 40× images qualitatively showed very minimal co-localization between catecholaminergic marker TH and noradrenergic marker NET (**Figure 12**). Therefore, our data strongly suggest that most, if not all, TH-positive fibers remodeled around the stroke were dopaminergic fibers.

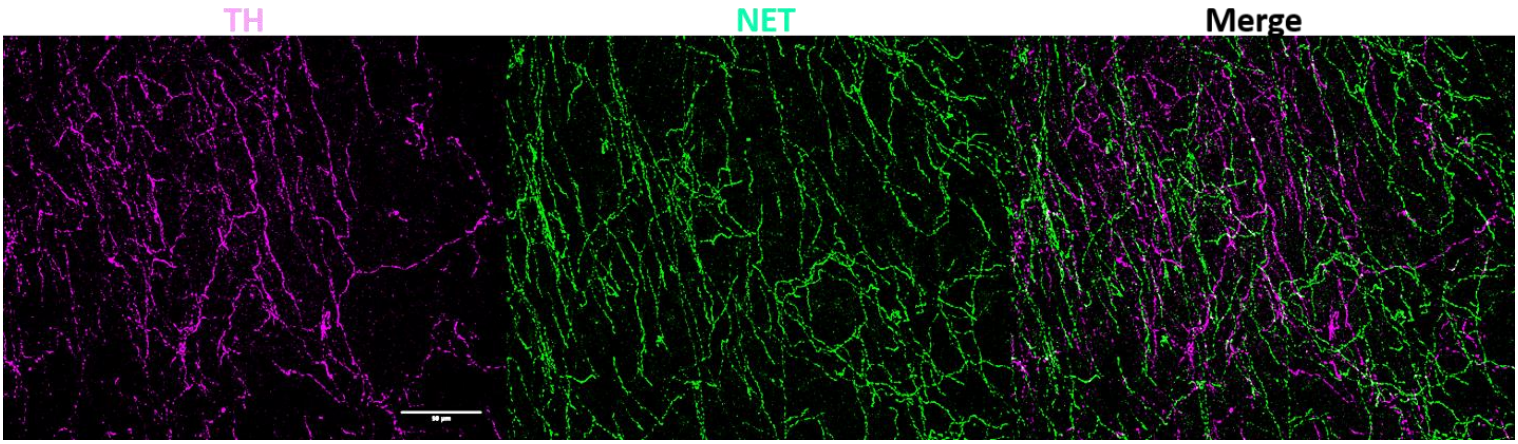


Figure 12: TH-positive fibers are dopaminergic rather than noradrenergic fibers: The images shown are 40× images taken adjacent to the stroke area. Merged images did not show co-localization between TH and NET.

4. TH is in an Active State in Dopaminergic Axons.

The enzymatic activity of TH is regulated by phosphorylation (Dunkley & Dickson, 2019; Lew et al., 1998). Although phosphorylation of serine residues (Ser-19, Ser-31, and Ser-40) has been observed both *in vitro*, *in situ*, and *in vivo*, however, only phosphorylation at ser31 and ser40 has been reported to correlates with stimulation of dopamine synthesis (Lindgren et al., 2001).

To determine the TH phosphorylation state in high-density fibers observed around the stroke, co-staining for both TH and phosphorylated TH (Ser31 or Ser40) was conducted. We observed a high level of co-localization between TH and either form of phospho-TH (**Figure 13**)

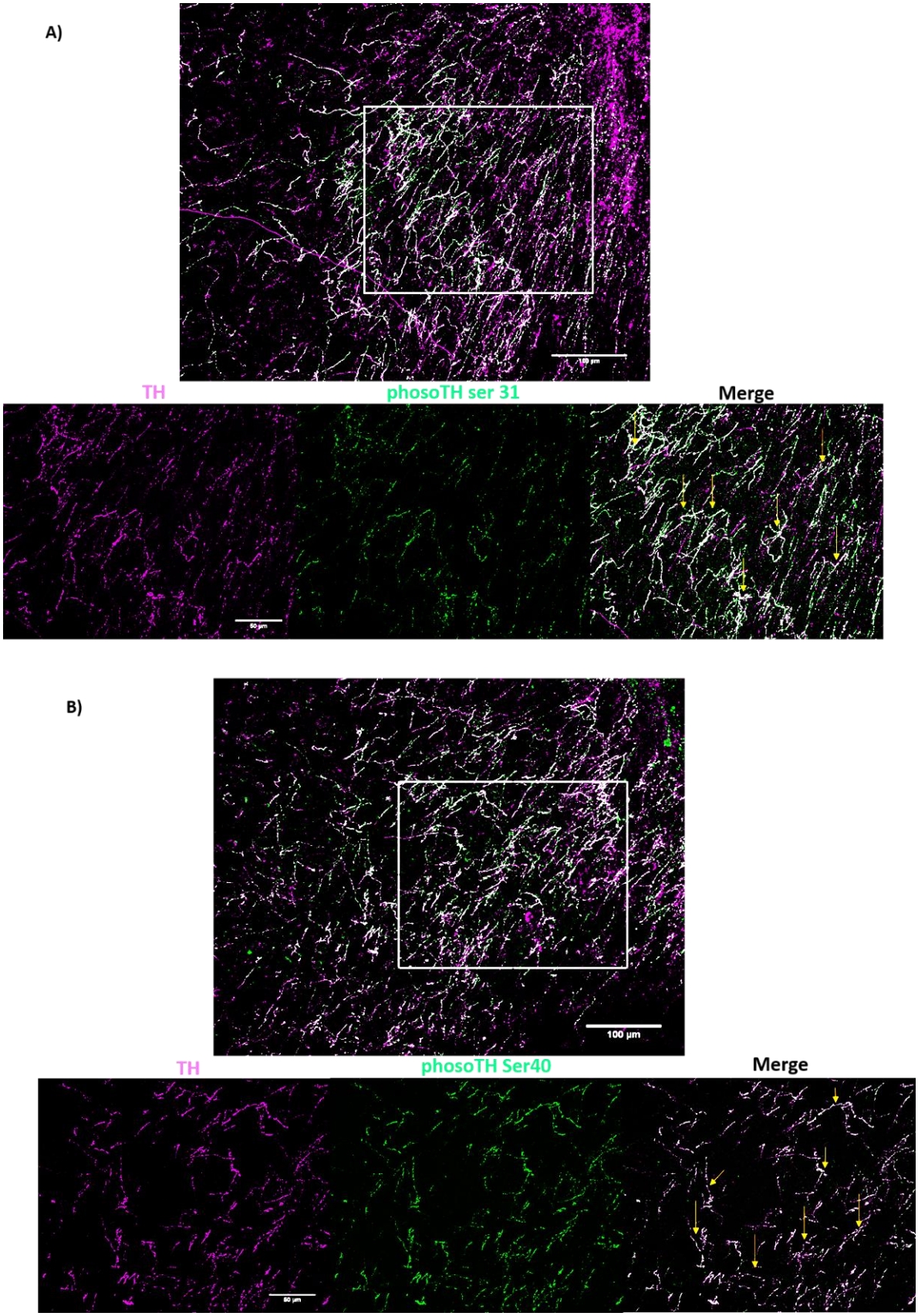


Figure 13: Dopaminergic axons harboring phospho-TH are present in the periinfarct area. A and **B**, Upper panels are 20× image for TH and phosphoTHser31 or TH and phosphoTHser40 co-staining, Lower panels are 40× images for the box indicated in the 20× images. Merged images show co-localization between TH and phospho-TH. The arrows in the two merged images indicates the co-localization between TH and phosphoTHser31 or TH and PhosphoTHser40 in **A** and **B**.

