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THE SPINAL TRANSCRIPTOME AFTER CORTICAL STROKE AND ITS IMPACT ON SPROUTING AND "SIDE-SWITCH" OF CONTRALESIONAL CORTICOSPINAL FIBERS

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“ *In adult centres the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree.* ”

Santiago Ramón y Cajal,
Degeneration & regeneration of the nervous system, 1959

Summary

Stroke leads to severe neurological disability, often affecting motor and sensory systems. Thus, stroke survivors often face a majorly decreased quality of life. Treatment options for chronic stroke patients are limited, in part because of the lack of knowledge about the pathophysiological processes underlying the limited spontaneous recovery that can be observed within the first weeks after the insult. In response to cortical injury, the brain shows a high degree of plasticity to restore or compensate for lost behavioral functions in humans and in animal models. This is evidenced by the remapping of functions to related areas. In rodents, increased compensatory sprouting has been observed on several levels along the neuraxis such as the brainstem and the spinal cord. This structural plasticity most likely is a key element of functional recovery, however, the mechanisms that shape the rewiring are incompletely understood. The present work aims at understanding tissue-derived factors and molecular mechanisms, which may influence the intrinsic neuronal growth capacity on the spinal level after cortical stroke.

In **Chapter 1**, we summarize the anatomical substrate of skilled motor movements across mammalian species with the corticospinal tract (CST) emerging as the major supraspinal command system in primates and humans. Ablation of the CST leads to structural changes within this circuitry required for skilled motor movements. Reparative and compensatory reorganization of the CST system can be strengthened by rehabilitative training. The presented findings suggest the existence of an underlying region-specific molecular machinery. **Chapter 2** summarizes the current knowledge about the extrinsic and intrinsic molecular mechanisms shaping structural plasticity after stroke with a focus on the time line of major events such as *(1)* trigger of growth *(2)* branching of axon collaterals *(3)* axon elongation / pathfinding *(4)* synapse formation / target interaction *(5)* synapse refinement by strengthening of active synapses and elimination of weak synapses.

In **Chapter 3**, we aimed to address the anatomical and molecular changes in the stroke-denervated spinal hemicord and how they relate to plastic changes of the contralesional CST. We show a reorganization of contralesional CST axonal arbors in the cervical stroke-denervated spinal hemicord. RNA-Sequencing of their target area at time points matching the previously defined key phases of structural plasticity revealed a time point specific differential gene expression. Two major phases emerged: An early inflammatory phase within the first week after stroke, as well as a highly dynamic phase at four to six weeks after injury,

during which upregulation of growth-promoting genes may influence rewiring of sprouting corticospinal motor neurons (CSMNs). We showed that three of these differentially regulated genes, Semaphorin 6a, Netrin G2 and TGF-β1, all of which were up-regulated at 28 days after injury, could represent potential modulators of the stroke-induced structural plasticity based on their potential to rescue neurite outgrowth in a growth-inhibitory setup *in vitro*. A number of additional genes have been found to be differentially regulated in this mRNA expression screen that may provide novel candidates as modulators of the different phases and processes leading to stroke recovery.

In **Chapter 4**, we further addressed the potential influence of the early immune response on stroke recovery. We found an early activation of spinal microglia after cortical stroke specifically in the projection area of the lesioned CST. Transgenic reduction of the CX3C fractalkine receptor 1, a chemokine involved in neuron-microglia communication, led to a decreased recovery rate in a fine motor-related behavioral task, while the stroke lesion size was unaffected. This may suggest a positive involvement of microglia on functional recovery after stroke.

In summary, fiber growth and structural plasticity can be observed on spinal levels, in particular the intermediate laminae, within six weeks after cortical stroke in adult mice. Early inflammatory responses may trigger a favorable tissue environment for axonal sprouting of the contralesional CSMNs after stroke. At later phases of the recovery, the stroke-denervated spinal grey matter may present an orchestrated growth-promoting environment to new, re-innervating contralesional CST branches. The transcriptomic data set described here will serve as a resource to uncover underlying molecular mechanisms for key steps leading to stroke recovery, that may ultimately lead to novel neuroregenerative treatment options also for stroke patients.

Zusammenfassung

Der Schlaganfall und seine Folgen zählen zu den führenden Ursachen von schwerwiegenden neurologischen Defiziten. Durch das Versagen von motorischen und sensorischen Systemen wird die Lebensqualität für den Patienten oft stark eingeschränkt. Die Behandlungsmöglichkeiten insbesondere in chronischen Stadien sind sehr limitiert. Dies ist zu großen Teilen darauf zurückzuführen, dass die Pathophysiologie der spontanen funktionellen Regeneration, die innerhalb der ersten Wochen nach dem Eintreten eines Schlaganfalls zu beobachten ist, bisher nur lückenhaft verstanden ist. Ein Grundelement dieser Regeneration ist eine anatomische Plastizität in Gehirnregionen, die funktionell mit den Bereichen des zentralen Nervensystems (ZNS) verwandt sind, welche durch den ischämischen Insult geschädigt wurden. Die Neurone in diesen Regionen bilden durch Aussprossen neue Nervenfasern und stellen so neue Verbindungen innerhalb des Gehirns, des Hirnstamms oder des Rückenmarks her. Die genauen Mechanismen, wie diese erhöhte Plastizität auf molekularer Ebene zustande kommt, sind nach heutigem Stand der Wissenschaft jedoch nur wenig verstanden. Die vorliegende Arbeit beschäftigt sich aus diesem Grund mit den Faktoren innerhalb des ZNS, die dieses Aussprossen initiieren und lenken können. Die hier erarbeiteten Resultate tragen zum Verständnis der molekularen Mechanismen bei, die die funktionelle Erholung nach einem Schlaganfall steuern.

In **Kapitel 1** beschreiben wir die anatomischen Grundlagen der Feinmotorik von Ratten und Primaten und wie diese sich durch einen ischämischen Insult verändern. Hierbei wird besonders der Kortikospinaltrakt (KST) als wichtiges supraspinales Kontrollsystem hervorgehoben. Eine Läsion des KST führt in Nagern zu einer anatomischen Umstrukturierung und einer Neubildung von Schaltkreisen, die durch rehabilitative Übungen gezielt gestärkt werden können. In **Kapitel 2** legen wir den heutigen Wissensstand um die molekularen Grundlagen dieser anatomischen Plastizität dar. Der Zeitverlauf dieses regenerativen Faserwachstums kann in verschiedene Phasen unterteilt werden: *(1)* Wachstumsanstoß, *(2)* Aussprossen von Kollateralen aus dem Axon des kompensierenden Neurons, *(3)* Wachstum und Wegfindung zu den Zielzellen, *(4)* Synapsenbildung mit der Zielzelle, *(5)* Feinjustierung des neu gebildeten Schaltkreises durch Stärken von nützlichen und Entfernungen von hinderlichen Synapsen.

Kapitel 3 fasst unsere Ergebnisse über die molekularen Veränderungen in der durch den Schlaganfall denervierten Hälfte des zervikalen Rückenmarks zusammen, einer Region, in der eine Umstrukturierung des kontraläsionalen KST zu beobachten

ist. Dieses Areal wurde mit Hilfe einer Gesamt-Transkriptom-Sequenzierung zu verschiedenen Zeitpunkten, die den oben definierten Phasen der anatomischen Plastizität entsprechen, untersucht. Es konnte eine sehr spezifische zeitliche Expressionsmodulation verschiedener Gene beobachtet werden. Auf Basis dieser Transkriptom-Analyse wurden molekular zwei wesentliche Phasen definiert, die das Gewebe für die neu innervierenden Nervenfasern bereitstellt: Eine frühe inflammatorische Phase innerhalb der ersten Woche nach dem Schlaganfall, und eine spätere Phase zwischen vier und sechs Wochen nach dem Schlaganfall, in der wachstumsfördernde Gene verstärkt exprimiert werden. Diese Faktoren können möglicherweise das Aussprossen der kontraläsionalen KST Neurone beeinflussen. Für drei dieser Gene, Semaphorin 6a, Netrin G2 und TGF-β1, die vier Wochen nach dem Schlaganfall im denervierten Rückenmark verstärkt exprimiert werden, konnten wir mittels *in vitro* Experimenten eine mögliche Rolle als Wachstumsmodulatoren bestätigen. Zusätzliche vermehrt exprimierte Gene aus unserem etablierten Datensatz könnten als weitere Kandidaten dienen, die in den verschiedenen Phasen einen modulatorischen Einfluss auf die Regeneration nach Schlaganfall haben. In **Kapitel 4** gehen wir auf die möglichen Einflüsse der beobachteten frühen Inflammationsantwort in der denervierten Hälfte des zervikalen Rückenmarks auf Reparationsprozesse nach Schlaganfall ein. Eine frühe Aktivierung der spinalen Mikroglia nach einem kortikalen Schlaganfall wurde innerhalb des Areals beobachtet, in das Nervenfasern des degenerierenden KST vor dem Insult projiziert haben. Eine reduzierte Expression des Fraktalkin Rezeptors CX3CR1, der in die Neuronen-Mikroglia Verständigung involviert ist, führte in heterozygoten knock-out Mäusen zu einer verlangsamten Verbesserung der Feinmotorik des Vorderbeins nach Schlaganfall, ohne dass ein Einfluss auf die Läsionsgröße festgestellt wurde. Dies deutet auf eine positive Rolle der Mikroglia für die Regeneration nach Schlaganfall hin.

Zusammenfassend konnten wir im Zeitverlauf von sechs Wochen nach einem induzierten Schlaganfall in der Maus eine Umstrukturierung der axonalen Projektion des kontraläsionalen KST innerhalb des zervikalen Rückenmarks, insbesondere in den prämotorischen Schichten der grauen Substanz, nachweisen. Eine frühe inflammatorische Reaktion innerhalb dieses Gebietes könnte den Weg für eine erfolgreiche Regeneration ebnen. Zu späteren Zeitpunkten stellt die durch den Schlaganfall denervierte Hälfte des zervikalen Rückenmarks eine wachstumsfördernde Umgebung für einwachsende Axone des kontraläsionalen KST dar. Der in dieser Arbeit etablierte Transkriptom-Datensatz wird in zukünftigen Studien als wichtiges Hilfsmittel dienen, um grundlegende Fragen über die Gewebereparatur und Faserregeneration nach Schlaganfall zu beantworten. Dies ermöglicht hoffentlich, in der Zukunft neue neuroregenerative Behandlungsmöglichkeiten für Patienten in chronischen Stadien entwickeln zu können.

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Abbreviations

ATRX	alpha thalassemia/mental retardation syndrome X-linked homolog
BBB	blood brain barrier
Bcl-2	B cell lymphoma/leukemia 2
BDA	biotinylated dextran amine
BDNF	brain derived neurotrophic factor
BMP	bone morphogenic protein
BSN	brainstem neuron
C1q	complement component 1q
C3	complement component 3
CAM	cell-adhesion molecule
cAMP	cyclic adenosine 3' , 5' - monophosphate
CAP23	cortical associated protein 23
cCSMN	contralateral corticospinal motor neuron
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CPA	CST projection area
CPM	counts per million mapped fragments
CREB	cAMP response element-binding protein
CSMN	corticospinal motor neuron
CSPG	chondroitin sulfate proteoglycan
CST	corticospinal tract
CTNF	ciliary neurotrophic factor
DCC	deleted in colorectal carcinoma
DEA	differential expression analysis
dpi	days post injury
DRG	dorsal root ganglion
ECM	extracellular matrix
ERK	extracellular- signal regulated kinase 1,2
FA	formaldehyde
FDR	false discovery rate
GAP43	growth associated protein 43
GDF	growth differentiation factor
GDF10	growth differentiation factor 10
GDNF	glial-cell-line-derived neurotrophic factor
GO	gene ontology
GSEA	gene set enrichment analysis
Iba-1	ionized calcium-binding adapter molecule 1
iCSMN	ipsilateral corticospinal motor neuron
IFN-γ	interferon γ
IGF-1	insulin like growth factor 1
IL	interleukin
Itga3	integrin subunit alpha 3
Itga7	alpha-7 integrin
KLF	krüppel-like factor
LIF	leukemia inhibitory factor
LPAR1	lysophosphatidic acid receptor 1
LPPR1	lipid phosphate phosphatase-related protein type 1
LRRTM	leucine rich repeat trans-membrane
LTD	long-term depression
LTP	long-term potentiation
MAG	myelin-associated glycoprotein
MAI	myelin-associated inhibitor
MAPK	mitogen-activated protein kinase
MCAO	middle cerebral artery occlusion

MHC1 class I major histocompatibility complex

MMP9 matrix metalloprotease 9

MN motoneuron

Mst3b mammalian sterile 20-like kinase-3b

MT-IIA metallothionein isoform IIA

mTOR mechanistic target of rapamycin

NEA network enrichment analysis

NGF nerve growth factor

NGL-2 netrin-G ligand 2

NgR nogo receptor

NL neuroligin

NRX neurexin

Ntng2 netrin G2

OMgp oligodendrocyte myelin glycoprotein

PI3K phosphoinositide 3-kinase

PKA protein kinase A

PKC protein kinase C

PlxnA1 plexin A1

PN propriospinal neuron

PNS peripheral nervous system

PTEN phosphatase and tensin homolog

RAG regeneration associated gene

RaST raphespinal tract

ReST reticulospinal tract

RFN reticular formation neuron

RGC retinal ganglion cell

ROCK rho-associated protein kinase

ROS reactive oxygen species

RTN reticulon

RuST rubrospinal tract

SCE spinal cord extract

SCI spinal cord injury

SDEG significantly differentially expressed gene

SEM standard error of the mean

Sema6a semaphorin 6A

Serping1 serine (or cysteine) peptidase inhibitor, clade G, member 1

sIN segmental interneuron

siRNA small interfering RNA

Sox9 SRY-Box 9

SPRR1a small proline rich protein 1a

Srgap1 Slit-ROBO Rho GTPase Activating Protein 1

STAT3 signal transducer and activator of transcription 3

TGF transforming growth factor

TNF-α tumor necrosis factor α

TREM-2 triggering receptor expressed on myeloid cells 2

Trk tropomyosin receptor kinase

TSP thrombospondin

WD wallerian degeneration

1

Tapping the Wires of Movement: Spontaneous Reorganization of Supraspinal Command Centers after Stroke

Writing a postcard, uncorking a bottle of wine, even laughing at a good joke – these activities of daily living seem, at first, easy tasks. The biological mechanisms of how the motor components of these activities are encoded and executed within the central nervous system (CNS) however are strikingly complex (Lemon, 2008) and depend on integration of descending motor and ascending sensory signals, ranging bidirectionally from the brain via the spinal cord to the muscle. The importance of this wiring is often seen only after an injury to the motor system affecting these activities of daily living. After insults to the brain, the CNS displays some degree of plasticity, which is causally linked to functional recovery. Fundamental knowledge about the wiring of the brain is pivotal to understand the anatomical changes occurring after injury and might allow for development of novel therapies for patients, ultimately enhancing their quality of life. In this chapter, a broad overview of the anatomy of skilled movement is provided with a special focus on rewiring after the most common lesion to the motor system, a cortical stroke.

1.1 | Anatomical Pathways Controlling Skilled Movement in the Healthy Adult Central Nervous System

The "motor infrastructure" used for executing movements is highly conserved across mammalian species (Grillner, 2003, Nudo and Masterton, 1988). Briefly, to coordinate motor behavior and initiate muscle contractions, motoneurons (MNs) receive descending input from spinal networks of interneurons as well as supraspinal centers such as the brainstem and the motor cortex (Eccles, 1967). Ascending sensory information from the periphery of the body refines movement by providing feedback onto these motor outputs (Rossignol, 2006, Windhorst, 2007). Even though these general building blocks of the "motor infrastructure" do not differ among mammalian species, the connectivity of them may have developed differently due to the individual needs of each species during evolution with an increasing importance of the motor cortex (Courtine et al., 2007, Isa et al., 2007, Lemon and Griffiths, 2005).

1.1.1 | The Motor Infrastructure: A Distributed Network on all Levels of the Neuraxis

In mammals, muscles are directly controlled by MNs within the brainstem (cranial nerves, head muscles) and the ventral grey matter of the spinal cord, with MNs controlling forelimb muscles found within the cervical enlargement, while hindlimb control is localized in the lumbar enlargement of the spinal cord. MNs are arranged topographically into clusters, with the pools controlling more distal muscles sitting more dorsally and caudally while proximal muscle MNs are located more ventrally and rostrally (Kuypers, 1981, Levine et al., 2012). The input for MNs stems from several levels of the neuraxis including the cortex, brainstem as well as spinal interneuronal networks, depending on the task at hand.

Basic motor functions such as locomotion and reflexes are mainly controlled by spinal networks, which include the so-called central pattern generators (Brown, 1914), as well as brainstem networks. The composition and circuitry of these networks are still under investigation (Arber, 2012, Grillner, 2003, Kiehn, 2016); however, it has emerged that they can act in a self-regulatory manner and independently of supraspinal command to some degree as shown in the famous experiment of the spinalized cat, which, after transection of the supraspinal levels, still was able to produce rhythmic locomotor patterns on the treadmill when a "memory trace" was formed by preceding training (Barbeau and Rossignol, 1987, de Leon et al., 1999). Voluntary interaction with the environment, e.g. skilled movement, on the other hand relies heavily on input from supraspinal levels (Barbeau and Rossignol, 1987, Courtine et al., 2009, Musienko et al., 2012).

Movement planning, initiation and refinement, which coordinate the execution of skilled movements, involves several supraspinal centers of the brain and brainstem (Armstrong, 1986, Drew et al., 2004, Jordan, 1998, Liang et al., 2011). Their descending pathways can be classified into four main categories based on their origin of projection as well as their termination field within the spinal cord (Figure 1.1) (Kuypers, 1981, Lemon, 2008). First, the emotional motor system (incl. raphespinal tract and locus coeruleus) projects ubiquitously exhaustively to the grey matter on all spinal levels, where it can modulate neuronal excitability. This system is responsible to regulate the overall activity level of spinal networks but also mediate emotional behaviors such as micturition and mating (Holstege, 1998).

Dorsolateral projecting brainstem pathways (pontospinal and rubrospinal tract (RuST)) descend mostly contralaterally and terminate in the dorsal and lateral regions of the intermediate zone of the spinal cord, where mostly short propriospinal neurons (PNs) and

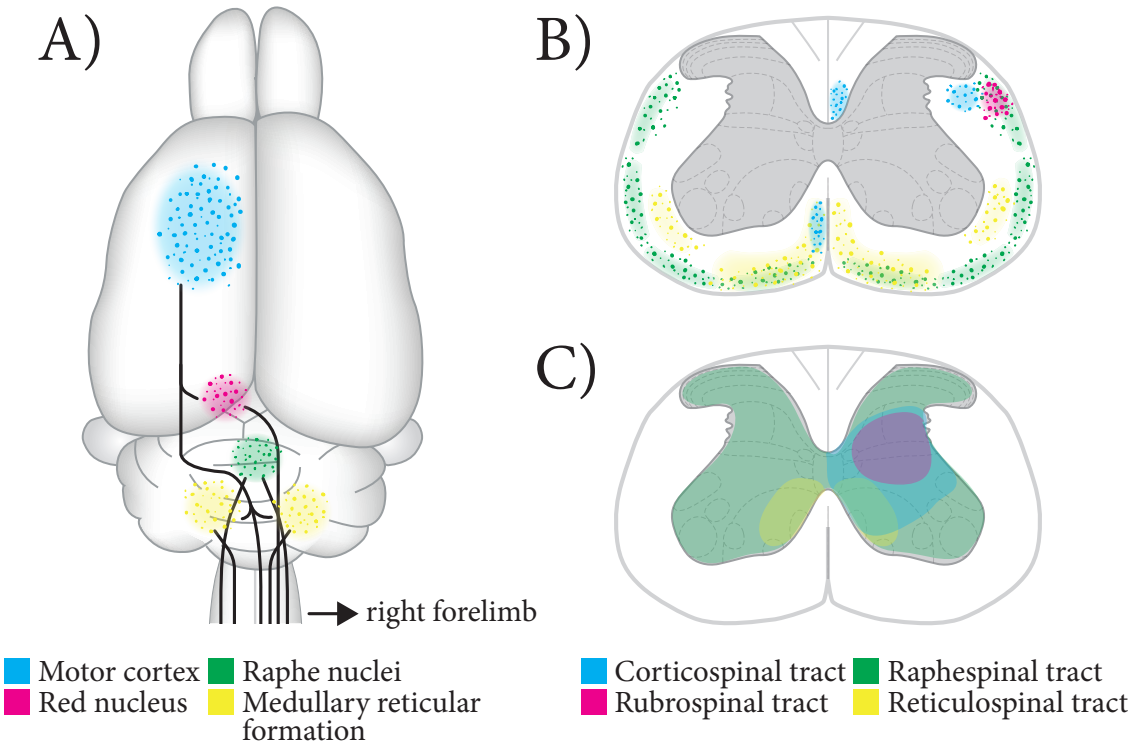


Figure 1.1 – Schematic organization of supraspinal command centers of the rodent brain and their projection fields within the spinal cord for right forelimb control. Supraspinal command centers involved in generation of movement defined by their origin in the brain (**A**), including the motor cortex (blue), ventromedial brainstem nuclei (medullary reticular formation, yellow), dorsolateral brainstem nuclei (red nucleus, red) and the emotional centers (raphe nuclei, green). Pathways of the respective supraspinal centers within the white matter (**B**) and their terminal projection field within the grey matter (**C**) of the cervical spinal cord. Adapted from (Kuypers, 1981, Lemon, 2008).

interneurons are located. These pathways are reportedly involved in control of flexion of distal limb segments, the elbow and the wrist and mediate mainly gross motor function (Kuypers, 1981, Lawrence and Kuypers, 1968b). Additionally, the RuST has also been implicated in control of skilled forelimb movements such as reaching (Iwaniuk and Whishaw, 2000, Martin and Ghez, 1988, Morris et al., 2015). Ventromedial projecting brainstem pathways (tectospinal, vestibulospinal and reticulospinal tract (ReST), part of the medullary reticular formation) terminate bilaterally on interneurons within the intermediate zone of the spinal cord. Additionally, they may connect directly to MNs supplying trunk and girdle muscles. In general, the ventromedial pathways are implicated in control of posture, head, neck and trunk as well as proximal limb movements (Lawrence and Kuypers, 1968b) and in respiratory control (Lane, 2011, Monteau and Hilaire, 1991).

Only recently, the contribution of the brainstem-spinal pathways to skilled forelimb movement have been uncovered with an elegant study by Esposito et al. (2014), in which, using modern tracing techniques, the brainstem nucleus MdV within the medullary reticular

formation was identified as a mediator of forelimb-innervating MNs as well as segmental interneurons (sINs). Furthermore, several upstream motor centers, amongst others the motor cortex, superior colliculus and red nucleus giving rise to the RuST, have been implicated to connect to the MdV and potentially influence its role in skilled forelimb tasks. This study suggests that indirect motor pathways modulate spinal computation on the level of the spinal interneuronal circuitry that is controlled by the direct pathways. Interestingly, the ReST has a higher amount of synapses onto cervical commissural interneurons and long descending PNs as compared to corticospinal inputs (Mitchell et al., 2016), highlighting the importance of the ReST. Further research is needed to unravel the specific functional role of the brainstem nuclei and other subcortical areas in motor performance and modulation.

1.1.2 | The Corticospinal Tract as a Major Command System of Skilled Movement

As the fourth and last descending pathway, the main contributor to skilled forelimb movements and dexterity is the motor cortex, giving rise to the corticospinal and corticobulbar projections (Alstermark and Isa, 2012, Isa et al., 2013, Lemon, 2008). The importance of the cortex for skilled movements has long been underestimated and has only been reevaluated after lesion experiments revealed a specific deficit of fine-motor movements in a challenging environment (Drew et al., 2004, Liddell and Phillips, 1944, Whishaw et al., 1993). The motor cortex is thought to be topographically arranged into movement representations as so-called motor engrams (Harrison et al., 2012), owing to the fact that stimulation of cortical neurons resulted not only in the activation of one particular muscle but evoked complex sequential movements resembling reaching and grasping movements.

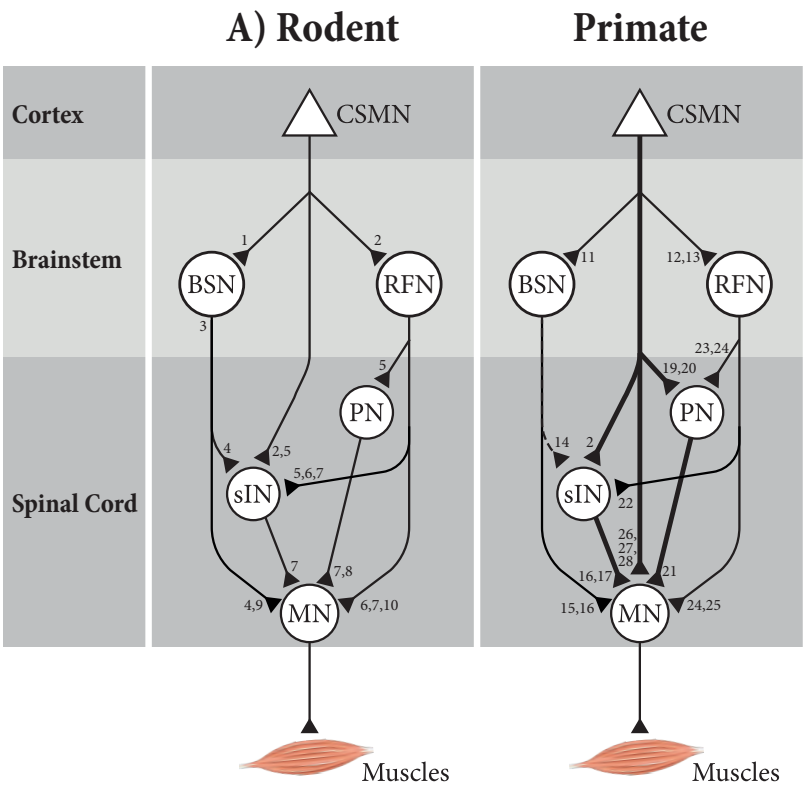
The pyramidal neurons located in layer V of the motor cortex project their axons through the internal capsule and the cerebral peduncle all the way down to the lower brainstem, where they innervate either multiple brainstem nuclei (corticobulbar tract) or proceed their projection through the pyramidal decussation into the spinal cord (corticospinal tract (CST), also called pyramidal tract). On the level of the pyramidal decussation, the majority of the spinally projecting fibers crosses over the midline and continues contralaterally within the dorsolateral funiculus in primates, or the dorsal funiculus in rodents, respectively (Kuypers, 1981). Axons of the CST leave their fiber bundle in every segment and terminate within the dorsal and intermediate horn of the grey matter (Casale et al., 1988). A small part of fibers (5 %) of the CST also innervates the spinal cord ipsilaterally in rodents (Terashima, 1995), descending in the ventral

(Brosamle and Schwab, 1997) and dorsolateral funiculus (Bareyre et al., 2005, Steward et al., 2004). Additionally, contralaterally projecting fibers have been shown to cross the midline segmentally within the cervical enlargement to influence spinal circuitries ipsilaterally in primates (Rosenzweig et al., 2009). Motor synergy encoder neurons as well as excitatory and to a lesser degree inhibitory interneurons have recently been identified as post-synaptic partners of the CST within the deep dorsal horn of the spinal cord (Levine et al., 2014, Ruder et al., 2016).

During the evolution of mammals, the CST has become increasingly important, as seen by the more devastating motor behavior deficits after lesions in higher order mammals (Lemon and Griffiths, 2005). It connects either monosynaptically or polysynaptically onto ventral horn MNs (Terashima, 1995), however, monosynaptic connections seem to be lacking in rodents or have so far not been proven to be functional (Alstermark, 2004, Babalian et al., 1993, Bannister and Porter, 1967, Bareyre et al., 2005, Terashima, 1995). It is generally believed that direct cortico-motoneuronal connections are the basis for the advanced finger/hand dexterity observed in primates (Heffner and Masterton, 1975, Isa et al., 2007, Lemon, 2008). Even so, rodents portray a high degree of skilled forelimb movements especially when handling food. Here, the motor cortex refines motor behavior indirectly via spinal interneuronal networks and brainstem pathways (Alstermark and Isa, 2012, Azim et al., 2014, Terashima, 1995, Whishaw et al., 1992).

1.1.3 | Interneuronal Networks on the Spinal Level Integrate Supraspinal Commands

The majority of supraspinal pathways do not project to MNs directly, especially in the rodent, but convey their information indirectly via interneuronal networks. These spinal microcircuits are not just mere hubs to forward signals from the cortex to the muscles, but they encode muscle synergies, e.g. for grasping movements, form spinal activity pattern circuits, in particular for repetitive, rhythmic movements, and integrate sensory feedback information from the body periphery (Azim et al., 2014, Bui et al., 2013, 2015). Especially PNs have been investigated for their role in skilled forelimb grasping in cats, monkeys as well as rodents (Alstermark and Isa, 2012, Isa et al., 2007). They are located in the cervical spinal cord at level C3-C4 and are characterized by their extensive input from several descending pathways such as the cortico-, rubro-, reticulo- and tectospinal tracts. As the PNs additionally possess an ascending connection to the medullary reticular formation (Arber, 2012, Isa et al., 2006), it stands to reason that they provide an efferent copy of the



ID	References
1	Brown (1974a)
2	Alstermark (2004)
3	Brown (1974b)
4	Kuypers (1981)
5	Mitchell et al. (2016)
6	Esposito et al. (2014)
7	Alstermark and Pettersson (2014)
8	Azim et al. (2014)
9	Küchler et al. (2002)
10	Lawrence and Kuypers (1968b)
11	Humphrey et al. (1984)
12	Borra et al. (2010)
13	Keizer and Kuypers (1989)
14	Isa et al. (2013)
15	Fujito and Aoki (1995)
16	Cheney (1980)
17	Fetz et al. (2002)
18	Takei and Seki (2010)
19	Alstermark et al. (1999)
20	Malmgren and Pierrot-Deseilligny (1988)
21	Isa et al. (2006)
22	Zaaimi et al. (2012)
23	Riddle and Baker (2010)
24	Riddle et al. (2009)
25	Soteropoulos et al. (2012)
26	Lawrence and Kuypers (1968a)
27	Porter and Lemon (1995)
28	Landgren et al. (1962)

Figure 1.2 – Comparison of descending motor pathways implicated in skilled forelimb motor control in rodents and primates. In rodents **(A)**, cortical input onto MNs is indirectly conveyed from corticospinal motor neurons (CSMNs) via brainstem neurons (BSNs) including the neurons giving rise to vestibulospinal and rubrospinal tract, or via reticular formation neurons (RFNs) giving rise to the ReST among others. Alternatively, interneuronal networks of sINs or PNs are contacted by the CSMNs. In primates **(B)**, the CST additionally gives direct input onto the MNs as well as PNs (blue lines). Pathways already existing in rodents might also be strengthened in primates (bold lines). Connections are labeled with the corresponding reference.

command of the MNs, subsequently modulating themselves as well as the commands that are forwarded to the MNs. Apart from the PNs, disynaptic corticomotoneuronal input can be conveyed via sINs, located in the same segment as the MNs they connect to (Illert and Wiedemann, 1984). The work by Alstermark and Isa highlighted the differential role these two subtypes of interneurons play in skilled forelimb movements in cats and monkeys, respectively. While lesions above spinal levels C3-C4 lead to impaired reaching and grasping, lesions below the propriospinal system only affect reaching (Alstermark and Isa, 2012). Also in the mouse, ablation of PNs by making use of their unique genetic background as members of the V2a interneuronal subtype leads to disturbed reaching movements with grasping movements not affected (Azim et al., 2014). Cutaneous feedback to MNs has recently been shown to be conveyed by sINs, as ablation of the dI3 interneuronal subclass led to deficits in tasks relying on tactile feedback (Bui et al., 2013). Since in the rodent system, indirect cortico-motoneuronal connections via PNs seem to be lacking, these findings highlight the importance of other tracts in reaching, such as the ReST, as well as the importance of efference copies to refine skilled forelimb movement.

The motor-related anatomical substrates of complex skilled forelimb movements for rodents and primates are summarized in Figure 1.2, while sensory circuitry, even though important for the execution of movement, is neglected for the sake of simplicity. Briefly, in the rodent, cortical control of MNs is polysynaptic with relays via spinal interneuronal networks of sINs and PNs as well as supraspinal brainstem pathways such as the ReST as part of the medullary reticular formation (RFN) or the RuST (BSN). In primates, the CST gives rise to direct connections onto the MNs as well as PN networks and spinal interneuronal networks, which are strengthened compared to the rodent motor system. Rubrospinal connections to sINs have not yet been demonstrated in primates, but are likely to exist given that they do in rodents and cats (Isa et al., 2013). In either case, the motor cortex provides strong control over forelimb muscles by direct projections or via connections over brainstem motor nuclei or spinal interneuron circuits.

In spite of the differing position of the CST in the dorsal (e.g. rodent) or dorso-lateral funiculus (e.g. cats, primates), the large majority of the CST axons cross the midline at the pyramids and project contralaterally, thereby controlling the contralateral side of the body in all mammalian species. Behaviorally, many aspects of skilled forelimb movements are comparable in rodents and humans as seen in kinematic analyses (Klein et al., 2012, Metz and Whishaw, 2000). The same building blocks are connected in a similar fashion with only the relative importance of the CST being strengthened from rodents to humans. Thus, it is not surprising that lesions to the motor system lead to defined deficits both in humans as well as animal models, and it can be assumed that a better understanding of the anatomical reorganization of the brain after lesion in rodents will lead to better treatment options for patients in the future.