5. NET Fibers Undergo Minor Remodeling during Poststroke Recovery in Motor Cortex of Saline and DHX-treated Mice.

In order to examine whether noradrenergic fibers undergo remodeling or not, we also quantified NET-positive fibers density and branching. The same procedure as described above was performed using an anti-NET antibody in IHC (**Figure 14**). Nine brains from each group (2 slices per each brain) were taken to perform NET staining. Saline and DHX-treated mice did not show significant difference in fiber density ($p=0.334$ and $p=0.998$ for saline and DHX-treated group, respectively), but a significant difference between the 2 hemispheres was detected in the number of branch points in saline-treated group ($p=0.038$) upon averaging the 2 ROIs (**Figure 14**C and **Supplementary Figure 2**). Overall, the data suggest that cortical NET-positive fibers does not extensively remodel during stroke recovery relative to dopaminergic axons in the periinfarct area.

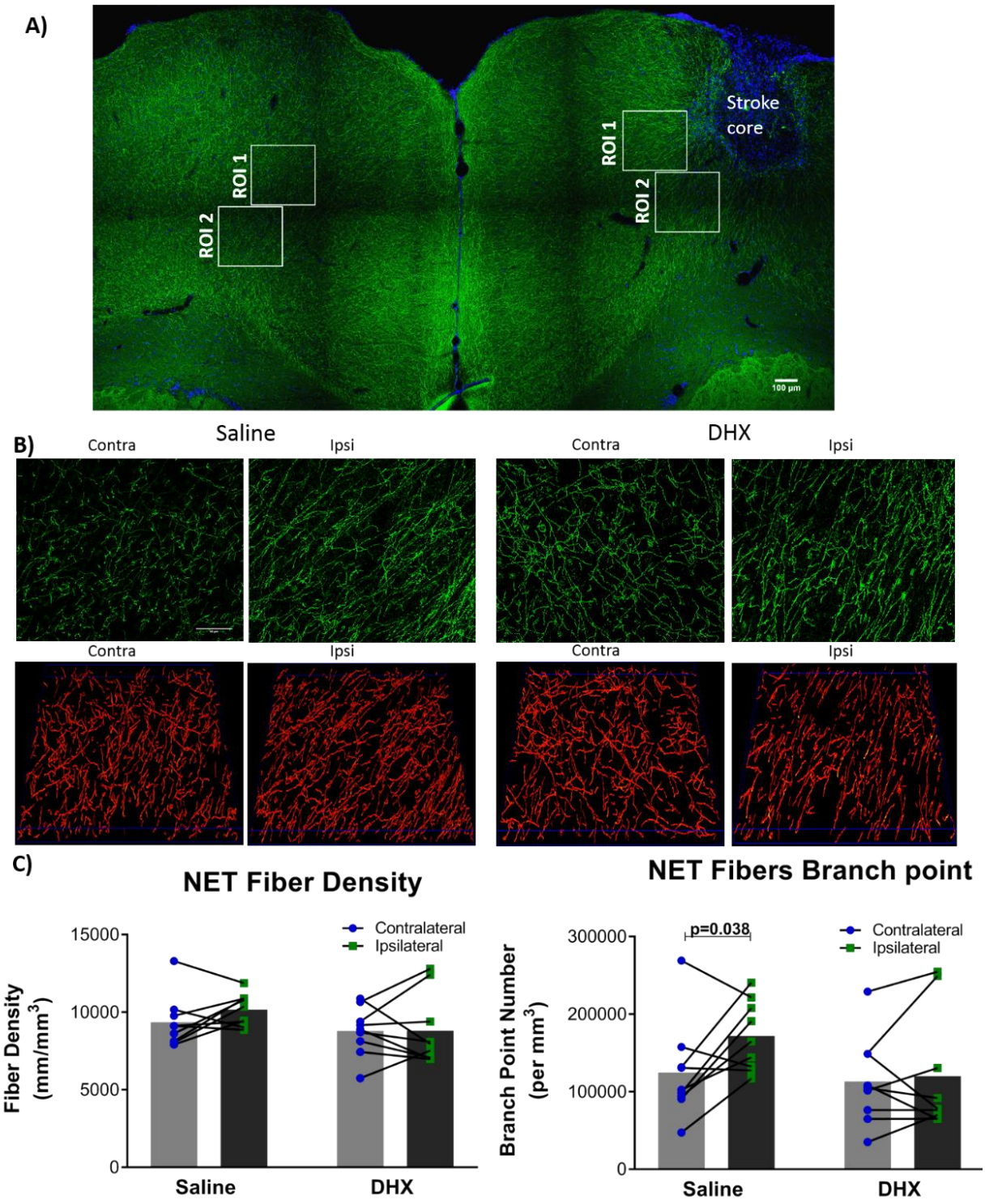


Figure 14: NET-positive fibers do not show hyper-innervation around stroke. A, Representative example of a 10× image magnification for NET immunostaining of saline-treated brain. The location of ROI1 and ROI2 on 10× image was taken at 40× magnification for the 3D reconstruction and quantification. **B,** Representative example of 40× images (upper panels) and their 3D reconstruction (lower panels) is shown. Images of contralateral (contra) and ipsilateral (IPSI) sides are shown in left (Saline group) and right (DHX group) panels. **C,** Graphs represent the averages of the two ROIs for the quantification of the density and number of branch point of NET-positive fibers. Significant p value is shown on the graphs. Values are reported as mean ± S.E.M, with n = 9 for both groups. There was no significant interaction between the treatment and the brain hemisphere (p=0.35 and p= 0.13 for Fiber density and number of branch points respectively). A two-way ANOVA using Sidak's multiple comparison test was performed with a significance level (α value) set at 0.05

II. Objective 2: Assessment of DHX effect on DA Fiber Remodeling in Naïve Mice.

The above data showed that DHX enhances dopaminergic axon density and branch point in the contralateral side while reducing those in the ipsilateral side. To establish if DHX also modulates TH-positive axonal remodeling in the motor cortex of naïve mice, or whether its effect is exclusively observed after stroke, the following experiment was conducted as depicted in timeline shown in **Figure 7.**

Staining with anti-TH antibodies of brain sections from both groups (n=6 for saline, n=5 for DHX) was performed and two 40× images of ipsi- and contralateral sides were taken (**Figure 15**A and B) It is worth mentioning that efforts were made to acquire images at a similar position of the images selected in stroke mice. The average of the 2 images quantified from ipsi- and contralateral sides of each brain slice did not show significant difference in the density and number of branch points of TH-positive fibers between saline and DHX-treated mice (p=0.93) (**Figure 15**).