1.2 | Spontaneous Recovery through Structural Plasticity of the Motor Cortex after Injury

The importance of motor behavior often is only appreciated after loss of the function. One of the most common conditions to disrupt the motor system is stroke. Drastically put, every 40 seconds someone suffers from a stroke in the United States alone while every 6^{th} person is killed by the incident (Benjamin et al., 2017). With improving treatments that have emerged over the last few decades, the mortality rate declines, but stroke survivors often suffer from long-term chronic disability (Center for Disease Control and Prevention, 2017, Koton et al., 2014, World Health Organization, 2011). Considering the age as an additional risk factor,

with the progressively aging population the incidence of stroke will intensify the burden on the patients themselves, on the community around them as well as the health care system (World Health Organization, 2004).

1.2.1 | Stroke: A Chronically Disabling Disease

The sudden reduction of blood flow, in most cases (80 %) due to ischemic strokes defined by transient or permanent blockage of arteries, has an irreversible impact on the surrounding tissue within minutes to a few hours (Deb et al., 2010, Dirnagl et al., 1999, Murphy et al., 2008). With oxidative phosphorylation as the main source of energy, the CNS has a high need for oxygen and glucose, which is usually supplied via the blood system. Once the local energy store is depleted and cannot be replenished through the blood, several energy-dependent processes necessary for cell survival are affected. These include, amongst others, failure of mitochondria, loss of membrane ion pump function, depolarization with consequent neurotransmitter release, intracellular calcium increase to cytotoxic levels as well as production of reactive oxygen species. A gradient of severity, depending on duration and location of the blockage, is generally seen in the affected area of the brain with a central core, where tissue is mainly necrotic, and a region with partial affection (penumbra), where cell death may be caused by apoptosis (Deb et al., 2010). In clinical care, the penumbra is the main target of intervention, as it retains blood supply at least partially (Donnan et al., 2008, Fisher and Garcia, 1996). With time being a major factor after stroke, quick therapies able to restore blood flow within the penumbra, mainly enzymatic thrombolysis and intravascular thrombectomy, are now routine in the minor population of patients who arrive in a stroke unit within less than 4-6 hrs after stroke onset and have no signs of hemorrhage (Ding, 2015, Elgendy et al., 2015, Kurz et al., 2013).

Only around 5-10 % of all stroke patients are eligible to early treatment due to restrictive exclusion criteria (Demaerschalk et al., 2016). Thus, with more than 80 % of all stroke patients experiencing motor impairments, a majority is left with a persistent disability affecting their activities of daily living (Gresham et al., 1975, Langhorne et al., 2009). Patients with milder strokes experience some degrees of spontaneous functional recovery after their initial injury, while patients with a more severe deficit are more restricted (Cramer, 2008). A period of spontaneous improvement has been described within the first months after stroke in patients as well as the first weeks in animal models (Cramer, 2008, Ward, 2017). This improvement can be further augmented by rehabilitative training, which currently represents the only treatment option for chronic stoke patients (Biernaskie et al., 2005, Murphy and Corbett, 2009).

1.2.2 | Spontaneous Recovery of Sensorimotor Function Depends on Structural Plasticity

The enhanced sensory and motor performance observed after stroke is often referred to as recovery, defined either as the re-emergence of the pre-stroke motor and sensory patterns or the development of compensatory movement strategies. In case of a large motor-cortical stroke, corticofugal neurons are destroyed completely and the descending pathways degenerate, thus functional improvements rely on compensatory mechanisms, i.e. intact pathways. This is in contrast to injuries to the spinal cord, e.g. by contusion, which lesion several descending pathways in the white matter, whereas cell bodies, residing in supraspinal command centers, remain intact. The axotomized fibers of the surviving neurons might be able to sprout and participate in a process of rewiring to restore function, additionally to the compensatory mechanisms of intact pathways. Therefore, after stroke injuries, it seems unlikely that the observed post-lesion behavior is identical to the pre-lesion behavior. Functionally, detailed kinematic analysis of post-stroke reaching movements in rats showed that adaptations in speed or even postural adjustments, e.g. change of body angle or shoulder rotation, are the reason for the observed success in grasping, much more than recovered aim, pronation and supination of the paw (Lai et al., 2014, Whishaw, 2000). This has also been seen in stroke patients where trunk movements accompanied the reaching movements regardless of the targets distance to the body as compared to healthy controls which used the trunk only when the target was placed far away (Levin et al., 2002). The term "recovery" thus often indicates compensation rather than true recovery of pre-lesion movement patterns in stroke patients and will be used in this thesis according to this definition.

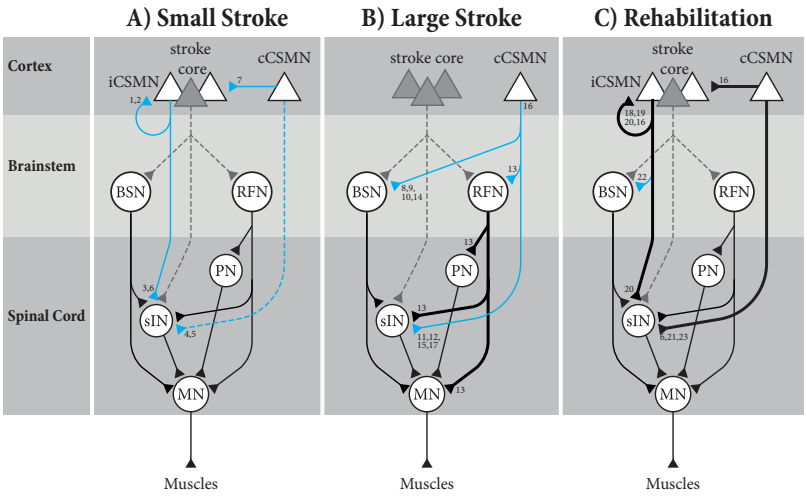
As motor behavior is organized by motor engrams which are lost due to the lesion, new engrams are needed to restore or compensate function. In accordance with Hebb's rule, that neurons that "fire together, wire together" (Hebb, 1949), redundant connectivity can be strengthened or disinhibited after an insult to the circuitry to restore function, e.g. by dendritic spine growth or modulation of GABAergic tonic inhibition (Benowitz and Carmichael, 2010, Brown et al., 2009, Clarkson et al., 2010, Mohajerani et al., 2011). Regarding the CST, this might include ipsilateral projections of the contralesional motor cortex (Chollet et al., 1991, Cramer, 2008, Rosenzweig et al., 2009). Additionally, reorganization of the circuitry by axonal sprouting might establish new routes for the motor cortex to participate in movements as seen in animal models of stroke (Carmichael et al., 2001, Murphy and Corbett, 2009, Starkey et al., 2012). This rewiring was proposed to occur also in humans, as indirectly identified by analysis of cortical remapping after stroke using

imaging techniques, and has been shown to occur in the same functional systems as in primates or rodents (Talelli et al., 2006). Additionally, *post-mortem* analysis of human post-stroke tissue showed an upregulation of growth associated protein 43 (GAP43), a marker commonly used to label growing fibers, in the peri-infarct region, suggesting that axonal sprouting was taking place (Calautti et al., 2003, Carmichael, 2016, Cramer and Crafton, 2006).

Thus, unaffected areas of the brain can, to some extent, take over functions that were previously maintained by the damaged areas. This remapping of cortical areas is heavily dependent on the location and the size of the stroke as shown by the correlation of the integrity of the CST to motor impairment after stroke (Dijkhuizen et al., 2003, Hsu and Jones, 2006, Zhu et al., 2010). According to the "vicariation concept" (Dancause, 2006), neighboring tissue of the affected area, unrelated to the function, plays an important part in post-stroke rewiring. However, it seems more reasonable that areas that share a connection with the stroke-affected tissue participate in remodeling than unrelated structures (Dancause, 2006, Murphy and Corbett, 2009), as postulated in the concept of "diaschisis" (von Monakow, 1910). In the rodent motor cortex, the strongest connections to premotor areas are found within the sensorimotor cortex surrounding this area and it has been shown that here, motor maps shift towards a new function after lesion (Rouiller et al., 1998, Starkey et al., 2012). Additionally, interhemispheric connections to homotopic areas are present and activated after stroke (Bauer et al., 2014, Feydy et al., 2002, Rouiller et al., 1994). Conclusively, structural plasticity might differ depending on the size of the lesion, i.e. in small lesion paradigms, where only part of the motor-related areas of one hemisphere are ablated, neighboring motor-related tissue might vicariate function. After a large lesion on the other hand, when all motor-related areas of one hemisphere are ablated, tissue with related function might be found only at distant sites to the infarct area, e.g. on the contralesional side of the brain.

1.2.3 | Structural Plasticity after Small Cortical Lesions: Peri-Infarct Cortex to the Rescue

In small strokes, which mostly have a good prognosis for recovery in humans (Cramer, 2008), the peri-infarct cortex has been identified as the major source of recovery in rodents as well as primates (Carmichael et al., 2001, Castro-Alamancos and Borrell, 1995, Clarkson et al., 2013, Dancause, 2005, Dijkhuizen et al., 2001, Harrison et al., 2013, Liu and Rouiller, 1999, Nudo and Milliken, 1996, Starkey et al., 2012). Initially, the peri-infarct region shows



ID	References
1	Carmichael et al. (2001)
2	Brown et al. (2009)
3	Starkey et al. (2012)
4	Lapash Daniels et al. (2009)
5	Mansoori et al. (2014)
6	Okabe et al. (2017)
7	Carmichael and Chesselet (2002)
8	Riban and Chesselet (2006)
9	Papadopoulos et al. (2002)
10	Seymour et al. (2005)
11	Wahl et al. (2014)
12	Lindau et al. (2014)
13	Bachmann et al. (2014)
14	Lee et al. (2004)
15	Wiessner et al. (2003)
16	Biernaskie et al. (2005)
17	Ueno et al. (2012)
18	Zeiler et al. (2013)
19	Nishibe et al. (2015)
20	Okabe et al. (2016)
21	Zai et al. (2011)
22	Ishida et al. (2016)
23	Zhao et al. (2013)

Figure 1.3 – Anatomical reorganization after stroke depends on lesion size and can be augmented by rehabilitation. After small cortical strokes **(A)**, cortical tissue rearrangements are found primarily within the ipsilateral hemisphere from ipsilateral corticospinal motor neurons (iCSMNs) with a disputed activation of contralateral corticospinal motor neurons (cCSMNs). Large cortical injuries **(B)** show a strong activation of brainstem nuclei (RFN) while cortical control relies on cCSMNs. Rehabilitative training **(C)** further deepens cortical control in both hemispheres by strengthening intra-cortical connectivity, projections to sINs as well as new corticobulbar connections. Respective lesions are depicted as light grey cells and degenerating connections as dotted grey lines. Newly formed anatomical changes due to the lesion paradigm are marked in blue, strengthening of pre-existing projections is depicted as bold lines. Connections are labeled with the corresponding reference.

a robust reduction of activity and responsiveness after the lesion in patients as seen by functional imaging, in the long-term, however, recovery of the function directly correlated with the later activation of the peri-infarct region (Rehme et al., 2011). In rodent stroke models, facilitation of long-term potentiation in the peri-infarct region was seen as early as seven days after injury (Hagemann et al., 1998), likely presenting an essential mechanism of lesion-induced plasticity necessary for map shifts. Neuroanatomical tracing studies revealed new projections formed by the peri-infarct cortex intracortically (Carmichael et al., 2001) as well as to spinal interneurons (Starkey et al., 2012). The functional relevance of these new connections was assessed by *in vivo* imaging during forelimb stimulation (Brown et al., 2009), by intracortical microstimulation and optical imaging (Nudo and Milliken, 1996, Silasi et al., 2013) and by silencing of pre-motor cortex optogenetically, pharmacologically or by secondary lesions after initial injury to the primary motor cortex, which resulted in the reinstatement of the original behavioral deficit (Liu and Rouiller, 1999, Wahl et al., 2017).

Clearly, the peri-infarct region is an important part of recovery after stroke. The role of the contralesional cortex in small lesion paradigms on the other hand is disputed. In patients, functional neuroimaging, mapping the activation of the contralesional areas post-stroke, showed an early enhanced activity lasting around ten days followed by a consequent decrease of this activity with a shift back to the damaged hemisphere and the peri-infarct

cortex, which was paralleled by functional recovery (Marshall et al., 2000, van Meer et al., 2010, Ward, 2003). These results were reproduced in animal models of stroke using functional imaging and silencing methods (Biernaskie et al., 2005, Dijkhuizen et al., 2001, Rouiller et al., 1998, Takatsuru et al., 2009) as well as tracing techniques to evaluate axonal sprouting of the contralesional cortex on cortical (Carmichael and Chesselet, 2002) and spinal levels (Lapash Daniels et al., 2009), indicating that the contralateral hemisphere at least transiently vicariates the function of the damaged hemisphere. Lateralized activation of the contralesional hemisphere has been shown to be an important factor of recovery at least in the acute phases of stroke as the activation correlates well with the amount of spontaneous functional recovery in patients with an initially worse deficit (Rehme et al., 2011). Nevertheless, functional outcome was shown to be highest in patients with normal lateralization patterns, i.e. activation of ipsilesional areas in later stages of the recovery (Cramer, 2008, Rehme et al., 2012, Ward, 2003). Recent experiments from Mansoori et al. (2014) showed that blocking the contralesional cortex over the course of recovery has a beneficial effect on the functional outcome, suggesting that in the small lesion paradigm reduced lateralization of function might be a maladaptation (Barry et al., 2014, Takeuchi and Izumi, 2012). Thus, inhibition of the contralesional M1 in patients by non-invasive brain stimulation such as transcranial magnetic stimulation may lead to increased motor recovery of the stroke-affected hand in the subacute and chronic phases (Di Pino et al., 2014, Nowak et al., 2008, Takeuchi and Izumi, 2012). These experiments underline the complex role of the contralesional cortex in the recovery of function after stroke with a high dependency on time, size or location of the lesion.

In summary, small stroke lesions seem to primarily involve cortical sprouting and rearrangements of connections to achieve functional recovery (Figure 1.3A). Considering subcortical centers, in primate and human studies both the ReST and RuST have been suggested as a substrate of recovery (Baker, 2011, Owen et al., 2017, Rüber et al., 2012). However, studies are lacking to report axonal sprouting from subcortical supraspinal centers in rodent stroke models, while it remains unclear whether this is owed to the failure of the subcortical centers to participate in axonal rewiring after small strokes in rodents or just a mere lack in conducted studies. Riban and Chesselet (2006) report that the contralateral cortex does not rewire to the red nucleus following small lesions, suggesting that corticobulbar pathways do not participate in post-stroke improvement after small insults, however, the roles of the ReST and the RuST remain to be elucidated.

1.2.4 | Structural Plasticity after Large Cortical Lesions: Multilevel Remapping to Restore Functionality

In contrast to what we described above for small lesions, in large lesions, the contralesional cortex might be the only area of the brain that shares connections and functional relation with the damaged tissue. Axonal sprouting of the contralesional cortex after large unilateral strokes has been observed in rodents and primates at spinal and brainstem levels (Lee et al., 2004, Lindau et al., 2014, Morecraft et al., 2016). The main source for the re-innervation of the denervated hemicord in rats was formation of new midline-crossing sprouts, paralleled by axonal sprouting of the pre-existing ipsilateral spinal projections (Lindau et al., 2014, Weidner et al., 2001). Additionally, ipsilateral spinal projections might also be strengthened by other mechanisms, such as increased neuronal firing rates, dendritic spine modulations or synaptic plasticity. Functional proof for the importance of the contralesional cortex in recovery has been provided in a recent study by Bachmann et al. (2014), in which an unilateral large stroke led to an initial impairment in selected motor-related behavioral paradigms with subsequent re-learning over the course of 4 weeks. After recovery levels reached a plateau, a second stroke was applied to the contralesional cortex, resulting in a reappearance of several of the initially observed deficits. This experiment, however, does not address the route the contralateral cortex takes to mediate post-stroke recovery. A more telling experiment therefore was the selective silencing of the fibers from the contralesional CST that had crossed the midline to innervate the denervated hemicord using modern pharmacogenetic techniques (Wahl et al., 2014). When these fibers were inactivated, the forelimb deficits which had disappeared during the recovery period reappeared acutely, demonstrating that direct spinal projections of the contralesional cortex are an important part of recovery.

Subcortically, the brainstem pathways have recently gained attention for their involvement in post-stroke recovery. In primates, medullary reticular formation cells have been implicated to play a role in fine finger movements (Soteropoulos et al., 2012), with reticulospinal projections contacting the same sIN as the CST (Riddle and Baker, 2010). Retrograde tracings from the stroke-denervated cervical spinal cord in rodents after spontaneous recovery showed enhanced projections from several brainstem nuclei (Bachmann et al., 2014), including several parts of the medullary reticular formation as well as the spinally projecting raphe nuclei. Additionally, increased corticobulbar connections from the contralesional cortex were found in these medullary nuclei, suggesting an increase in indirect corticobulbospinal pathways to regain control over the motor functions. Given the brainstem's contribution to skilled forelimb movement, it seems reasonable to assume that

indirect pathways, which are considered as phylogenetically old systems, will be recruited after large lesions to mediate recovery.

In summary, large cortical strokes induce a multilevel reorganization of connectivity (Figure 1.3B), which is associated with and may in part form the basis for functional recovery. At the same time, maladaptive plasticity such as hyperexcitability of especially the ReST has been linked to post-stroke spasticity (Li, 2017), which is a common limitation to recovery in human stroke survivors. One explanation for this might be provided by the work of Zaaimi et al. (2012), revealing an imbalance in strengthening of the reticulospinal connections to the flexors as compared to the extensors in primates after corticospinal tract specific lesions (pyramidotomy), which is also often seen in stroke patients (Kamper et al., 2003). Therefore, not all connections endogenously formed by the damaged CNS might be desirable and clinical interventions need to address this by designing therapies to selectively enhance restorative circuits (Takeuchi and Izumi, 2012).

1.2.5 | Rehabilitation: Reinforcement of a Task-Specific Circuitry

Despite the anatomical changes outlined, the adult CNS shows only limited endogenous capacity to rearrange circuits owing to the many growth-inhibitory molecules such as myelin-associated inhibitors (MAIs) and extracellular matrix-associated chondroitin sulfate proteoglycans (CSPGs) within the CNS tissue (Schwab and Bartholdi, 1996, Thallmair et al., 1998, Yiu and He, 2006) as well as missing trophic signals for the neuron (Liu et al., 2011). However, skilled motor tasks such as playing the piano can be acquired even in adulthood by repetition and training, a process which strengthens connectivity of circuits and results in movements becoming faster and less variable (Dayan and Cohen, 2011). In the healthy brain, training was associated with upregulation of genes influencing synaptic efficacy, synaptogenesis and cytoskeletal dynamics (Cheung et al., 2013). After stroke, repetition of movements in form of rehabilitative training often leads to a better outcome in behavioral tasks (Maldonado et al., 2008, Nishibe et al., 2015, Wahl et al., 2014, Zeiler et al., 2013). The observed improvement was intensity-dependent and transient if training was not continued further (Bell et al., 2015). Rehabilitative training seems to be mostly effective in a time window after the insult, as exposure to enriched environments, a natural way of rehabilitative training in animal models, resulted in significant recovery only in the early rehabilitation groups (Biernaskie, 2004). The exact timing and dosing of rehabilitative training for stroke patients is still under debate, with recent studies showing a rather detrimental

effect of very early intense training for stroke patients (Avert and Collaboration, 2015). Data from animal models indicate that very early activation might lead to exacerbated neuronal loss, thereby worsening the initial injury (Kozlowski et al., 1996). Nevertheless, due to the observed beneficial effect on functional outcome, training-based therapy accompanied by general exercise remains the gold standard of post-stroke care for patients.

Rehabilitation in form of forced limb use increased contralateral corticospinal sprouting within the denervated cervical spinal cord while MAI and associated genes were down-regulated and growth-promoting genes upregulated (Maier et al., 2008, Okabe et al., 2016, Zai et al., 2011, Zhao et al., 2013) (Figure 1.3C). Additionally, interhemispheric and intra-hemispheric connectivity of the motor cortex was strengthened in several studies in small stroke paradigms (Biernaskie et al., 2005, Nishibe et al., 2015, Okabe et al., 2016, Zeiler et al., 2013). Intriguingly, rehabilitation seems to induce training-specific circuit changes, as for the contralateral cortex sprouting into the red nucleus was only observed for rats which were forced to use their paretic limb but not when allowed to recover without any further treatment (Ishida et al., 2016, Riban and Chesselet, 2006). This enhanced corticorubral sprouting has recently been associated with functional recovery (Ishida et al., 2016). Using retrograde tracing in stroked rats, the contralateral cortex has been confirmed to increase projections into the intermediate layers of the cervical spinal cord levels C7-C8 due to rehabilitative training. On the other hand, brainstem-spinal projections seem not to be influenced by rehabilitative training, as the number of neurons in any of the brainstem nuclei projecting to spinal levels was unchanged (Okabe et al., 2017). This suggests a selective cortico-fugal resp. cortico-spinal enhancement due to rehabilitative skilled forelimb movement (Figure 1.3C), sufficient to evoke a task-specific recovery. Overall, due to the activity-dependent formation of motor engrams, rehabilitative training may only induce task-specific recovery, which is also confirmed by the finding that only skilled training but not endurance exercise i.e. running was able to restore reaching behavior after ischemic lesions in rats (Maldonado et al., 2008).

The mechanisms of how rehabilitation can influence post-stroke recovery are still under investigation. As seen in motor learning in a healthy state, changes of CSMN properties such as dendritic branching, synaptogenesis and neuronal sprouting have been observed (Adkins et al., 2006, Biernaskie and Corbett, 2001, Greenough et al., 1985, Jones et al., 1999). Activation of CSMNs by direct stimulation results in enhanced rewiring accompanied by improved recovery (Benecke et al., 1991, Brus-Ramer et al., 2007, Carmel et al., 2014, Carmel and Martin, 2014, Nowak et al., 2008, Wahl et al., 2017). These findings indicate that increased neuronal activity can lead to activation of growth-enhancing gene programs. Additionally, molecular changes within the environment have been found to

be modulated by training, including upregulation of growth-promoting (Himi et al., 2016, Ishida et al., 2015) and downregulation of growth-inhibitory molecules (Choi et al., 2016, Li et al., 2015, Zhao et al., 2013) or of their receptors, respectively. Thus, rehabilitative training addresses both features needed to restore recovery: the intrinsic growth state of the neuron as well as the permissiveness of the extrinsic environment. The clinical limitation of rehabilitative training however is the task-specificity of the training. Future directions are therefore to combine rehabilitation with additional interventions that influence the permissiveness of the tissue environment for a best-possible outcome for patients. This might include blockage of MAIs such as Nogo-A or CSPGs (Soleman et al., 2012, Wahl et al., 2014), administration of growth-promoting factors such as brain derived neurotrophic factor (BDNF) or Inosine (Chen et al., 2002, Schabitz et al., 2004), transplantation of neural stem cells (Liang et al., 2013) or electrical stimulation of the CSMNs (Carmel and Martin, 2014, Wahl et al., 2017).

1.3 | Conclusion

The traditional view that the adult CNS is mostly hardwired has long been overturned. Important functional and structural recovery after lesions to the brain and spinal cord has been documented in numerous cases across several species as outlined above. Multilevel structural axonal sprouting was identified as one of the basic elements causally linked to the observed functional recovery. New transgenic models (Bareyre et al., 2005, Fink and Cafferty, 2016), improved tracing techniques (Ciabatti et al., 2017, Fink et al., 2015, Wickersham et al., 2007) and advances in imaging techniques, such as CLARITY (Tomer et al., 2014) or *in vivo* imaging during behavior (Sekiguchi et al., 2016) will allow for more detailed analyses of the complete neuraxis for post-stroke structural plasticity in the future, adding to and highlighting the structural changes observed so far.

Taken together, available data on post-stroke anatomical plasticity suggest that recovery after stroke is in first line dependent on the size of the lesion and the remaining tissue that might vicariate function (Murphy and Corbett, 2009, Rehme et al., 2011). With small lesions, most changes are seen in the remaining motor cortex of both hemispheres with little adaptations of corticobulbar projections. In large lesion cases, however, adaptations of the cortical projections have been observed on several levels. Ipsilateral corticospinal projections by midline crossing, sprouting CST fibers from the intact, contralesional cortex are significantly increased. Their inactivation leads to reappearance of the initial functional deficits (Bachmann et al., 2014, Wahl et al., 2014). Additionally, corticobulbar as well as

brainstem-spinal projections are found to be increased. Studies investigating the specific roles of these indirect pathways to functional recovery of skilled movement control are mostly lacking, however.

The structural plasticity induced by stroke in the studies briefly summarized here suggests the existence of an underlying region-specific molecular machinery. Rewiring requires that the neurons are in an elevated plastic growth state intrinsically, while in the extracellular environment growth-inducing, attractive factors should be up-, and growth-inhibitory factors should be down-regulated. How rehabilitative training enhances fiber growth and/or refinement and stabilization of the new synaptic connections remains a question of key importance. Future therapies need to take as many of these factors as possible into account in order to reach the highest possible level of functional recovery. The next chapter (**chapter 2**) will outline the molecular mechanisms currently known to shape CST rewiring after stroke.

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2

The Timetable of Recovery — The Molecular Basis of Reorganization in the Lesioned Central Nervous System

Post-stroke motor recovery relies on spontaneous reorganization of intact circuits to compensate for lost functions. The motor cortex undergoes map shifts in response to injury and rewires on several levels along the neuraxis. Rehabilitative training can furthermore induce activity-dependent plasticity, as can other interventions that shape molecular mechanisms such as growth-promotion or blockade of growth-inhibitory molecules. The goal of these interventions is to modulate the two key elements that shape structural plasticity: intrinsic and extrinsic regulators of axonal regeneration (Liu et al., 2011, Yiu and He, 2006). Nevertheless, the improvements of post-stroke deficits in patients after rehabilitation, the only treatment option for chronic stroke patients, are limited, and do not compare to the observed good recovery in animal models of stroke (Krakauer et al., 2012, Ward, 2017). A better understanding of the molecular mechanisms underlying the spontaneous recovery, especially in regards of timing and how interventions modify these mechanisms, is key to designing better treatment options for the future.

2.1 | Setting the Mood for Growth — Key Features of Structural Plasticity

After injury to the central nervous system (CNS), some neurons reorganize on a structural level to establish functional connections and re-innervate targets that have lost their input (Figure 2.1). In the event of a stroke, this reorganization is compensatory (Figure 2.1B), i.e. intact neurons change their mode of action and start to grow new axon branches towards the target region. In comparison, after spinal cord injury (SCI), additionally to this compensatory sprouting, regenerative sprouting of the axotomized axons can be observed over a short distance (Figure 2.1C). However, spontaneous long-distance elongation of axons of the adult CNS is rarely seen, while axons of the peripheral nervous system (PNS) are able to display a robust long-distance regeneration followed by functional recovery after peripheral nerve injury (Giger et al., 2010, Kaplan et al., 2015, Mar et al., 2014).

Transplantation experiments of peripheral nerve bridges into the lesioned CNS collectively unveiled two important key elements of axonal regeneration: *(1)* the environment of the PNS but not the CNS supports long-distance axon regeneration *in vivo*, and *(2)* subpopulations of CNS neurons retain some degree of capability for long-distance growth into adulthood (Giger et al., 2010).

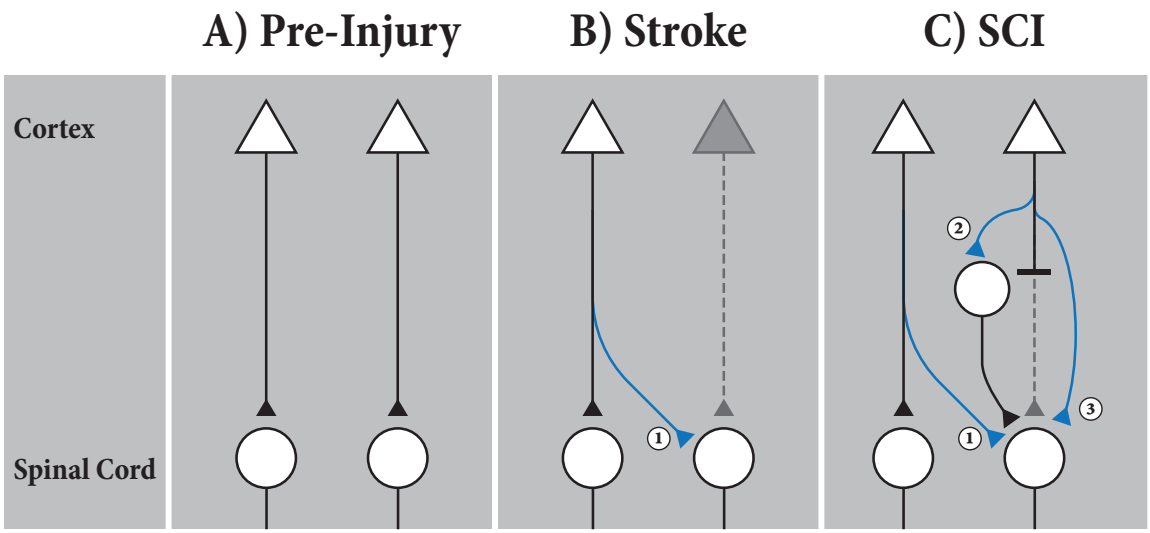

Figure 2.1 – Strategies for re-innervation after stroke and spinal cord injury. Injury to the CNS might elicit three distinct types of strategies to re-establish connectivity to the pre-injury targets. After stroke **(A)**, due to cortical cell death, compensatory sprouting of intact neighboring tissue is the only option for re-innervating pre-injury targets ①. In comparison, after SCI, additionally short-range regenerative sprouting ② or long-range regenerative sprouting ③ might re-establish connectivity.

2.1.1 | Extrinsic Mediators: The Growth-Inhibitory Environment

In a pioneering study, David and Aguayo (1981) demonstrated that the intrinsic properties of some CNS axons allowed for long-distance growth when presented with a growth-permissive environment, such as a peripheral nerve graft transplanted into the injured spinal cord. This led to the hypothesis that failure of regeneration is due to the lack of neurotrophic factors in the adult CNS, a concept originally formulated by Ramón y Cajal (1928). This concept became questionable by the finding and subsequent isolation of neurotrophic activities in the adult CNS. Several neurotrophins have been shown to be expressed in the adult CNS, in particular nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) (Barde et al., 1978, Turner et al., 1982), but also ciliary neurotrophic factor (CTNF), glial-cell-line-derived neurotrophic factor (GDNF), leukemia inhibitory factor (LIF) as well as interleukins (ILs) (Giger et al., 2010), while increased expression levels of selected neurotrophic factors were found especially at lesion sites as an early response to the injury (Carmichael, 2012, Hayashi et al., 2000). Exogenous application of neurotrophins increased axon growth of multiple neuron types *in vitro* (Cui, 2006) and neurotrophic support for axonal sprouting has been described *in vivo* after SCI and stroke (Bradbury et al., 1999, Hayashi et al., 2000, Lanfranconi et al., 2011, Liu and Rouiller, 1999, Mocchetti and Wrathall, 1995). Nevertheless, this support was found to differ for the different spinal cord projections (Blesch et al., 2012), e.g. BDNF may elicit growth of raphespinal tract (RaST)

and rubrospinal tract (RuST) fibers but does not promote corticospinal axonal regeneration after spinal or cortical lesion (Lu et al., 2001). Additionally, in an *ex vivo* experiment using optic and sciatic nerve explants and spinal cord frozen sections, addition of neurotrophic factors proved insufficient to induce regenerative long-distance axon growth (Schwab and Thoenen, 1985).

A new hypothesis was therefore formulated about the existence of specific growth inhibitory factors in the adult CNS (Savio and Schwab, 1989, Schwab and Thoenen, 1985). Subsequently, CNS myelin and their cells of origin, oligodendrocytes, could be shown to be strongly inhibitory for neurite outgrowth (Caroni and Schwab, 1988a,b, Schwab and Caroni, 1988). The activity was attributable to several myelin-associated inhibitors (MAIs) including myelin-associated glycoprotein (MAG), the member of the reticulon (RTN) family Nogo-A and oligodendrocyte myelin glycoprotein (OMgp) (Chen et al., 2000, Schwab, 2004, Spillmann et al., 1998, Yiu and He, 2006). These factors lead to growth cone collapse, preventing axon elongation. Both in stroke as well as SCI, blockade of the most potent of these factors, Nogo-A, using genetic manipulations or function-blocking reagents has resulted in increased axonal regeneration and functional recovery in rodent studies (Bartsch et al., 1995, Cafferty and Strittmatter, 2006, Cash et al., 2016, Lee et al., 2004, Lindau et al., 2014, Schnell and Schwab, 1990, Thallmair et al., 1998). Considering the remarkable diversity among the MAI components, it is difficult to define their exact contribution to outgrowth inhibition, especially since they may display a high degree of functional redundancy. In addition, MAIs are not the only inhibitory factors in the CNS environment. Several repulsive molecules of the axon guidance families are present, such as Netrins, Ephrins and Semaphorins, as well as chondroitin sulfate proteoglycans (CSPGs), whose respective roles in inhibition of neurite outgrowth have been extensively described in numerous reviews (Bolsover et al., 2008, Giger et al., 2010, Low et al., 2008, Thiede-Stan and Schwab, 2015). In conclusion, the inhibitory influence of CNS myelin and the repulsive factors therein are important components to restrict outgrowth of neurons, providing a target for clinical intervention to enhance axonal regeneration after injury.