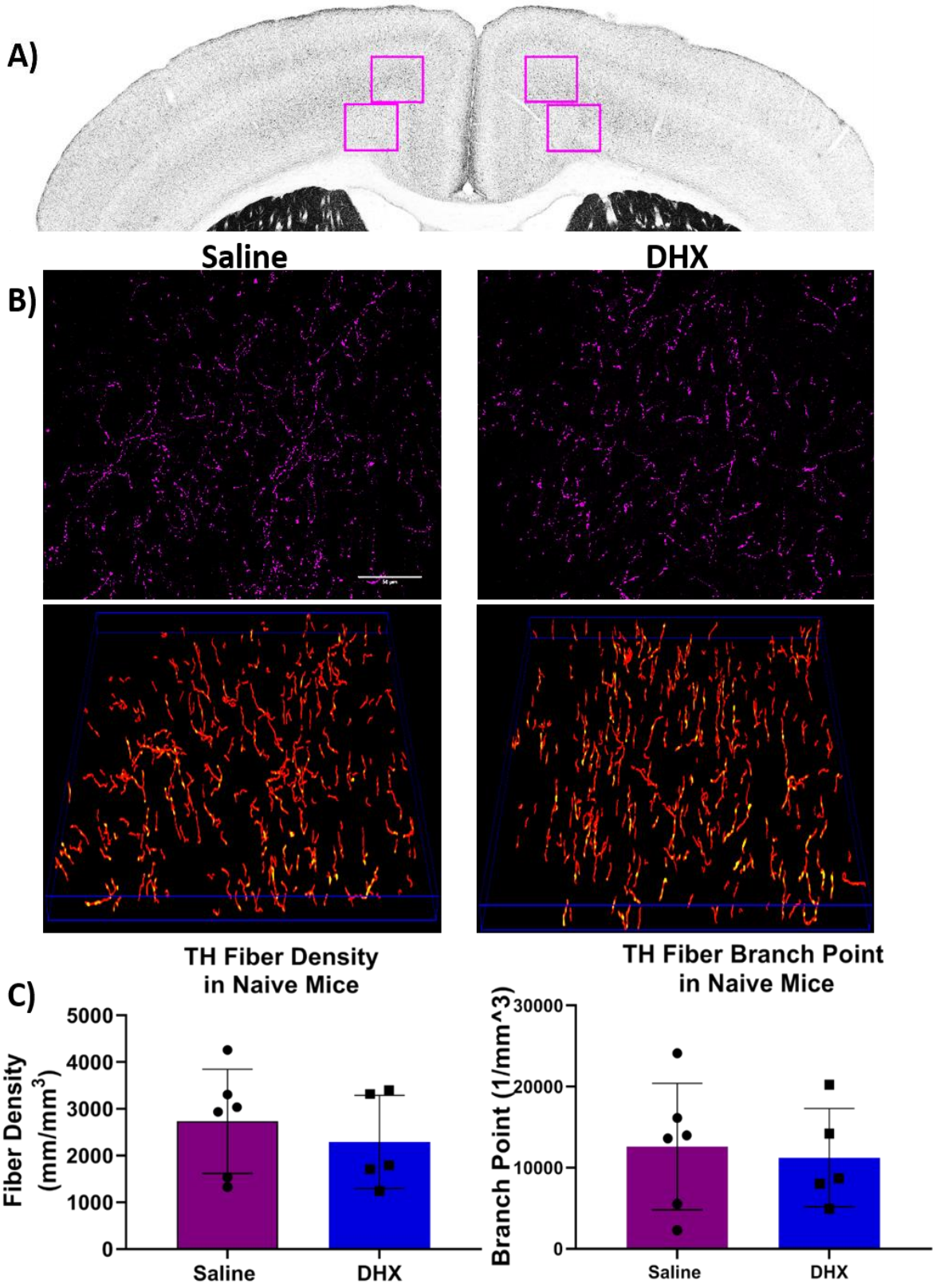


Figure 15*:* **Effect of DHX on dopaminergic axon remodeling in normal mice. A**, represent the position of ROIs. **B,** represent 40X images from saline and DHX treated mice stained for TH (upper panel) and their 3D reconstruction (lower panel). Four images (2 from each brain side) were taken for quantification. **C,** is the quantification of fiber density and density and branch points of TH fibers (n=6 and 5 for saline and DHX respectively). Mann–Whitney U test was performed with a significance level (α value) set at 0.05

III. Objective 3: Determination of Remodeling Timing of Dopaminergic Axons in Untreated Mice after Stroke.

We next wanted to determine the timing of occurrence of TH-positive fiber remodeling during poststroke recovery without any pharmacological and/or physical intervention. For this purpose, we conducted a study using healthy mice that underwent stroke after 2 weeks of acclimation and sacrificed at different time points as depicted in timeline shown in (**Figure 8**).

1. Infarct Sizing

Infarct size was measured for the 3 different groups 4, 10 and 28 days poststroke using neurotrace staining. There was significant decrease in the infarct size between 4 and 10 days also between 4 and 28 days poststroke (p= 0.007 and p=0.0065, respectively) (**Figure 16**).

2. Temporal Profile of Dopaminergic Fiber Remodeling.

In order to examine TH-positive fiber remodeling following ischemic stroke, a time course study was performed. It is worth mentioning that animals subjected to stroke did not undergo any pre- or post-stroke behavioural testing, drug injection or physical exercise (e.g. running wheels). Mice were sacrificed at 4, 10 and 28 days postroke to assess their TH fiber density and branching. The 40× images were taken from the same location used in DHX study (i.e. ROI2 in **Figure 10** and **Figure 14**).

At 4 days poststroke, there was a significant decrease in the density of TH-positive fibers that were adjacent to periinfarct area (p=0.0042) compared to the contralateral side. However, at 10 days poststroke, there was no significant difference between the two brain hemispheres and also no significant difference in the periinfarct side compared to 4 days poststroke group (p=0.55 and p=0.51, respectively). TH fiber density reaches a significant difference at 28 days poststroke

compared to 4 days poststroke in the periinfarct area (P=0.0057). The results suggest a damage in TH fibers in the periinfarct zone, which is mitigated by a remodeling of TH-positive fibers occurring between 4- and 10-days post-stroke. Furthermore, results suggest TH-positive fiber remodeling was significantly manifested at 28 days poststroke in the periinfarct area. Interestingly, although a drop-in branch points was evidenced at 4 days poststroke between the 2 hemispheres albeit not statistically significant (p=0.33). Furthermore, the number of branch points on the periinfarct side at 4 days poststroke was less than that at 28 days but it was also not statistically significant (p=0.085). At all-time points, there was almost no change in density and in branch points of the TH-positive fibers of the contralateral side (**Figure 17**).

N.B. Sham data (control group for 28 days poststroke) was excluded from the statistical analysis due to the missing controls to the other 2 groups (4 and 10 days poststroke). Nonetheless, the quantification of sham group for 28 days poststroke is reported on the graphs (**Figure 17**B).