Apart from inhibitory molecules in the adult CNS tissue, additional obstacles arise after lesion that are associated with limited axon growth and plasticity. Glial scar formation by reactive astrocytes, microglia and meningeal fibroblasts migrating into the lesion site can be seen as early as five days after the insult. Scarring is thought to mainly contribute to wound repair, however, it may interfere with axonal remodeling (Kawano et al., 2012). In SCI, the scar presents a physical barrier which axons cannot penetrate to rewire to targets caudal of the lesion. Furthermore, in stroke and SCI, reactive astrocytes secrete a gradient of scar-associated molecules such as CSPGs, which can additionally suppress neurite outgrowth

(Fitch and Silver, 2008, Properzi et al., 2003). Treatment with Chrondroitinase ABC, an enzyme digesting the CSPGs, enhanced sprouting and improved recovery after SCI and stroke in the long-term (Bradbury, 2002, Kwok et al., 2008, Moon et al., 2001, Soleman et al., 2012), further highlighting the limiting action of the CNS on axon regeneration.

After axotomy or cell death of corticospinal motor neurons (CSMNs), the distal part of the axons undergoes progressive degeneration, a process known as Wallerian degeneration (WD) (Waller, 1850). In the PNS, increase in blood-tissue barrier permeability leads to infiltration of macrophages to the lesion site, resulting in the clearance of myelin debris distal to the lesion (Vargas et al., 2010), taking about one to two weeks in mammals. In the CNS, however, macrophage infiltration and activation is much more restricted (Gaudet et al., 2011) and clearance of debris is substantially slower, taking months to years (Griffin et al., 1992, Vargas and Barres, 2007). The remaining myelin debris limits axotomized fibers' re-growth due to the aforementioned inhibitory molecules. These observations suggest that the immune-privileged status of the CNS might contribute to the lack of axon regeneration in the adult CNS.

In contrast to the injured axons of the adult CNS, newly outgrowing axons of immature neurons of the CNS display robust axon regeneration after lesion (Kartje-Tillotson et al., 1985). These developing neurons face a strikingly different environment compared to regenerating adult neurons. Myelination by oligodendrocytes is a relatively late event in the development of many tracts, taking place after connections have been successfully established (Canty and Murphy, 2008). The work of Silver et al. (1982) suggested that immature astrocytes, but not mature astrocytes provide trophic cues to enhance axonal regeneration within the CNS environment, a function seen for PNS glia as well (Smith et al., 1986). Thus, a growth-promoting support provided by glial cells might exist in the immature CNS that is lacking in the adult CNS.

As discussed above, many obstacles are presented to the adult CNS neurons that may limit axonal regeneration. Additionally to the inhibitory influence of the environment, the intrinsic neuronal component may contribute to success of axonal regeneration.

2.1.2 | Intrinsic Neuronal Mediators: Brakes on Axonal Growth and Regeneration

Sensory and motor axons of adult PNS neurons are able to regenerate over long distances, in contrast to CNS axons. Though peripheral nerve grafts allow CNS axons of multiple

origins to grow, several studies have observed the different growth capabilities of neuronal subpopulations, as treatment with neurotrophic factors such as insulin like growth factor 1 (IGF-1) or BDNF resulted in axonal rewiring of the raphespinal tract (RaST) but not the corticospinal tract (CST), where only the survival of neurons was influenced by the treatment (Grill et al., 1997, Hollis et al., 2009, Li et al., 2010, Lu et al., 2001). Thus, the question arises whether different subpopulations are intrinsically prone to participate in or resign from axon regeneration.

During development, immature CST neurons connect to their targets by relying on a growth-promoting program to extend axons all the way along the neuraxis, finally innervating their target region in the cervical and lumbar spinal cord. They show pronounced rerouting capability when facing a lesioned-primed spinal environment (Bregman et al., 1989), but only when the lesion occurs before the innervation of the grey matter is complete. After the establishment of functional connections around five to six days postnatally, the ability to elongate axons declines, with axonal outgrowth of various adult CNS neurons slowing substantially as compared to their immature counterpart (Blackmore and Letourneau, 2006, Dusart et al., 1997, Goldberg et al., 2002a,b). This indicates a post-developmental switch within the CNS neurons towards a stable connectivity and away from the capability to grow.

Contrary to the CNS, PNS neurons retain their regenerative ability even in adulthood. After peripheral nerve injury, PNS neurons show marked expression of regeneration associated genes (RAGs), a response mostly lacking in CNS neurons (Mar et al., 2014, van Kesteren et al., 2011). Remarkably, the subpopulations of CNS neurons that were able to successfully grow into a transplanted peripheral nerve graft were also shown to upregulate some of the RAGs following axotomy (Anderson et al., 1998, Mason et al., 2002), highlighting the importance of a neuron-intrinsic growth-program. As already stated, the subpopulations able to mount the growth response in the CNS are, unfortunately, very low in numbers, with the majority of CNS neurons not able to induce RAG upregulation spontaneously (Fernandes et al., 1999). In dorsal root ganglion (DRG) neurons, which extend axons into both the CNS and the PNS, injury to the peripheral nerve but not the central nerve results in a robust regeneration, accompanied by upregulation of RAG expression (Bonilla et al., 2002, Costigan et al., 2002, Tanabe et al., 2003). A "conditioning" of the DRG neuron by cutting the peripheral nerve has a beneficial effect on axonal regeneration also of the central axon branch: Due to the subsequent upregulation of RAG expression, the central branch projecting into the CNS environment shows robust axonal outgrowth after a second lesion to the central branch (Blesch et al., 2012, Neumann and Woolf, 1999). This effect of the conditioning lesion was shown to be transcription-dependent (Smith and Skene, 1997), indicating that intrinsic factors may be responsible for this. However, manipulation of

the expression of individual RAGs such as growth associated protein 43 (GAP43), small proline rich protein 1a (SPRR1a), c-Jun or alpha-7 integrin (Itga7) did not result in the expected axonal regeneration in CNS neurons (van Kesteren et al., 2011). The quest to find responsible mechanisms to induce growth within the neurons therefore continues.

In addition to the lack of robust RAG expression, intrinsic molecules have been identified that suppress the neuron's capability to elongate axons in the adult CNS. Administration of the transcriptional inhibitor actinomycin D to the perinatal retina enabled retinal ganglion cells to elongate axons on a growth-inhibitory substrate, suggesting that adult CNS neurons have an intrinsic "brake" on axonal growth once they are established in their post-developmental niche (Moore et al., 2009). Using loss-of-function experiments, several cell-autonomous factors such as cyclic adenosine 3' , 5' - monophosphate (cAMP) and cAMP response element-binding protein (CREB) (Cai et al., 2001, Gao et al., 2004), B cell lymphoma/leukemia 2 (Bcl-2) (Chen et al., 1997, Cho et al., 2005), RhoA/rho-associated protein kinase (ROCK) (Lehmann et al., 1999), phosphatase and tensin homolog (PTEN)/mechanistic target of rapamycin (mTOR) (Liu et al., 2010, Park et al., 2008a), mammalian sterile 20-like kinase-3b (Mst3b) (Lorber et al., 2009), signal transducer and activator of transcription 3 (STAT3) (Bareyre et al., 2011), and several members of the krüppel-like factor (KLF) family (Li et al., 2017, Moore et al., 2009, Wang et al., 2007) were suggested to control the intrinsic capability of adult axon regeneration either positively or negatively. Indeed, manipulation of these internal brakes may influence axonal regeneration, e.g. PTEN deletion promoted axon regeneration of retinal ganglion cells (RGCs) after optic nerve crush via activation of the phosphoinositide 3-kinase (PI3K)/mTOR pathway, thereby controlling cell growth (Park et al., 2008b). Many MAIs signal via the RhoA/ROCK pathway, with blockage of this pathway also resulting in increased axonal regeneration (Lehmann et al., 1999). Elevated levels of cAMP lead to desensitization of DRG neurons towards MAIs (Cai et al., 2001). Thus, intrinsic brakes may be important factors for the neuronal capacity for growth. However, the question of whether different subpopulations of CNS neurons are restricted by different "brakes" still remains open.

Extrinsic factors are integrated by intrinsic downstream signaling influencing the neuronal growth response. Thus, the intrinsic state of the CNS neuron can influence the response to the inhibitory environment, blurring the lines of distinction between intrinsic and extrinsic mechanisms. This additional level of complexity was presented by Cai et al. (2001) as they found an age-associated decline of intrinsic cAMP accompanied with a gain of sensitivity towards MAGs, suggesting a switch of responsiveness towards inhibitory cues. Furthermore, injection of cAMP into the DRG was sufficient to induce axon regeneration

after dorsal column lesion in the same study. In summary, this temporally and spatially orchestrated influence of the environment and intrinsic neuronal properties on axonal regeneration provides a challenge for finding treatment options to promote recovery after injury that has to be met in the future.

2.2 | The Timeline of Stroke-Induced Spontaneous Structural Plasticity

Post-stroke axonal rewiring, as observed especially in the CST (see chapter 1), has been a focus of pre-clinical research in recent years. A major goal has been to elucidate underlying mechanisms, and how they might limit a successful axonal sprouting. Functional recovery in pre-clinical models of stroke can be observed within the first month after insult as forelimb connectivity is re-established (Dijkhuizen et al., 2003), suggesting an early and transient phase of heightened plasticity (Murphy and Corbett, 2009). A precisely timed series of events is necessary to allow CST neurons to re-establish routes to innervate pre-injury target regions after such an insult (Figure 2.2, upper panel). In the case of compensatory sprouting, neurons that are already established in a motor circuit must be triggered (Figure 2.2.1) to participate in structural plasticity. This triggering mechanism most likely differs from the triggering mechanisms for regenerative sprouting, where axotomy of the axon presents a strong signal for the injured cell, setting intrinsic mechanisms for axonal regeneration in motion (Abe and Cavalli, 2008, Cavalli et al., 2005, Michaelevski et al., 2010). In both cases, however, a growth-promoting transcriptome (Figure 2.2.2) must be initiated, allowing them to elongate an axon into the denervated area (Figure 2.2.3), where connections to target cells must be established (Figure 2.2.4). Afterwards, this connectivity may be refined by strengthening of active and pruning of inactive synapses (Figure 2.2.5), resulting in a newly established connectivity that allows for additional degrees of function. Each of these steps requires an individual molecular mechanism in which the neurons, the environment and, as a last step, the target neuron engage in a perfect interplay. Here, we will review the current literature on the known mechanisms of each of the steps of compensatory axonal sprouting focused on CSMNs.

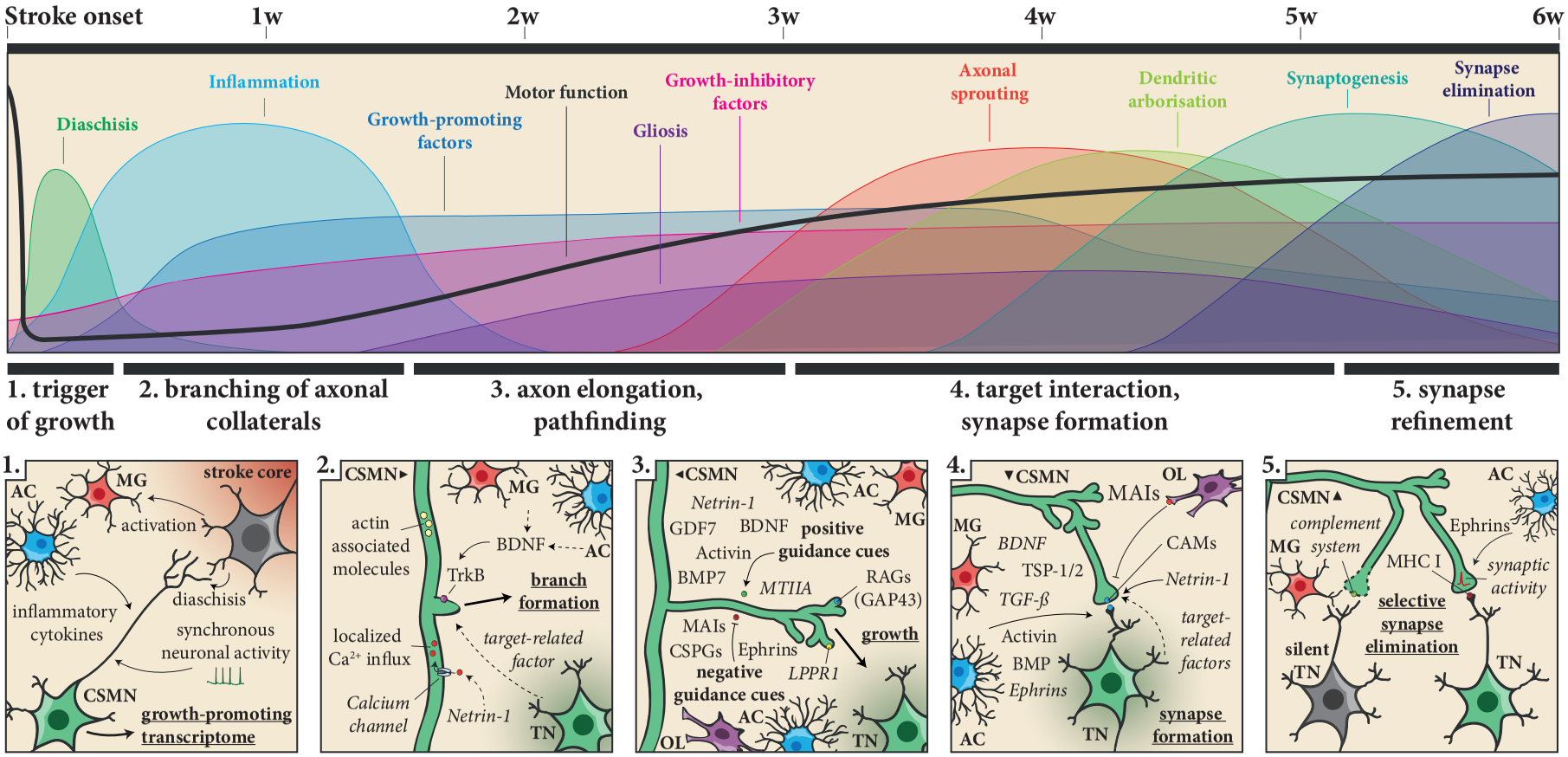

Figure 2.2 – Schematic illustration of molecular and cellular events after stroke. The temporal sequence of events over the course of recovery in mice within the period of heightened plasticity of 6 weeks after stroke onset. Observed molecular and cellular events are plotted over the timeline of recovery. Motor function (bold line) is impaired initially after injury but recovery over the course of 6 weeks to a stable plateau. Events that might influence this are: diaschisis, inflammation, gliosis, growth-promotion and inhibition, axonal sprouting, neurotrophic factors, dendritic arborization and synaptogenesis. Note that most studies were conducted in the peri-infarct cortex. Molecules that were only indirectly indicated are italicized. Adapted from (Cramer, 2008, Dalise et al., 2014, Murphy and Corbett, 2009). These events can be summarized into five distinct categories according to the proposed mechanism they exert: trigger phase, initiation phase, maintenance phase, maturation phase and pruning phase. Schematic illustrations of factors known to shape the defined phases are depicted in the lower panels accordingly. Abbreviations: microglia (MG), astrocyte (AC), corticospinal motoneuron (CSMN), target neuron (TN), oligodendrocyte (OL), myelin associated inhibitors (MAIs), regeneration associated genes (RAGs), cell adhesion molecules (CAMs).

2.2.1 | The Bat-Signal for Help — Triggering Fully Integrated Adult Neurons

The stroke-induced compensatory structural plasticity of CST neurons is a remarkable event considering that adult brain tissue was previously thought to be mostly hardwired. It indicates that adult neurons already fully differentiated and integrated into a sensory motor circuit, give up on their original function and undergo a demanding mechanisms of growth along growth-inhibitory paths, in the end competing with other neurons for the re-innervation of the pre-injury targets of stroke-ablated neurons. Participating neurons can be found within the peri-infarct cortex, but also at more distant sites such as the contralesional cortex, making the question for how they are recruited even more interesting. One possibility is presented by diaschisis, i.e. the loss of a chain of neurons, as a fast way to relay such a trigger to the recruits (Cheng et al., 2015). Interestingly, a large area of cortical hypometabolism was found after induction of a small stroke, which was later indicated to undergo axonal sprouting (Carmichael et al., 2004). Thus, a loss of a synaptic partner might provide a sufficient intrinsic trigger to initiate a growth-promoting transcriptome.

Additionally, synchronous cortical rhythms may contribute to triggering remote areas. Two forms of rhythmic activity have been described after stroke, emerging as early as one day after the insult. The early pattern, which can be observed in the peri-infarct cortex in a frequency range of 0.2 - 2 Hz, is primarily associated with polymorphic delta waves that are typically induced by brain lesions (Gloor et al., 1977, Sharbrough, 2005). Intriguingly, a second type of synchronous activity can be seen with a peak at three days post injury (dpi) in a slower frequency range of 0.1-0.4 Hz. This rhythm was relayed more widespread across cortical areas, reaching more distant sites such as the contralesional hemisphere. The low frequency range is consistent with the infra-slow spontaneous neuronal activity and suggests involvement of network activity of cortical and subcortical structures (Aladjalova, 1957, Colonnese et al., 2010, Steriade, 1998). Blockage of rhythmic slow wave activity of the peri-lesional cortex using the sodium channel inhibitor tetrodotoxin prevented axonal sprouting of the contralesional corticostriatal projections after a small stroke (Carmichael and Chesselet, 2002). Even though this study indicates synchronous neuronal activity as a mediator of axonal sprouting, it is not clear whether it plays a permissive role by altering the inhibitory environment or an inductive role, inducing an neuronal intrinsic growth-promoting transcriptome.

Not only neurons, but also glial cells in the penumbra and peri-infarct region, are triggered by the stroke onset to participate in post-stroke recovery (Carmichael, 2016). Sec-

ondary injury cascades induced by the ischemic event lead to early activation of microglia and neutrophils, and later trigger the invasion of macrophages to the lesion site (Iadecola and Anrather, 2012). The release of inflammatory cytokines by these immune cells can influence repair mechanisms such as neurogenesis and axonal regeneration (Ekdahl et al., 2009, Gensel et al., 2009, Popovich and McTigue, 2009). Furthermore, astrocytes are activated by these cytokines, which in turn can influence synaptogenesis as well as axonal sprouting (Becerra-Calixto and Cardona-Gómez, 2017, Gleichman and Carmichael, 2014, Liddelow et al., 2017). Whether these secondary injury cascades may ultimately lead to beneficial or a detrimental effects on axonal sprouting is still under debate (Sierra et al., 2013), especially since molecules are often shared amongst several key elements of stroke progression including, but not limited to, neuroprotection, neurogenesis, angiogenesis, blood brain barrier breakdown and scar formation. Microglia have been shown to activate neurotoxic reactive astrocytes by a specific cocktail of molecules (Liddelow et al., 2017), while additionally releasing pro-inflammatory cytokines such as IL-1, IL-6, and tumor necrosis factor α (TNF-α). Reactive astrocytes release CSPGs as well as Ephrins (Gleichman and Carmichael, 2014) which suppress axonal regeneration, as discussed above. The common consensus in the literature is that the net outcome of functional recovery depends on the balance between secreted molecules and their pro- and/or anti-inflammatory function.

Considering the region-specific structural plasticity that is observed after stroke (Nudo and Milliken, 1996), it seems plausible to conceive a multi-step process which needs several conditions to be met. The hypometabolic state induced by the loss of a post-synaptic partner might prime the neuron to be more susceptible to neuronal activity rhythms and inflammatory cytokines and blockage of any of these steps might result in loss of sprouting capability. Studies should focus on the many open questions, namely: (1) whether subpopulations of CSMNs have a predisposition to participate in structural plasticity after stroke and are thus more susceptible to sprout, (2) how the switch to a growth state of the CSMNs might be conveyed intrinsically, and (3) how these mechanisms are influenced by extrinsic factors.

2.2.2 | Neuronal Reprogramming — Switching Gears towards Growth

To participate in structural plasticity, CSMNs must first, once triggered, be able to initiate a new branch that will then evolve into a new axon to re-establish connections to a new target. In regenerative sprouting after SCI, the cut axon forms a retraction bulb which develops into a growth cone, only to be suppressed thereafter by the inhibitory signals of the environment

(abortive sprouting) (McKerracher and Selzer, 2006). Regenerative CSMNs seem to possess an intrinsic mechanism for a switch towards growth, thereby establishing new functional circuits by short-distance sprouting (Bareyre et al., 2004, Fouad et al., 2001). This detour circuitry formation has been associated with upregulation of the aforementioned RAGs, such as SPRR1a, which is found localized in the growth cone after axotomy lesion (Bareyre and Schwab, 2003, Bonilla et al., 2002).

In contrast to SCI-induced regenerative axon growth, compensatory sprouting, as observed after stroke, relies on an established neuron that is fully integrated into a circuit. This neuron extends a new axonal branch along its axon shaft, which can e.g. extend across the spinal cord midline. This process can lead to a transitory innervation of left and right spinal cord at the same time (Lindau et al., 2014). Seminal work by Li et al. (2010) analyzed the transcriptome of sprouting cortical neurons, which show pronounced axonal sprouting in the peri-infarct region after small strokes (Carmichael et al., 2001), by labeling sprouting neurons using a sequential tracer approach before and after the lesion. The results showed a marked upregulation of molecules associated with cytoskeletal rearrangements and growth cone formation at 7 dpi. Several factors affecting actin turnover, actin interactions with cell adhesion signals or actin-associated motility were induced, and upregulation of integrin signaling molecules linked this cytoskeletal remodeling to extracellular matrix binding. Microtubule-modifying genes were also found to be upregulated, suggesting that at 7 days after injury, sprouting neurons express a growth-promoting transcriptome that allows them to form a collateral branch. The upregulation of epigenetic modifying proteins, such as alpha thalassemia/mental retardation syndrome X-linked homolog (ATRX), further indicates an intrinsic rearrangement towards a growth-promoting state, as overexpression led to increased axonal outgrowth *in vitro* (Li et al., 2010). The transcriptomic profiling in the study of Li et al. (2010) was however restricted to the soma of cortical sprouting neurons. Additional analyses on the level of the growth cone or branch tip, which possess their own set of mRNA transcripts (Hatch et al., 2017, Zivraj et al., 2010), might provide further insight into the molecular mechanisms underlying collateral sprouting after stroke and how neurons may react to external cues promptly.

The growth cone formation in regenerative sprouting has been shown to be dependent on calcium (Ca^{2+}) signaling (Bradke et al., 2012, Goldberg et al., 2002b) as axotomy of cultured DRG neurons in a Ca^{2+}-free environment or after blockage of downstream extracellular- signal regulated kinase 1,2 (ERK) or protein kinase A (PKA) signaling does not result in regenerative growth cone formation. Thus, formation of a growth cone with subsequent activation of downstream growth-inducing pathways seems to be induced by Ca^{2+} influx (Chierzi et al., 2005), and developmental branch formation has also been linked

to Ca^{2+} influx evoked by e.g. Netrin-1 (Spitzer, 2006, Tang and Kalil, 2005). An auxiliary subunit of the voltage-gated calcium channel complex, Alpha2delta2, has recently been identified as an neuronal inhibitor of regenerative sprouting (Tedeschi et al., 2016). Intriguingly, several components linked to Ca^{2+}-dependent signaling have been found to be upregulated starting from 7 dpi after stroke in cortical sprouting neurons (Buga et al., 2012, Li et al., 2010). Paradoxically, endogenous levels of the subunit Alpha2delta1 have been found increased in the peri-infarct region in young animals after stroke, while levels were down-regulated in aged animals (Buga et al., 2012, Li et al., 2010). As in this lesion model compensatory sprouting can be observed, a difference between regenerative and compensatory sprouting may exist in terms of Ca^{2+} dependency. Future work should address this potential link of Ca^{2+} influx to neuronal growth-induction for compensatory sprouting.

During development, branching through the formation of filopodia, lamellipodia and growth cones has been linked to two closely related receptors, tropomyosin receptor kinase (Trk) A and B. By binding to its ligand NGF, TrkA activates downstream signaling to the cytoskeleton, including activation of PI3K, which leads to actin polymerisation, and therefore stabilization of the actin skeleton for branch formation (Gallo and Letourneau, 1998). In stroke and SCI models, NGF has primarily been described for its function in neuroprotection (Cheng et al., 2009, Zhang et al., 2014) and while NGF can induce axonal branching in DRG axons, no effect of NGF on adult CSMNs was found *in vitro* (Özdinler and Macklis, 2006). TrkB and its ligand BDNF, on the other hand, have been implicated in functional recovery in both stroke and SCI. TrkB and BDNF levels are elevated one week after pyramidotomy in the denervated hemicord (Bareyre et al., 2002) as well as in the peri-infarct cortex and spinal cord three days after stroke (Clarkson et al., 2011, Sist et al., 2014), suggesting a neurotrophic attractive role of this signaling pathway on contralesional sprouting CSMNs. Downregulation of spinal BDNF by use of small interfering RNA (siRNA) led to a reduction in midline crossing fibers after a large stroke, coupled to abrogated functional recovery (Ploughman et al., 2009, Ueno et al., 2012). In contrast, systemic administration of BDNF resulted in a better motor recovery (Ploughman et al., 2009). However, caution in interpreting these results should be taken, as BDNF also shows neuroprotective effects (Schabitz et al., 2004). In development, BDNF/TrkB signaling has been linked to microtubule destabilization in the early branching stages (Jeanneteau et al., 2010), a mechanism by which collateral branching might be influenced in the injury context as well.

The signal for sprouting CSMNs to induce branching along the axon shaft might be conveyed within the target region, as sprouting is primarily observed in a region-specific manner. Involvement of glial cells such as astrocytes or microglia seems plausible, as e.g. BDNF may be released primarily by microglia (Parkhurst et al., 2013). Recent studies have

looked at activated microglia (Wattananit et al., 2016) and astrocytes (Liddelow et al., 2017) in more detail, and further analysis of the produced transcriptome data sets might yield more information about specific molecular contributions towards this step of recovery. Additionally, target-derived factors released by the neuron in search for synaptic input might induce branch formation within the sprouting CSMNs.

2.2.3 | Extending a Helping Hand — Axon Formation after Lesion

Initiation of a growth structure, either at the tip as a growth cone or along the axon as a collateral branch, is only the first step in successful formation of a new axon needed for recovery. Axon growth towards the target region, as a fundamental part of recovery, can be divided into two distinct modes: elongation and arborization (Rossi et al., 2007, Smith and Skene, 1997).

During development, elongation is the first step for a neuron to project its axon rapidly across long distances towards the target region (Canty and Murphy, 2008, Kalil and Dent, 2014). The growth cone of immature CSMN axons will react to guidance cues in its vicinity (Canty and Murphy, 2008). Steering through the tissue is primarily organized by attractive and repulsive guidance cues provided by the glial cells and neurons of the CNS at given choice points, such as the medullary decussation. Here, a highly complex set of guidance cues re-routes the axon to first take a dorsal turn, decussate at the midline and subsequently take a turn into the dorsal funiculus in rodents (Canty and Murphy, 2008, Kolodkin and Tessier-Lavigne, 2011) or the dorso-lateral funiculus in primates or cats. Elongation of growth requires the upregulation of cytoskeletal elements, membrane constituents and growth cone components (Fawcett, 2001, Lasorella et al., 2006, Rossi et al., 2007). Insults such as axotomy can trigger neurons to undergo transcriptional changes to initiate the elongation mode (Smith and Skene, 1997). Axotomized CSMNs are, however, rarely able to resume this growth status of elongation spontaneously (Bradke et al., 2012, Rossi et al., 2007).

Once elongation through the neuraxis is established in development, collateral branching of CST axons can be observed at distinct levels in the brainstem and spinal cord. Some axons first bypass their spinal targets to later arborize in a topographically arranged manner by formation of approximately three to seven branches with subsequent elimination of the axon distal to the target (Akintunde and Buxton, 1992, Canty and Murphy, 2008, Kalil and Dent, 2014, Kuang and Kalil, 1994, Luo and O'Leary, 2005, O'Leary et al., 1990). Recent

studies furthermore identified a subpopulation of genetically distinct CSMN that directly innervate cervical levels (Arlotta et al., 2005, Molyneaux et al., 2005). Arborizing is a common feature seen in the adult CNS, with subtle reorganizations at the terminal arbors occurring throughout life, possibly shaped by learning and memory formation (Purves et al., 1987, Smith and Skene, 1997). The observed switch from elongation to arborization mode is coincident with a down-regulation of growth-associated proteins (Skene, 1989), whereas synaptic assembly and transmission mechanisms are activated (Hökfelt et al., 1994). As such, arborizing mode is thought to be less dependent on the intrinsic molecular machinery controlling cytoskeletal rearrangements that are on the other hand necessary for elongation of an axon (Fawcett, 2001, Skene, 1989, Smith and Skene, 1997).

After stroke, GAP43, a regulator of cytoskeletal rearrangements amongst other functions, is upregulated in cortical neurons at 14 dpi in the peri-infarct cortex, the contralesional cortex and at more distant sites, such as the denervated spinal cord (Kawamata et al., 1997, Liu et al., 2013, Sist et al., 2014, Stroemer et al., 1995). Even though GAP43 is commonly referred to as a growth cone marker, it has been shown to be involved in collateral formation as well (Benowitz and Routtenberg, 1997, Lin et al., 1992). GAP43 levels peak at 14 dpi after complete forelimb cortical lesions within the cervical spinal cord while growth-promoting genes induced at earlier time points (7 dpi) are dampened at this time point. Thus, given the lesser extent of cytoskeletal rearrangement machinery needed, the compensatory structural plasticity in spinal levels after stroke might rather be based on arborizing growth than on elongation mode (Carmichael, 2005, Stroemer et al., 1995).

At 28 dpi after pyramidotomy, the transcriptome of sprouting CSMNs of Nogo receptor (NgR)1 *null* mice, which show increased connectivity on spinal levels as compared to wildtype mice, showed an enrichment of molecules of pathways that facilitate axon growth (Fink et al., 2017), such as HIPPO signaling, mTOR signaling and 3PI degradation. By comparison of sprouting to quiescent neurons, lipid phosphate phosphatase-related protein type 1 (LPPR1) and lysophosphatidic acid receptor 1 (LPAR1) were identified as intrinsic axon growth modulators. Overexpression of LPPR1 enhanced neurite outgrowth *in vitro* as well as sprouting of CSMNs after pyramidotomy *in vivo*. Whole tissue analysis of the peri-infarct cortex after stroke showed marked upregulation of additional RAGs, such as cortical associated protein 23 (CAP23), c-Jun and SPRR1a, as well as several additional genes implicated in growth during development (Bonilla et al., 2002, Buga et al., 2012, Carmichael, 2005). However, here, it is not clear whether these genes are expressed by sprouting cortical neurons or by other cells, e.g. glia or other neurons, in the analyzed area.

It seems reasonable to assume that sprouting CSMNs, once triggered, also depend on guidance cues within their vicinity to navigate through the spinal cord in a topographically

meaningful manner. The final termination area of midline crossing CST collaterals is well defined, with neurons re-innervating the same areas as the pre-injury CSMNs (Lindau et al., 2014, Ueno et al., 2012). This innervation pattern can either be established by targeted innervation or by excessive sprouting followed by selective refinement. Both processes, however, would depend on guidance cues within the environment considering the growth-permissive environment the spinal midline poses to the sprouting CSMNs. Remarkably, after cortical aspiration lesion, transplanted embryonic stem cells that still retain their developmental growth capacity were shown to project axons all the way along the neuraxis to subcortical and spinal targets, where they form synaptic contacts to target cells (Gaillard et al., 2007). Astrocytes activated by the lesion were postulated to form a supportive substrate for the observed outgrowth, while myelinated areas were avoided (Gaillard and Jaber, 2007). Thus, environmental factors, potentially provided by glial cells, might play important roles to steer the projecting axons to their respective target area.