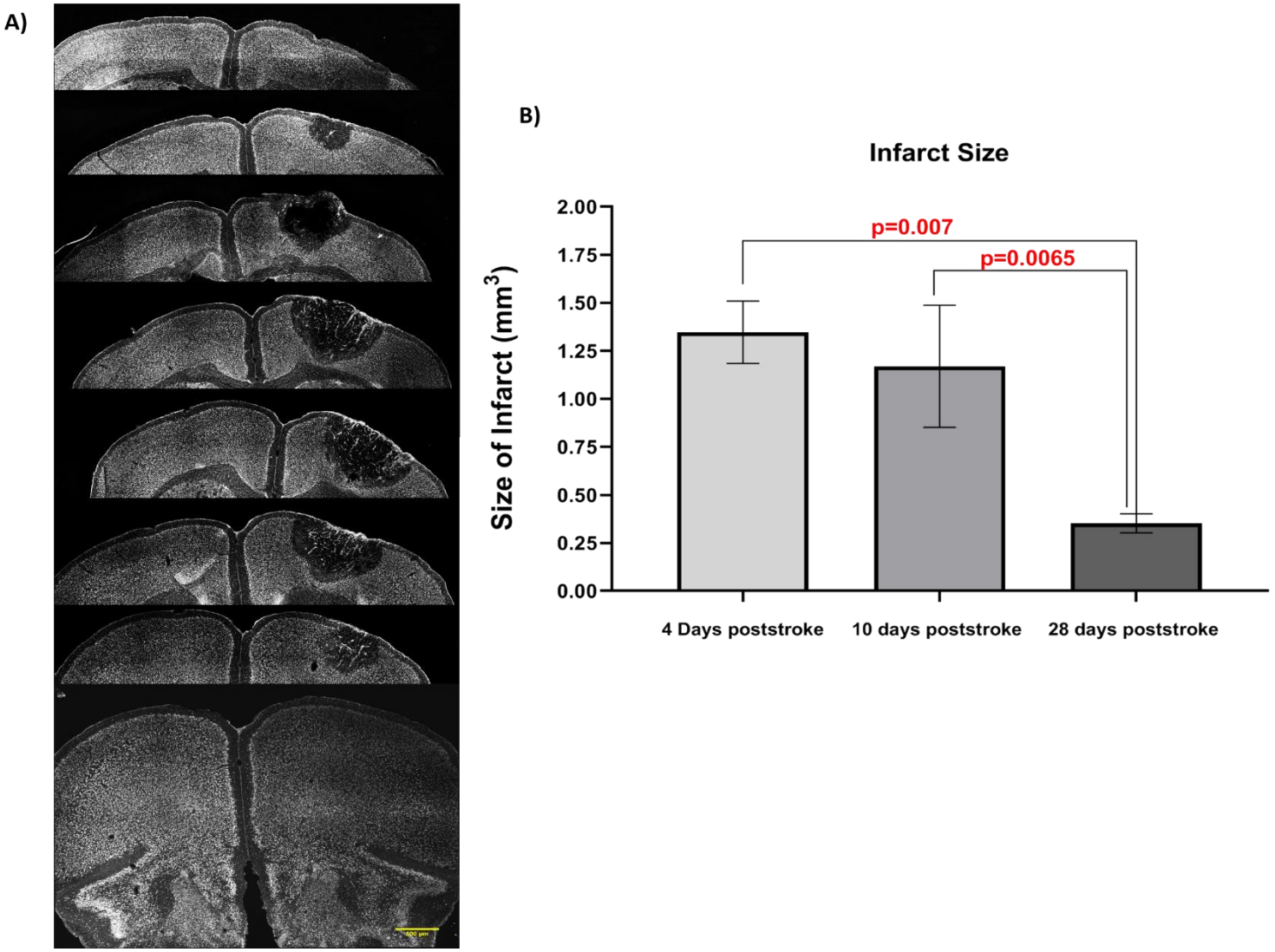


Figure 16: Time course of infarct size. A, shows a representative image of neurotrace staining for 4 days poststroke group. **B**, shows the results of the infarct size done for 3 groups. n= 6, 11 and 17 for 4, 10 and 28 days poststroke, respectively. Significant p values are shown on the graph. One-way ANOVA using Tukey's multiple comparison was performed with a significance level (α value) set at 0.05.

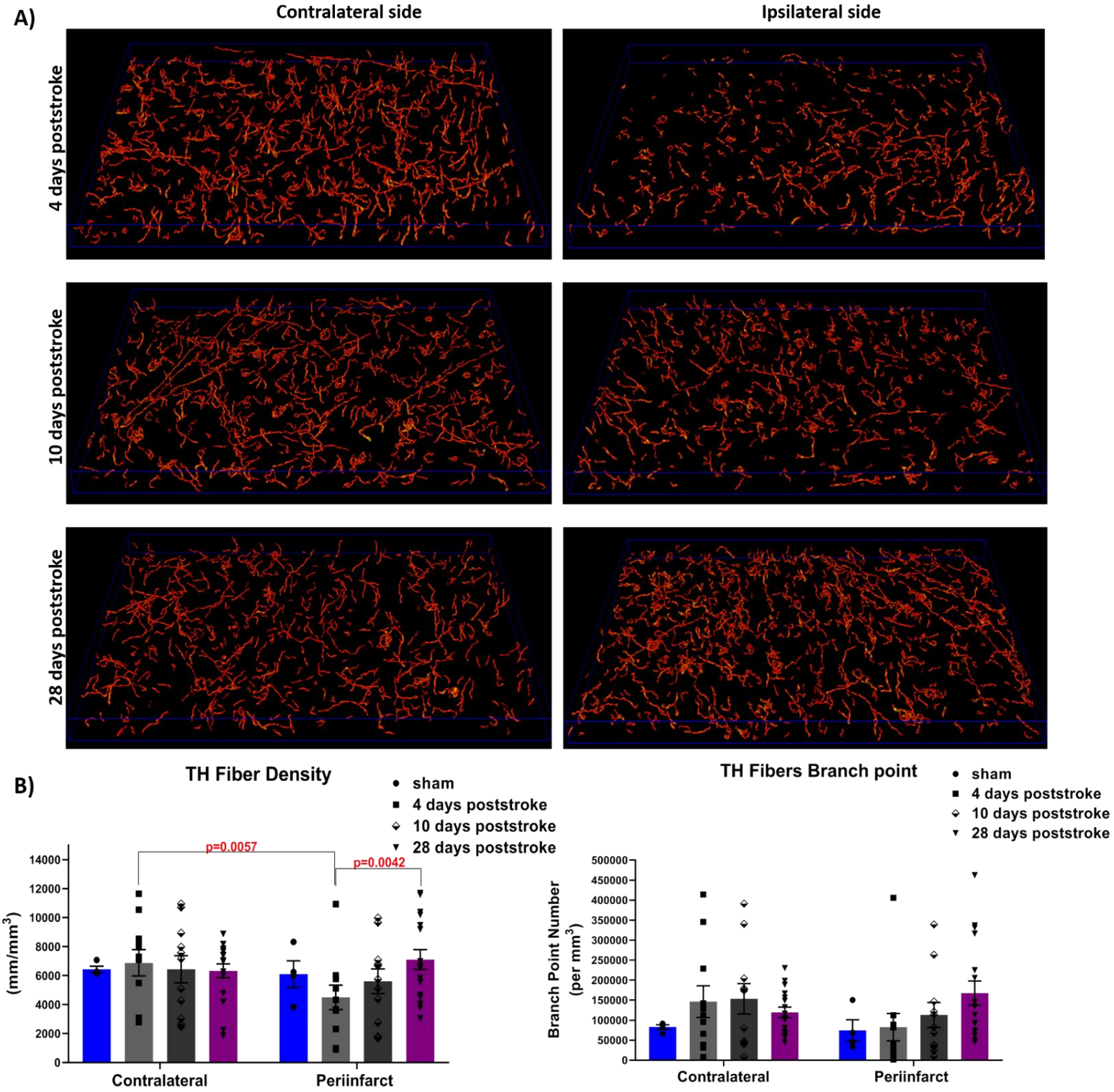


Figure 17: Long-term remodeling of TH-positive fibers after focal stroke. One 40× images are taken from each brain side, which represent the position of ROI2 in figure**. A**, Images represent the 3D reconstruction for TH-positive fibers at 4 (upper panels), 10 (middle panels) and 28 days (lower panels) post-stroke for contralateral (left panels) and ipsilateral side (right panels). **B,** represent the quantification for the density and number of branch points of TH-positive fibers. The significant p values are shown on the graph. For both TH fiber density and branch point measurements Values are reported as means ± S.E.M. (n= 11, 11, 17 for 4, 10- and 28-days post-stroke, respectively and sham n=4). There was significant interaction between the brain side and post-stroke days (p=0.0088 and p=0.036 for Fiber density and number of branch points respectively). A two-way ANOVA using Sidak's multiple comparison test was performed with a significance level (α value) set at 0.05.

Discussion

I. Summary of Findings

Our study, focusing on the involvement of catecholaminergic systems in stroke recovery, demonstrates a DA axon-specific remodeling in the periinfarct region in a mouse model of unilateral ischemic stroke, with or without therapeutic intervention. Increase of dopaminergic, but not noradrenergic, fiber density was reliably observed within the periinfarct region. In mice used to assess spontaneous DA axon remodeling (i.e. without therapeutic intervention), recovery of dopaminergic axon density was evidenced over a period of 28 days. In our mouse stroke model subjected to an asynchronous combination of pharmacological (DHX) intervention and physical activity (running wheel), DA fiber density was further remodeled within the periinfarct region but was also in the contralateral hemisphere. Interestingly, DHX did not affect DA fiber density in a naïve, intact brain, suggesting that the therapeutic intervention used in our study was only effective following ischemic brain injury.

II. Axonal Remodeling is a Fundamental Phenomenon Following Stroke.

Although several studies have investigated axonal remodeling after stroke in different brain regions, to our knowledge our work represents the first attempt to investigate catecholaminergic fiber remodeling, and specifically DAergic axons. We present the first anatomical characterization of dopaminergic and noradrenergic fiber remodeling following stroke in the mouse cerebral cortex. The DA system plays an important role after stroke. Hence, understanding the mechanisms underlying its remodeling following stroke is an important step prior to developing new therapies that target the DA system to promote recovery.

Results of my M.Sc. thesis show that TH-positive structures are axons and not dendrites, as visualized by double staining with MAP2 and DAPI. Indeed, cell bodies of DA neurons are not in the cortex, but located in the brainstem, as documented by other studies on the origin of DA innervation in the cortex (**Figure 11**)(Björklund & Dunnett, 2007).

Our study demonstrates a loss in TH-fibers around the infarct compared to the contralateral, intact side when measured 4 days post-stroke (**Figure 17**). This is consistent with other studies reporting acute and subacute axonal damage following stroke (Hinman, 2014). The axonal damage occurs primary within minutes to hours, as a result of disruption in axonal homeostasis and calcium-dependent proteolysis events, secondary due to disruption of glial-axon communications that impair the supportive role of glial cells (Matute et al., 2013; Rishal & Fainzilber, 2013). This axonal damage does not only occur in the stroke core but it also continues over days in the surroundings of the infarct (Baron et al., 2014).

Days-to-weeks post-stroke, a series of axonal regrowth events, or sprouting, occurs in the periinfarct area (Carmichael, 2006). Recovery is accompanied by formation of new neuronal connections that ultimately ensure rewiring between brain regions (Hinman, 2014). In our study, following reduction in TH-positive fibers in the cortex (periinfarct area), axonal density goes back to the same level as the contralateral side after 3 weeks, which can be explained by a sprouting of DA axons upon appropriate stimulation (endogenous mechanisms triggered in the brain after stroke). Stimulants such as neurotrophic factors, that contribute in nourishing dopaminergic axonal, have been shown to be upregulated after stroke (Benowitz & Carmichael, 2010; Carmichael, et al., 2017; Overman & Carmichael, 2014).

An important factor to consider for promoting recovery is to find the best time for therapeutic/rehabilitation intervention, implying that detecting the timing for DA fiber remodeling is a key element in stroke recovery research.

III. Poststroke-specific Remodeling of Dopaminergic Axons

Catecholaminergic axons express TH, but TH-positive axons are not necessarily dopaminergic. Our data show that TH-positive axons in the periinfarct area are mostly dopaminergic as they do not express NET (**Figure 12**). This is consistent with studies reporting that most of catecholaminergic fibers expressing TH in the motor cortex are DA fibers (Lewis et al., 1987; Vitrac & Benoit-Marand, 2017). Furthermore, NET-positive axons are denser in the rodent prefrontal cortex compared to motor cortex, and higher in layer I than in layers V and VI, while DA axons are denser in deep layers (i.e. layer VI) (Gaspar et al., 1991; Agster et al., 2013; Vitrac & Benoit-Marand, 2017). In our study, selected ROIs for fiber quantifications (**Figure 10**A and **Figure 14**A) were located in the deeper layers of the motor cortex, which could further support the dopaminergic nature of remodeled TH-positive axons.

In contrast to noradrenergic axons (**Figure 14**), our data show a significant increase in the density of DA axons in selected ROIs (periinfarct) of saline-treated mice (undergoing only physical rehabilitation) relative to the contralateral side (**Figure 10**C). Studies have also reported a similar DA fiber remodeling in the deep layers (layer V) of the anterior cingulate cortex (ACC) but not in superficial layers nor in the parietal cortex of aged rats, where the density of dopaminergic fibers increases compared to young animals. In contrast, noradrenergic fibers do not remodel with aging (Allard et al., 2011). In our study, the ROIs referred to periinfarct areas are covering parts

of the deep layers of ACC that are prone in normal aging for dopaminergic remodeling. Moreover, 28 days after traumatic brain injury (TBI), the number of TH-positive axons and TH levels, but not DBH levels, were significantly higher in the frontal cortex (Yan et al., 2001). Furthermore, another study has reported a significant acute decline in DA levels but not norepinephrine levels over 2 weeks post-TBI (Mcintosh et al., 1994). Therefore, it seems that in the several areas of cerebral cortex, the DA system is more susceptible to alteration than the noradrenergic system after cerebral damage.

Although this is an interesting observation, the physiological and behavioural consequences of DA fiber remodeling on post-stroke recovery is not completely understood. One possible explanation are the adaptive mechanisms that the brain engages after stroke to promote recovery. A study conducted by Windle et al. in 2007 showed that depleting the noradrenergic innervation in the sensorimotor cortex did not impede enriched environment-induced recovery but facilitated recovery for standard housed animals, which may suggest that the norepinephrine system does not participate in motor recovery to the same extent as the DA system. This could explain the minimal remodeling of noradrenergic axons we observed. These findings support the hypothesis that DA innervation and/or the DA system may be more involved in post-stroke motor recovery than the noradrenergic system.

IV. An Important Question: Where do the new or regenerated DA axons come from?

There are two possible sources.

- **The first possibility,** that is worth discussing and we cannot rule out is the existing, damaged but not destroyed DA axons may exhibit a significant reduction of TH levels, which may have led to undetectable expression by immunostaining. Moreover, DA levels are also reduced, and these damaged DA cells and axons only recover with time. To rule out this possibility further studies have to be done to address this issue. However, this possibility can somehow be excluded as reported by other studies that TH and DA levels change also during days-to-weeks following stroke in the contralateral side, not just in the periinfarct side (Obi et al., 2018; Gower & Tiberi, 2018). In our study, the density of DA axons in the contralateral side did not change and this was consistent throughout our time course study and similar relative to sham animals (**Figure 17**).
- **The second possibility,** which is more likely, is the sprouting of new cortical axons from the existing (severed) axons. This phenomena has been seen after DA toxin lesions to the DA innervation in the striatum of rodents and monkeys (Bezard et al., 2000; Stanic et al., 2003a;b). Our data favours this possibility since the branching points in saline-treated mice were significantly increased a month after stroke induction (**Figure 10**C). In addition, our time course study shows that, even if the increase in branching points between 4- and 28-days post-stroke was not statistically significant, there was a reduction followed by an increase in the following weeks (**Figure 17**B).