Post-stroke axonal sprouting has been shown to be stimulated by inflammatory signals, especially by members of the transforming growth factor (TGF) superfamily of cytokines. Bone morphogenic protein (BMP) 7 can promote behavioral recovery after stroke (Ren et al., 2000), while its primary action seems to be stimulation of dendritic growth (Withers et al., 2000). Recent work has indicated that the TGF-β family member growth differentiation factor 10 (GDF10) may be a potent stimulant for axonal sprouting in the peri-infarct cortex after stroke in rodents, primates as well as humans (Li et al., 2015). TGF-β1 has mainly neuroprotective effects (Pang et al., 2001), but also shows growth-promoting properties in *in vitro* studies of hippocampal neurons (Abe et al., 1996) as well as on neocortical neurons during development (Yi et al., 2010). Activin, another member of the TGF-β family, can furthermore control the intrinsic susceptibility of neurons towards myelin, with neurons expressing less activin being able to extend longer axons on a myelinated substrate *in vitro* (Omura et al., 2015). In development, BDNF/TrkB as well as Netrin/deleted in colorectal carcinoma (DCC) binding leads to increased arborization (Drinjakovic et al., 2010, Granseth et al., 2013, Manitt et al., 2009), providing another mechanism for BDNF to influence functional recovery after stroke, in addition to its effect on the induction of branching. Netrin-1 has neuroprotective effects after stroke, as it ameliorates blood-brain barrier impairment by increasing angiogenesis after stroke, but its direct role in axonal regeneration has not been addressed thus far (Chen et al., 2017, Lu et al., 2012, Wu et al., 2008, Yu et al., 2017). IGF-1, which also signals through PI3K downstream pathways, was shown to increase axon elongation of adult CSMNs *in vitro*. Blockade of IGF-1 with function-blocking antibodies during development resulted in misrouting and defasciculation of axon outgrowth (Özdinler and Macklis, 2006). After stroke, increased levels of IGF-1 were found in compensatory sprouting CSMNs, however, local administration of the protein into the

peri-infarct cortex after stroke did not influence compensatory sprouting of horizontal peri-infarct cortical neurons (Li et al., 2010). Blockade of IGF-1 signaling using an antagonist resulted in reduced sprouting caused by retraction of arbors or neuronal cell death, even when applied after 7 dpi, indicating that neurons develop a dependency towards growth factors, possibly due to the growth-promoting transcriptome. Thus, a variety of growth factors and inflammatory cytokines play important roles in guidance and enhancement of compensatory sprouting of neurons after stroke.

As mentioned above, CSPGs and Ephrins released by astrocytes have shown to have a negative influence on sprouting of axons (Gleichman and Carmichael, 2014, Omoto et al., 2011, Overman et al., 2012). Additionally, many positive guidance factors have been identified, with metallothioneins emerging as interesting candidates. Metallothionein isoform IIA (MT-IIA) has been shown to be highly upregulated as early as 14 hours after stroke and SCI (Carmel et al., 2001, Carmichael, 2003, Trendelenburg et al., 2002). Mainly produced by astrocytes (Neal et al., 1996), MT-IIA has been implicated in increased survival of neurons that are weakened by the stroke incident (Eidizadeh et al., 2015, Trendelenburg et al., 2002), and an influence on initial neurite elongation and reactive axonal growth after axotomy has been established *in vitro* (Chung et al., 2003). *In vivo* studies investigating the effect on axonal regeneration are missing thus far in the literature.

Further studies into the molecular mechanisms underlying the balance between intrinsic and extrinsic factors are needed to fully understand the molecular regulation of the different stages of sprouting and CNS hardware plasticity and their relation to the establishment of new, functional circuits.

2.2.4 | Do You Copy? — Establishing Connectivity with the Target

After arrival of new axonal branches at an appropriate target area, synaptic connectivity with target cells will provide functionality to the new axons of sprouting cortical neurons. During development, the final innervation pattern of the CST relies on precise connections between the CST and the interneurons in laminae IV-VII in rodents (Asante and Martin, 2013, Brosamle and Schwab, 1997). The established synaptic connectivity is concomitant with decreased levels of GAP43 at a time point corresponding to initial target contact and decreased axon growth (Curfs et al., 1995, 1994). In contrast to the better known molecular components suggested to act as guidance cues during axon formation, little is known about

target recognition and the initiation of synaptogenesis involved in stroke-induced circuitry reformation.

In development, target-dependent factors were proposed to instruct the newly innervating axons for correct positioning and adherence as a first step of synaptogenesis (Fox and Umemori, 2006). Binding of neuroligins (NLs) to their respective binding partners, neurexins (NRXs), leads to coupling of the two compartments of the synapses (pre- and post-synaptic) (Scheiffele et al., 2000). Additionally, the leucine rich repeat trans-membrane (LRRTM) protein family has been identified as a synaptic organizer able to instruct excitatory pre-synaptic differentiation (Linhoff et al., 2009). Together, both NLs as well as LRRTMs can regulate synaptic strength *in vivo* (Soler-llavina et al., 2011). Pre- and post-synaptically expressed factors such as Netrin-G ligands, ephrinBs and members of the cell-adhesion molecule (CAM) family, including NCAM, cadherins and integrins, might influence synapse formation (Aoto and Chen, 2007, Fields and Itoh, 1996, Graf et al., 2004, Henkemeyer et al., 2003, Kim et al., 2006), highlighting the importance of cooperation of factors in both compartments in development, maintenance and function of synapses.

Extrinsic factors released by glial cells can further influence synaptogenesis during development. Immature astrocytes, as opposed to their mature counterpart, have been shown to express thrombospondins (TSPs)-1 and -2, which induce synapse formation both *in vitro* and *in vivo* (Christopherson et al., 2005). Many of the factors described above for their role on collateral branching have also been implicated in synapse formation. Members of the TGF superfamily, such as TGF-β and BMPs, influences dendritic sprouting *in vitro*, providing a mechanism to prime the post-synaptic target cell to participate in synaptogenesis (Bae et al., 2011, Chen and Panchision, 2007, Hocking et al., 2008). Administration of Netrin-1 and BDNF increased the number of pre-synaptic sites in retinal ganglion axons (Alsina et al., 2001, Hu et al., 2005, Manitt et al., 2009, Parkhurst et al., 2013).

While extrinsic factors are clearly important in promoting synapse formation during development, the switch between the growth mode and the establishment of connectivity observed in development may also be influenced by inhibitory factors. Nogo-A represses synaptic plasticity *in vitro* (Mironova and Giger, 2013, Tews et al., 2013), and neutralization of its function led to marked improvements of motor learning in healthy adults (Zemmar et al., 2014). Activity-dependent synaptic strength was found reduced after depletion of a functional receptor for Nogo-A, the Nogo-66 receptor 1 (NgR1), in hippocampal neurons (Lee et al., 2008). During development, myelination of the CST occurs later than synaptic plasticity (Canty and Murphy, 2008), however, the negative influence of MAIs on synaptogenesis might hinder a successful regeneration after injury in the adult CNS.

After stroke, increased levels of synaptophysin, a marker for pre-synaptic terminals, has been found in the cortex and the spinal cord (Liu et al., 2013, Stroemer et al., 1995). This may indicate a similar switch from growth towards synaptogenesis in the adult, sprouting CSMNs as observed during development. Furthermore, dendritic spine turnover was observed in the peri-infarct region, showing plasticity also at the post-synaptic level (Brown et al., 2007). On the spinal level, CSMNs were shown to synapse on segmental interneurons and propriospinal neurons within laminae VI+VII, and these connections were functional as assessed by intracortical microstimulation, EMG of the forelimb muscles and selective silencing during skilled motor behavior assessment (Ueno et al., 2012, Wahl et al., 2014).

The transcriptomic profile of cortical sprouting neurons provided by Li et al. (2010) indicates an upregulation of synaptic proteins at 21 dpi suggesting their involvement in synapse formation. For sprouting CSMNs, the transcriptome has recently been established at 28 dpi after pyramidotomy, and pending analysis should provide further insight into synaptogenesis of CSMNs after axotomy (Fink et al., 2017). In the CAST/Ei mouse which possesses genetic propensity for enhanced axonal sprouting after stroke and SCI (Omura et al., 2015), activin can, additionally to its observed function as a growth-promoter, increase dendritic complexity (Ishikawa et al., 2010), modify spine morphology and lead to an overall increase in the number of synaptic contacts on dendritic spines (Shoji-Kasai et al., 2007). This suggests an important role of activin also during synapse formation. TSP-1 and -2 levels, usually low in the adult brain, are increased following stroke within the peri-infarct cortex, potentially as a result of their release by reactive astrocytes and activated microglia (Lin et al., 2003). Double knockouts of these proteins showed pronounced defects in synaptogenesis and axonal sprouting within the cortical penumbra, ultimately leading to a worse functional recovery after stroke (Liauw et al., 2008, Tyzack et al., 2014). Reactive astrocytes have also been shown to secrete Ephrin-A5 in the peri-infarct region (Overman et al., 2012). Even though Ephrins are negatively implicated in axonal sprouting (Overman and Carmichael, 2014), they have been associated with developmental synapse formation, especially when coinciding with neuronal activity (Pfeiffenberger et al., 2005). Therefore, Ephrin signaling might provide an additional mechanism to affect synaptogenesis and thereby recovery after stroke. Protein levels of Nogo-A were found to be reduced in the first two weeks after stroke in the peri-infarct cortex (Li and Carmichael, 2006), however, after 28 dpi Nogo-A is significantly increased (Cheatwood et al., 2008), potentially posing a limiting factor to synaptogenesis.

In conclusion, many of the guidance cues that steer navigation of elongating axons through the tissue are also able to influence synaptogenesis either on the pre- or the post-synaptic level in development and potentially also after stroke. Interestingly, selective roles

of single molecules have been proposed for development of excitatory versus inhibitory synapses, suggesting a diversity of molecular signals for the variety of synapse types seen in the CNS (Chih et al., 2005, Chubykin et al., 2007). Target-released factors might influence this diversity already during target recognition, as development of GABAergic synaptic transmission was shown to be independent on the type of target neuron whereas glutamatergic synapse development was strongly affected by the target neurons *in vitro* (Mohrmann et al., 1999). Deciphering the role and timing of induction of these pre- as well as post-synaptic signals during development might provide new ideas for the mechanisms of post-stroke synaptogenesis.

2.2.5 | Should I Stay or Should I Go? — Activity-Dependent Stabilization or Elimination of Synapses

As a last step, after CST axons have successfully established synapses to pre-injury targets, a refinement of the connectivity is needed to establish fully functional patterns of movement. Connections must either be stabilized by the recruitment of CAMs and turned into functional synapses, which contain a pre-synaptic vesicle pool and corresponding receptors at the post-synaptic sites, or eliminated when functionally irrelevant (Goda and Davis, 2003).

Hebbian or related mechanisms could reinforce stabilization of pre-synaptic and post-synaptic elements (Hebb, 1949) comparable to mechanisms observed during learning and memory (Whitlock et al., 2006). Strengthening of connections occurs after a fast train of coinciding activity of pre-synaptic and post-synaptic neurons (long-term potentiation (LTP)) (Bliss and Lømo, 1973, Bliss and Collingridge, 1993, Song et al., 2000). Another important aspect of learning and memory is the refinement of functionally irrelevant synapses, a process that can either be activity-dependent following low-frequency activity of weak synapses (homosynaptic long-term depression (LTD)); or activity-independent, occurring at inactive synapses (heterosynaptic LTD) (Escobar and Derrick, 2007, Malenka and Nicoll, 1993). Following stroke, this might provide a means to strengthen connections that can successfully elicit meaningful motor movements, or removal of those that cannot. Hebbian plasticity has been proposed to underlie the observed sensory remapping of peri-infarct cortex, as prolonged neuronal responses have been measured after stroke, which potentially facilitate coincident activation with post-synaptic targets (Brown et al., 2009). This increased neuronal activity furthermore correlated with dendritic changes and axonal sprouting into subcortical areas, suggesting establishment of a functionally relevant circuit (Brown et al., 2009, Murphy and Corbett, 2009). Thus, neuronal activity determines whether strengthen-

ing or elimination of specific synapses occurs in learning and memory, and might potentially do so after stroke.

The success of rehabilitation training in increasing functional outcome furthermore indicates involvement of Hebbian mechanisms in post-stroke recovery (Biernaskie, 2004, Biernaskie and Corbett, 2001, Nudo and Milliken, 1996). In stroke patients, the improvement of arm use after constraint-induced movement therapy, a specific form of rehabilitation, was directly correlated to increased neuronal activity of cortical areas (Gauthier et al., 2008). In monkeys, remapping of cortical representation from elbow and shoulder to hand functions could only be observed after reach training-based rehabilitation (Nudo and Milliken, 1996), suggesting a coinciding activity of pre-synaptic cortical neuron and post-synaptic motoneuron induced specifically by this type of training. Furthermore, a high degree of competition is suggested by studies showing that training of both the paretic and nonparetic hand resulted in decreased motor outcome of the paretic hand (Jones and Adkins, 2015, Kim et al., 2015). Use-dependent rehabilitation could therefore potentially enhance coincident activity of the newly formed connections after stroke, thereby strengthening selective synapses while reducing activity of others, leading to elimination thereafter. Nevertheless, direct implications of Hebbian mechanisms after stroke are lacking and the analogy between learning and memory and stroke recovery remains an area for future studies.

On a molecular level, strengthening of synapses can be induced by the ephrin family. EphA4 overexpression in immature neurons *in vitro* enhanced network activity (Clifford et al., 2011), and depletion of the ligands ephrin-A5 or ephrin-A2 in the adult brain led to decreased LTP (Gerlai et al., 1999, Yu et al., 2013), possibly mediated by alterations of NMDA receptor mechanisms. Thus, the primarily astrocytic Ephrin signaling might provide an additional method for strengthening of synapses to dodge elimination (Overman and Carmichael, 2014).

Even though the exact mechanisms of synaptic refinements are suggestive at best, stroke-induced synapse elimination is implied by the reduction of fibers present in the newly innervated grey matter after 42 dpi as compared to earlier time points (Lindau et al., 2014, Ueno et al., 2012). As this reduction in CST branches did not influence motor performance, a refinement of meaningful connectivity can be assumed. The pruning of inappropriate synapses has been shown to be driven through neuronal activity in many systems studied thus far, such as the neuromuscular junction and the visual system (Hua and Smith, 2004, Katz and Shatz, 1996). Also in the CST, neuronal activity plays a crucial role in development of connectivity, as silencing of activity through local infusion of the GABA agonist muscimol during the postnatal refinement period led to disturbances in topographical distribution

as well as altered morphology of axon terminals with a decreased bouton density (Friel and Martin, 2005, Martin et al., 2010, 1999). As the active contralateral cortex was firmly connected with the spinal target of the silenced cortex, this suggests an activity-dependent competition between the two hemispheres.

Work by Stevens et al. (2007) has unraveled molecular mechanisms steering synapse elimination in development, with the classical complement cascade being implicated as a major factor. Mice lacking the protein complement component 1q (C1q) or the downstream protein complement component 3 (C3) show sustained defects in CNS synapse elimination and thus connectivity (Stevens et al., 2007). Astrocytes might induce production of C1q in developing neurons through yet unknown mechanisms, thereby tagging weak or inactive synapses for microglial phagocytosis (Chung et al., 2015, Stephan et al., 2012). Interestingly, this seems to be activity-driven, as microglia engulfment of synapses has been primarily shown for less active terminals (Hong et al., 2016, Paolicelli et al., 2011, Schafer et al., 2012, Tremblay et al., 2010, Wake et al., 2009). After stroke, a marked upregulation of several components such as C3 and serine (or cysteine) peptidase inhibitor, clade G, member 1 (Serping1) is seen in cortical sprouting neurons within the first week, reflecting the involvement of the complement cascade in neuroprotection (Arumugam et al., 2009, Li et al., 2010). However, less is known about its role in long-term stroke recovery and whether it fulfills the same role in synapse elimination after stroke as during development.

Apart from the innate immune system, which the classical complement cascade belongs to, components of the adaptive immune system have also been implicated in developmental synaptic refinement such as neuronal class I major histocompatibility complex (MHC1) molecules (Boulanger, 2009, Huh et al., 2000). MHC1 mRNA and protein levels are found to be increased in spinal motoneurons (MNs) after axotomy of the peripheral nerve (Lindå et al., 1998, Maehlen et al., 1988). Interestingly, a link between MHC1 expression and successful regeneration was proposed, as nerve crush injury, a lesion model that allows for regeneration, led to a transient increase in MHC1 expression with a temporal profile according to the time course of regeneration. On the other hand, transection of the nerve, which allows no regeneration of the axon, led to a persistent upregulation of the protein (Maehlen et al., 1989). MHC1-deficient mice showed disturbances in axon regeneration after peripheral nerve axotomy together with reduced levels of synaptophysin, a marker for synaptic connectivity, establishing a link between synapse stabilization and regeneration after CNS injury (Oliveira et al., 2004). After stroke, MHC1 was found to be upregulated in sprouting cortical neurons at 7 dpi (Li et al., 2010), but whether this protein plays part in neuroprotection or also in synapse stabilization/elimination in the peri-infarct cortex needs further evaluation. Additionally, several members of the ephrin family and their receptors

are upregulated at 28 dpi in reactive astrocytes of the peri-infarct cortex after stroke (Choi et al., 2016, Overman et al., 2012), potentially facilitating synapse strengthening by increasing neuronal activity.

Little is known about synapse stabilization/elimination of new CST sprouts in the spinal cord after stroke, but as summarized above developmental studies as well as single observations after stroke suggest an involvement of the complement cascade as well as the adaptive immune system, where especially MHC1 was identified as a critical factor. Further studies should address pruning and the suggestive microglial involvement, as synapse strengthening, e.g. by rehabilitative training, provides a strong means to increase recovery of selective tasks.