V. Role of DA System on Axonal Growth

Stroke induces series of regenerative events to promote axonal sprouting. However, therapeutic intervention is needed to promote optimal axonal remodeling (Carmichael et al., 2017; Obi et al., 2018; Carmichael et al., 2001; Li et al., 2015).

The present study sought to determine the mechanism by which D1-class receptor agonist DHX modulates TH-positive axons following stroke. Cohort 1 data showed that DA fibers substantially remodeled by increasing the density and the branch points mainly in the periinfarct area compared to contralateral side of saline-treated mice. However, DHX–treated mice show a modulation of axonal remodeling mainly in the contralateral side. This remodeling could explain the recovery on our behavioural tests. Nevertheless, associating the recovery seen on the behavioral tests and the observed axonal remodeling induced by DHX should be further investigated by impeding the remodeling of the cortical dopaminergic axons following drug treatment.

Dopamine also plays important roles during development, as it regulates neuronal structure and function, as well as formation of neural circuits (Money & Stanwood, 2013). DA receptors activation induce changes in architecture of neurites. *In vivo* and *in vitro* studies demonstrate that D1-class receptors exert different effects on neurite outgrowth in a regional- and receptor subtype- specific manner during brain development (Money & Stanwood, 2013). *In vitro* experiments D1-class receptors reduces neurite outgrowth in cortical neurons but increases neurite outgrowth in striatal neurons (Money & Stanwood, 2013). Depleting midbrain DA neurons during development induce changes in the structure of the fronto-parietal cortex and

decrease expression of neurite guidance and growth genes (Krasnova et al., 2007). Studies suggest that, after stroke, several mechanisms are awaken to mimic the developing brain at both the molecular and cellular levels (Cramer & Chopp, 2000). Although, regenerative mechanisms following stroke have not yet been fully clarified, the DA-induced modulatory mechanisms in the neuronal and network structure could be triggered after stroke.

It is unclear how D1-class receptor activation regulates axonal remodeling. In fact, multiple signaling cascades have been reported to regulate axonal remodeling. Further studies are required to explore the underlying mechanisms of axonal remodeling controlled by D1-class receptors. Meanwhile, activation of D1-class receptors increases intracellular cAMP that activates PKA, which in turn phosphorylates CREB. CREB is a transcription factor that regulates axonal growth and regeneration, and it promotes recovery after stroke by inducing axonal sprouting and remapping of the periinfarct areas, and enhances formation of new connections (Moore & Goldberg, 2011; Caracciolo et al., 2018). Another possible mechanism that D1-Class agonist regulate remodeling through regulating axon guidance molecules. It has been shown in vitro that DHX induces changes the axon guidance gene expression (EphrinA1, EphrinA5, EphB1, and Semaphorin3C). Axon guidance molecules contribute to synaptic function and shaping neural circuits (Stoeckli, 2018).

VI.Effect of Physical Exercise on DA-Related Neuronal Repair

There is growing interest in research on physical exercise and its beneficial effects on neurodegerative diseases (i.e. Parkinson's disease, Alzheimer's disease, stroke) (Hou et al., 2017; Liu et al., 2019) . Forced use of the DA lesion-impaired limb, running wheels, and treadmill are

forms of exercise used in several studies to demonstrate enhanced motor recovery from the PD-like motor deficit symptoms. Interestingly, exercise increases DA innervation in the striatum after damage nigrostriatal projections. Although studies have documented the neuroprotective effect of exercise on DA innervation, evidence also exist for a role of exercise in promoting axonal sprouting (Hou et al., 2017).

A possible explanation for the increase in density and branching of periinfarct DA axons in saline-treated mice may due to the physical rehabilitation that these animals went through (i.e. free access to running wheels). Indeed, several studies have reported that physical rehabilitation induces axonal sprouting. Running wheels as a form of exercise can increase expression of brain-derived VEGF and IGF-I following tissue damage by whole-brain irradiation (Wong-goodrich et al., 2010). VEGF stimulates axonal outgrowth *in vitro* in response to axonal injury that could act in an autocrine fashion (Sondell et al., 2000). IGF-I enhances axon outgrowth of corticospinal motor neurons both *in vivo* and *in vitro,* and blocking IGF-I receptor causes axon outgrowth defects in the corticospinal tract (Lee et al., 2014; Zdinler & Macklis, 2006).

Another known neurotrophic factor that is expressed in the brain that participates in neuronal growth and differentiation is the brain-derived neurotrophic factor (BDNF). Aerobic exercise increases BDNF levels mainly in the cortex, which is associated with increased motor learning and cortical reorganization during post-stroke recovery (Quirie et al., 2012; Alcantara, et al., 2018). A study performed by Zhang et al. in 2013 assessed the effect of treadmill, as a form of physical exercise, on axonal regeneration in a MCAo stroke model. They showed that physical exercise enhanced axonal regeneration of newborn striato-nigral and cortico-nigral projection neurons. This form of exercise significantly up-regulates BDNF expression and down- regulates

Nogo-A expression, which impedes axonal regeneration (GrandPre et al., 2000; Zhang et al., 2013).

VII. Other Possible Mechanisms Underlying Post-Stroke DA Axon Remodeling

Astrocytes are glial cells present in the brain, with a supportive role for neurons by regulating cerebral blood flow and energy metabolism (Pellerin et al., 2007; Barres, 2008). Upon neuronal injury or damage, these glial cells change their molecular and structural profile and turn into reactive astrocytes that form glial scar around the damaged area (Eddleston & Mucke, 1993; Ridet et al., 1997). Studies on the role of astrocytes on repair mechanisms after stroke are contradictory and inconclusive. Several studies have used ablation or attenuation of the reactive astrocytes after stroke or TBI. These experimental approaches cause many detrimental effects on the neuron regeneration and repair that include an increase of lesion size and neuronal loss, demyelination, and exacerbated loss of function (Bush et al., 1999; Myer et al., 2006; Herrmann et al., 2008; Drögemüller et al., 2008; Li et al., 2008).

Astrocytes secrete neurotrophic factors like GDNF, which promotes the survival of neurons, and enhance axonal regeneration and neurite outgrowth following damage in vivo and in vitro (Scharr et al., 1993; Zhang et al., 2010). Culturing DA neurons with astrocyte-conditioned medium that contains GDNF, enhances neuronal survival (Lin et al., 1993). In addition, astrocytes express dopaminergic receptors and are thought to secrete trophic factors in response to DA (**Figure 18**). Moreover, GDNF is found to increase phosphorylation of TH at Ser-31 and Ser-40, which enhances dopamine synthesis in rat primary mesencephalic neuron cultures (Kobori et al., 2004).

These studies may explain the contribution of reactive astrocytes via GDNF factor in activating the TH in the remodeled TH-positive fibers in the periinfarct area as demonstrated in our data (co-localization of TH-positive fibers with either form of phosphorylated TH).

Our immunohistochemical studies showed that extensions of reactive astrocytes are intermingled with the high-density TH-positive fibers around stroke, which could suggest interaction between the cell types (**Figure 19**). However, future molecular studies are needed to characterize better the effects of the astrocytes on DA axon remodeling following stroke.

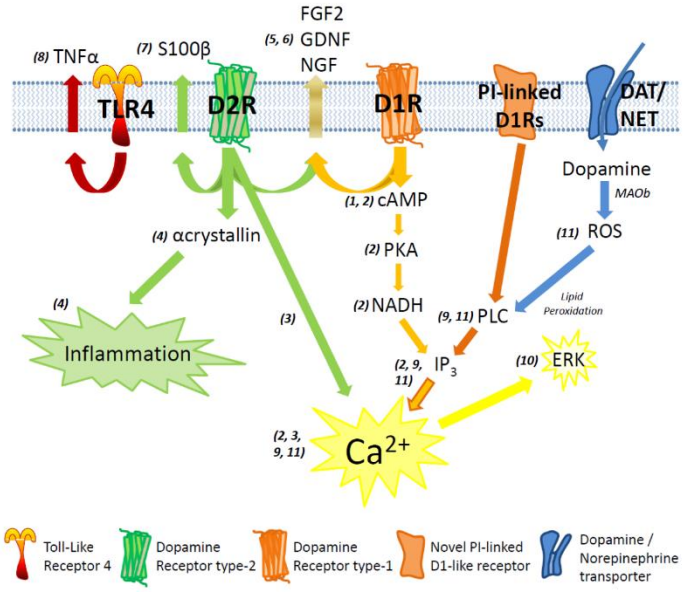


Figure 18: Possible dopaminergic signalling pathways in astrocytes. Dopaminergic receptors stimulate different signaling pathways within astrocytes that eventually triggers increase in the intracellular Ca^{2+} and the release of tropic factors. D1-type receptor (D1R), cyclic AMP (cAMP), Protein Kinase A (PKA), NADH, IP3 and Ca^{2+}. D2-type receptor (D2R), S100β secretion, αcrystallin. Fibroblast Growth Factor 2 (FGF2), Glial-Derived Neurotrophic Factor (GDNF) and Nerve-Growth Factor (NGF), Monoamine Oxidase B (MAOb), Reactive Oxygen Species (ROS). Adapted from Jennings & Rusakov, 2016

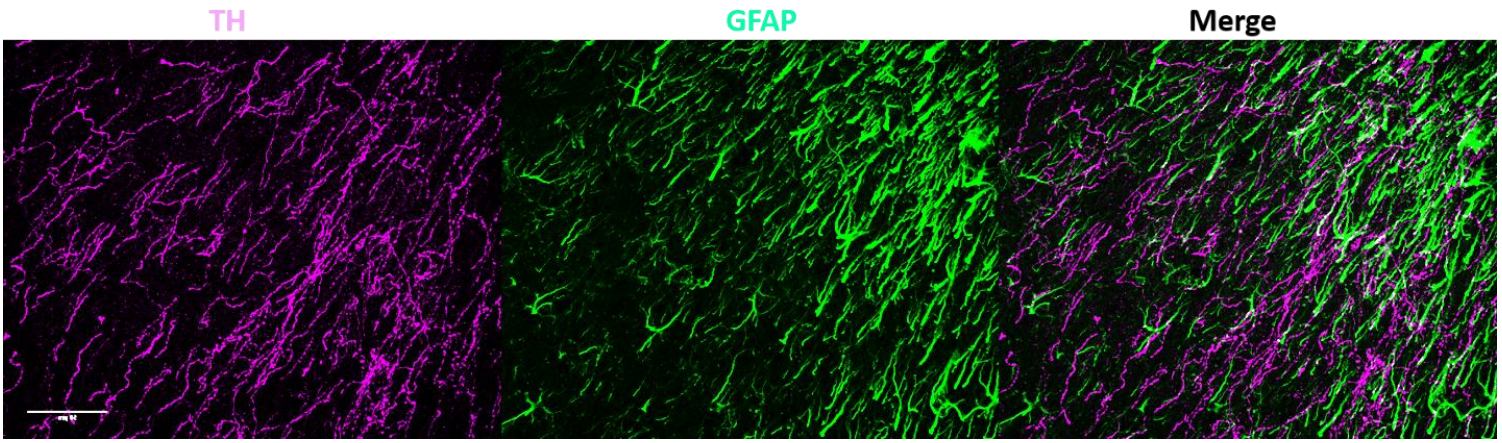
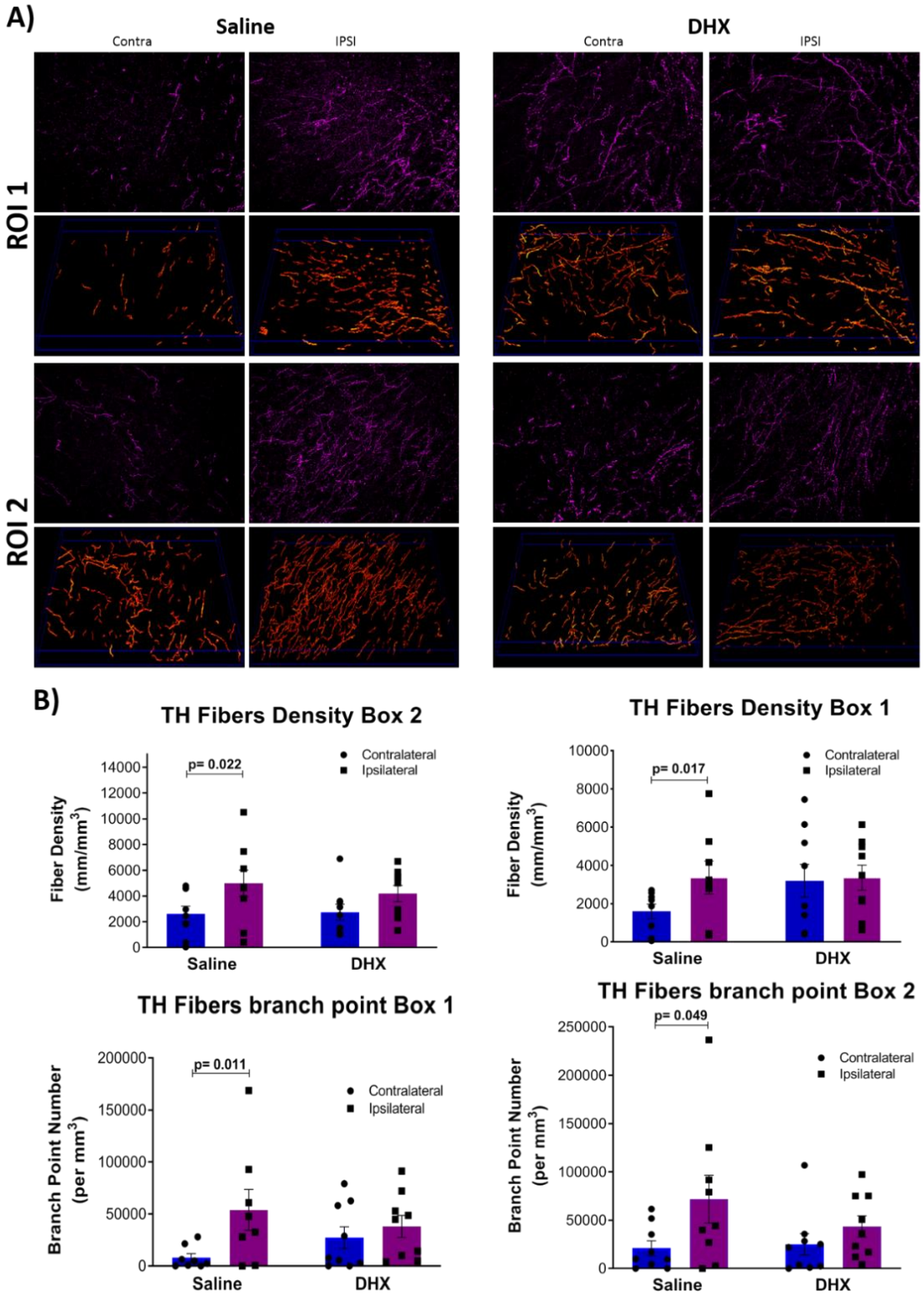
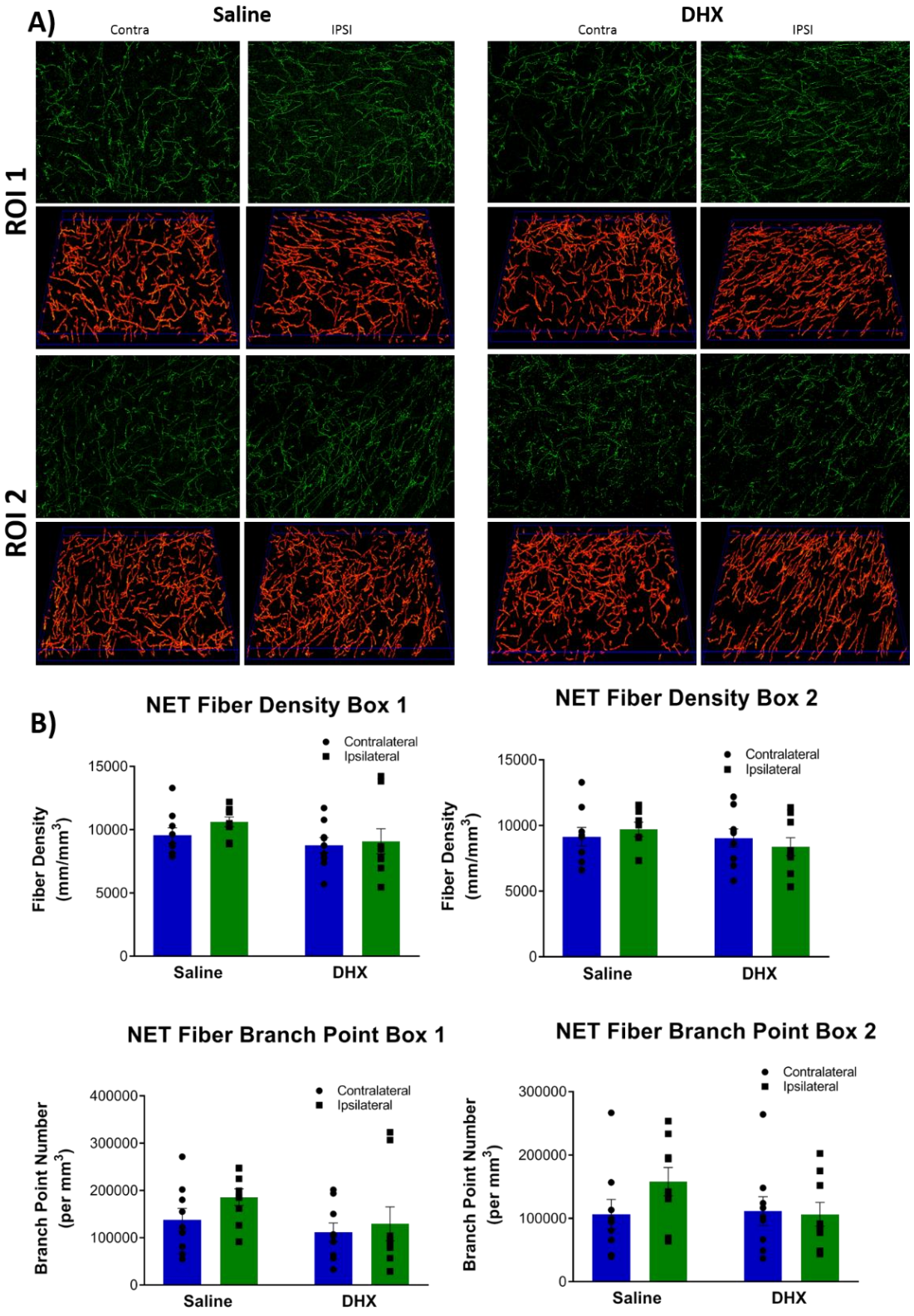


Figure 19: Astrocytes and TH-positive fibers intermingling after stroke induction. GFAP (Glial fibrillary acidic protein, marker for reactive astrocytes) and TH-positive cells are prominently expressed in the peri-infarct region

Appendix.



Supplementary Figure 1: TH-positive fiber remodeling. A, Representative example of 40X images and their 3D reconstruction (ROI 1 and ROI 2 second rows) is shown. Images of contralateral (contra) and ipsilateral (IPSI) sides are shown in left (Saline group) and right (DHX group) panels for box 1 (upper two rows) and box 2 (lower two rows). **B,** Quantification of the density and number of branch point of TH-positive fibers for each ROI. Values are reported as mean ± S.E., with n = 9 for both groups. A two-way ANOVA using Sidak's multiple comparison test was performed with a significance level (α value) set at 0.05.



Supplementary Figure 2: NET-positive fiber quantification. A, Representative example of 40X images and their 3D reconstruction (ROI 1 and ROI 2 second rows) is shown. Images of contralateral (contra) and ipsilateral (IPSI) sides are shown in left (Saline group) and right (DHX group) panels for ROI1 (upper two rows) and ROI 2 (lower two rows). **B,** Quantification of the density and number of branch point of NET-positive fibers for each ROI. Values are reported as mean ± S.E., with n = 9 for both groups. A two-way ANOVA using Sidak's multiple comparison test was performed with a significance level (α value) set at 0.05.

Conclusion

Our findings demonstrate that, after stroke, axons display a very specific remodeling. The present study supports previous work showing post-stroke axonal remodeling, but also extends previous findings to a specific class of neurons, i.e. dopaminergic neurons. Indeed, we show that DA axons are more prone to remodel following ischemic stroke. DA axons are highly dynamic after stroke, especially in the periinfarct area, while noradrenergic axons showed minimal remodeling. We also investigated the dynamics of DA fiber remodeling in the days and weeks following stroke. We investigated the contribution of an asynchronous combination of pharmacological (DHX, D1-class receptor) intervention and physical activity (running wheel), on DA axonal remodeling following stroke, which was correlated with the positive behavioral outcomes.

Future Directions: The present study set the foundation for future work that could help understanding the contribution of DA axonal remodeling to functional stroke recovery. It remains to be addressed if physical activity is required for DHX effect on axonal remodeling. Future studies could address this issue by investigating axonal remodeling, without any form of physical activity. In addition, it would be important to understand to what extent DA fiber remodeling is implicated in motor recovery that could be addressed by damaging the new DA fibers during their recovery period. Also, it would be important to know where these new/remodeled fibers are coming from using tracing techniques. Finally, it will also be critical to understand whether other cell types in the brain, such as astrocytes, play a regulatory role in DA axon remodeling.

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