2.3 | Conclusion

Stroke induces axonal sprouting locally and at distant sites connected to the area of damage. This process, lasting for about six weeks after injury in rat and mice, is initiated by triggering fully integrated CSMNs within the first three days by loss of post-synaptic partner, synchronous neuronal activity as well as inflammatory cytokines released by activated glial cells, which may collectively lead to an intrinsic growth-promoting transcriptome. Initiation of a collateral branch is proposed to be calcium-dependent, and influenced by target-derived factors, e.g. BDNF or Netrin-1, on the spinal level. Axonal growth towards the target is shaped by both positive (e.g. GDF10, BDNF, Activin) as well as negative (e.g. MAIs, CSPGs, Ephrins) guidance cues. Upon establishment of a contact to potential target cells, synapse formation might be mediated by inflammatory cytokines as well as target-derived factors that are yet unknown. Neuronal activity, increased specifically by rehabilitative training, is likely a key factor for the selection, survival and stabilization of newly established synapses. Synapse elimination for refinement of circuits in development is highly dependent on complement cascades and induced by microglia and astrocytes. However, little is known about circuit refinement after stroke.

Overall, each step must be orchestrated by neuronal intrinsic as well as tissue-derived extrinsic factors and requires a high degree of interplay of both. The defined key phases of stroke recovery are often overlapping and share many molecules, making research into the exact mechanisms of single molecules challenging. Furthermore, many developmental molecules are suggested to be re-purposed after stroke (Cramer, 2008); nevertheless, the intrinsic transcriptome of sprouting cortical neurons does not match with the developmen-

tal properties of growing neurons but instead displays a unique post-stroke transcriptome (Carmichael et al., 2017, Li et al., 2015). Additionally, sprouting CSMNs have been shown to cross the growth-inhibitory midline to innervate the contralateral hemicord and establish functionally meaningful connections (Bachmann et al., 2014, Lindau et al., 2014, Ueno et al., 2012, Wahl et al., 2014), a process which they do not have to undergo during development in rats, suggesting that recovery does not necessarily recapitulate ontogeny after stroke. Thus, further care should be taken when conducting *in vitro* studies for the potential of single molecules to enhance neuronal sprouting, as most of the studies conducted used embryonic neurons, when they should be focused more on cultured adult neurons, which can e.g. be accomplished by CSMN purification methods (Özdinler and Macklis, 2006).

Taken together, research on the timeline of structural plasticity and functional recovery is advancing fast with more and more molecules influencing either step of this process being unraveled. Recent transcriptomic studies have provided key information on the molecules involved in sprouting of cortical neurons after stroke (Li et al., 2010) as well as CSMNs after pyramidotomy (Fink et al., 2017), presenting an unbiased means to identify key factors for specific phases of recovery. Environmental factors are being investigated on a single cell level, with studies highlighting the importance of astrocytically (Liddelow et al., 2017, Zamanian et al., 2012) and microglially released molecules (Hirbec et al., 2017, Wattananit et al., 2016) on survival and sprouting of neurons. Nevertheless, as outlined above, structural plasticity is highly dynamic and depends on a sequence of steps that need to be taken before a new connection can be successfully established. Interfering with this coordinated course of recovery can result in detrimental sprouting and lead to a worse functional outcome (Wahl et al., 2014). Future experiments therefore need to address molecular changes both intrinsically and extrinsically within each step of the recovery process and furthermore detangle the effects of candidate molecules on structural plasticity, angiogenesis, neurogenesis and other elements of stroke recovery as they are often involved in several of these key processes. Only then can the obtained pre-clinical knowledge be used to optimize treatment plans using combinatorial approaches for stroke patients in the future.

2.4 | References

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Context & Aims

Stroke patients often suffer from permanent damage to the motor and sensory areas of the central nervous system (CNS), severely affecting their quality of life (Cramer, 2008). While care in the hyperacute and acute phase is improving (Ding, 2015, Elgendy et al., 2015, Kurz et al., 2013, Ward, 2017), the majority of patients arrive too late or are not eligible for thrombolytic interventions. Here, the chance for recovery depends on the stroke size and location, with the integrity of the motor cortex being a determining factor for recovery success (Cramer, 2008). Spontaneous recovery may be observed within the first three months after small strokes, but is limited in the case of larger strokes (Cramer, 2008, Ward, 2017). To date, the only treatment option for chronic stroke patients is rehabilitative training, which may result in limited recovery of motor function in the best case (Langhorne et al., 2009). The substantial burden of chronic disability in stroke has therefore motivated scientists to understand the mechanisms of spontaneous recovery in the hope to develop targeted therapy plans for chronic stroke patients.

One key element of post-stroke recovery has been identified using functional neuroimaging methods and brain mapping: Brain-wide modifications in connectivity may lead to functional improvements (Rehme and Grefkes, 2013). In animal models of stroke, the insult triggers spontaneous structural plasticity of functionally related areas such as the peri-infarct cortex (Carmichael et al., 2001, Dancause, 2005, Dijkhuizen et al., 2001, Nudo and Milliken, 1996) or, in case of substantial tissue loss, the contralesional hemisphere (Bachmann et al., 2014, Lee et al., 2004, Lindau et al., 2014, Morecraft et al., 2016, Ueno et al., 2012). In the latter, circuitry reformation can be observed also on brainstem and spinal levels, where midline crossing fibers re-innervate the stroke-denervated hemicord to establish new, functional motor circuits (Bachmann et al., 2014, Ueno et al., 2012). Intriguingly, selective silencing of these re-innervating contralesional corticospinal motor neurons (CSMNs) leads to an acute re-appearance of initial deficits (Wahl et al., 2014), highlighting the importance of structural plasticity for post-stroke functional improvements. While a substantial body of research has defined the anatomical correlates of stroke recovery, the molecular mechanisms underlying this structural plasticity are only beginning to be unraveled.

Considering the observed spontaneous recovery mediated by structural plasticity, the endogenous molecular machinery seems to be capable of initiating and steering axonal

growth and to establish a functional motor circuitry. The intrinsic properties of sprouting neurons have been addressed in the past (Fink et al., 2017, Li et al., 2010), providing an insight into the neuronal capability to re-initiate growth in the adult lesioned CNS. Furthermore, several compounds have been probed for their potential to increase the neuronal growth-potential (Chen et al., 2002, Li et al., 2015, Liauw et al., 2008, Overman et al., 2012, Schabitz et al., 2004, Sist et al., 2014, Ueno et al., 2012, Zai et al., 2009). None of these factors could be translated into clinical care so far (Ward, 2008, 2017). Thus, a better definition of the endogenous molecular machinery, especially of the factors produced by the stroke-denervated tissue promoting growth of axons through the growth-inhibitory adult CNS, is pivotal for the development of new treatment options for chronic stroke patients. This thesis aims to provide an insight into the molecular changes in the spinal cord tissue after ischemic cortical injury, with a focus on factors that might influence the rewiring process of contralesional CSMNs associated with functional recovery.

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3

The Spinal Transcriptome Following Cortical Stroke — In Search of the Molecular Changes Regulating Spontaneous Structural Plasticity

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Author contributions: J.K. conceived and designed the study, performed all surgeries, performed behavioral experiments, perfused and dissected tissue, performed immunohistochemical staining, analyzed behavioral and staining data, prepared the RNA samples for further analysis, further analyzed the differentially expressed gene list and gene set enrichment analysis, produced all figures and wrote the manuscript.

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3.1 | Introduction

Stroke is a leading cause of neurological disability in the elderly population and often impairs the motor and sensory systems, resulting in severely decreased quality of life (Murphy and Corbett, 2009). While patients with smaller strokes often show some spontaneous functional recovery, which can be further enhanced by rehabilitative training (Adkins and Jones, 2005, Alaverdashvili et al., 2008), large strokes affecting very large parts of motor-related areas within one hemisphere lead to permanent major functional impairments with limited potential for recovery (Biernaskie et al., 2005).

Functional improvements, if they occur, are mostly seen within the first few weeks to months after the stroke in human stroke survivors, highlighting a period of heightened plasticity early after injury (Cramer, 2008). Reorganization of the motor circuitry has been causally linked to the improved outcome in stroke patients (Chollet et al., 1991, Cramer, 2008, Dimyan and Cohen, 2011, Sharma and Cohen, 2012, Ward, 2017), primates (Morecraft et al., 2016, Murata et al., 2015, Rouiller et al., 1998) as well as rodents (Carmichael and Chesselet, 2002, Murphy and Corbett, 2009). In animal models, structural plasticity of spared brain regions that exert a related function can be seen in remote areas, e.g. the peri-infarct cortex (Carmichael and Chesselet, 2002) or the contralesional cortex in the case of larger strokes (Bachmann et al., 2014, Lindau et al., 2014, Wahl et al., 2014). Compensatory rewiring of the contralesional corticospinal tract (CST) occurs on several levels with collateral branching observed in the peri-infarct cortex (Carmichael et al., 2001, Carmichael and Chesselet, 2002), the striatum (Napieralski et al., 1996), the brainstem (Bachmann et al., 2014, Lee et al., 2004, Papadopoulos et al., 2002, Riban and Chesselet, 2006) and the spinal cord (Kartje-Tillotson et al., 1985, Lindau et al., 2014, Ueno et al., 2012, Wahl et al., 2014, Wiessner et al., 2003), and is established within four weeks after initial injury (Biernaskie et al., 2005, Carmichael, 2006, Murphy and Corbett, 2009). The re-innervation on spinal levels is mainly driven by midline crossing sprouts and increase of pre-existing ipsilateral spinal projections of the contralesional CST (Lindau et al., 2014, Ueno et al., 2012, Weidner et al., 2001). Selective silencing of these spinal midline crossing sprouts leads to acute re-appearance of the initial post-stroke forelimb deficits in a skilled forelimb assessment task in rats (Wahl et al., 2014), linking the contralesional cortex to functional recovery after large lesions.

Distinct phases for compensatory rewiring have been defined in the cortex, with a trigger phase within the first three days during which fully integrated, differentiated neurons are activated to participate in the structural plasticity. This is followed by a phase during which axonal arbors are initiated and elongated within the following two weeks (Carmichael et al., 2017), as evidenced by the growth-promoting transcriptome expressed by sprouting cortical neurons as early as 7 days post injury (dpi) (Carmichael, 2005, Li et al., 2010). Four weeks after initial injury, the newly formed circuitry can be detected neuroanatomically and functionally and is presumably refined by synapse stabilization/elimination in the following two weeks (Carmichael, 2006, 2016, Li et al., 2010, Ueno et al., 2012). Nevertheless, the spontaneous recovery observed in large stroke cases is limited even with the active growth-program of sprouting neurons, suggesting additional factors that influence the structural plasticity, e.g. the growth-inhibitory factors present in the adult central nervous system (CNS) (Savio and Schwab, 1989, Schwab and Caroni, 1988, Schwab and Thoenen, 1985). While after a small stroke, down-regulation of growth-inhibiting genes, including chondroitin sulfate proteoglycans (CSPGs) and EphrinA5, was found in the peri-infarct region concomitant with the upregulation of growth-promoting molecules such as SPRR1a, GAP43, SCLIP and SCG10 (Carmichael et al., 2005), much less is known about the conditions at more distant sites such as the spinal cord.

Sprouting corticospinal motor neurons (CSMNs) have been shown to be able to cross the midline into the denervated hemicord to re-innervate pre-injury targets (Lindau et al., 2014, Ueno et al., 2012, Weidner et al., 2001). Such fiber growth must be attracted and guided by appropriate factors within the target region. We therefore analyzed the intermediate laminae of the spinal grey matter, representing the main target area of midline crossing CSMNs, for differential gene expression at selected time points that match the key steps along the course of their structural changes after stroke, i.e. growth initiation (4 dpi), branching and elongation of collaterals and pathfinding through tissue (7-14 dpi), target interaction and synaptogenesis (28 dpi) with subsequent synapse refinement to fine-tune the newly established circuitry (42 dpi) (Carmichael et al., 2005, Ueno et al., 2012). Differential expression analysis (DEA) revealed 955 genes to be differentially expressed over all time points, with the most pronounced changes seen after 28 dpi. Using network enrichment analysis (NEA), we provide evidence for an early inflammatory phase within the first week after stroke, as well as a highly dynamic phase at later time points, during which upregulation of growth-promoting genes might influence rewiring of sprouting CSMNs. Particularly interesting candidate genes include TGF-β1, Semaphorin 6a and Netrin-G2, which rescue neurite outgrowth under growth-inhibitory *in vitro* conditions. The data obtained in this study may serve as a starting point for further studies concerning key

elements of stroke recovery on the spinal level, including inflammation, synaptogenesis and circuit reformation.

3.2 | Methods

Animals.
A total of n = 80 adult C57BL/6J mice (2-3 month, 20-25 g, mixed sex, Charles River) were used in this study. For the transcriptomic analysis, only female mice were used. By constraining the study to one sex only, we could decrease lesion size variability due to less variation in body size. Animals were housed in groups of four to five under a constant 12 h light/dark cycle with food and water ad libitum. All experimental procedures were approved by the veterinary office of the canton of Zurich, Switzerland.

Photothrombotic stroke.
For all surgeries, mice were anesthetized using 3-4 % isoflurane and transferred in a stereotactic frame (David Kopf Instruments, USA). Anesthesia was maintained at 1-2 % isoflurane throughout the surgery. Body temperature was maintained at 37 °C on a heating pad. All animals received a unilateral photothrombotic stroke to lesion the sensorimotor cortex as previously described (Bachmann et al., 2014, Watson et al., 1984). Briefly, the skull was exposed by a midline incision of the scalp, blood and periosteum was cleaned off to ensure a homogenous light application. An opaque template with a defined opening (3x5 mm) was aligned to the midline so that the right motor and pre-motor cortex was exposed (i.e., -2 to +3 mm a/p, 0 to 3 mm m/l related to Bregma)(Tennant et al., 2011). Five minutes after intraperitoneal injection of 0.1 ml Rose Bengal (10 mg/ml in 0.9 % NaCl, Sigma-Aldrich), the skull was illuminated with a cold light source (Olympus KL1500LDC, 150W, 3000K) placed firmly on top of the skull for 10.5 minutes at maximal output. Control animals received a sham operation according to this protocol without illumination of the skull. Post-operative care included recovery on a heating mat, sustained analgesia provided via drinking water (Novalgin, 2 mg/ml with 5 % sucrose) and antibiotic treatment where necessary for 3 days.

Anterograde tracing of the forelimb cortex.
The contralesional corticospinal tract was labeled using the anterograde tracer biotinylated dextran amine (BDA) (MW 10.000 , 10 % (w/V) solution in 0.01 M PBS, Invitrogen). Two weeks prior euthanasia, mice were fixed in a stereotactic frame (David Kopf Instruments, USA) and the scalp was re-exposed. A craniotomy was performed over the left motor cortex with the dura being kept intact. Five injections of BDA were made at specific forelimb related coordinates (±1.25|-0.5 mm, ±2|-0.5 mm, ±1.25|+0.5 mm, ±2|+0.5 mm, ±1.25|+2 mm, in relation to Bregma) at two given depths each. Two 40 nl injections were given with a 33 g needle on a 10 µl Nanofil syringe (Hamilton, Switzerland) driven by an electrical pump with a constant flow rate of 6 nl/s (World Precision Instruments, Germany) at 1 mm and 0.8 mm below the cortical surface. To avoid backflow of the tracer, the needle was kept in position for 2 min after each injection.

Behavioral testing.
Behavioral tests were performed prior surgery (baseline) as well as 4, 7, 14, 21 and 28 dpi after photothrombotic stroke of the right motor cortex. The horizontal ladder walk test was used as a sensitive assessment of skilled limb placement (Metz and Whishaw, 2002). Three trials on a 40 cm long stretch on the irregularly spaced rungs of 1 cm distance were recorded on each testing day (Panasonic HDC-SD800 High Definition Camcorder) and evaluated using frame-by-frame analysis (VLC media player, VideoLAN). Blinding was not possible due to

obvious fine motor deficits induced by the photothrombotic stroke. Forepaw placement was scored as previously described (Maier et al., 2008). Briefly, a full success (score 4, 1 point) was counted when all four digits were placed correctly in front of the rung. Misplacement of one or more digits or of the palm of the paw was counted as partial success (score 2 resp. 3, 0.5 points), while a complete misplacement and subsequent slip was counted as no success (score 1, 0 points). The success score was expressed as percentage of success (sum of success points) in relation to maximal possible outcome (total amount of steps taken). The success rate and complete misses represents the number of correct paw placements (score of 4) or misplacements (score of 1), respectively, divided by the total amount of steps taken.

Perfusion and tissue procession.
For the tracing studies, animals were deeply anesthetized using pentobarbital (i.p., 0.2 ml, Streuli Pharma AG) and transcardially perfused with Ringer's solution (105 IU/L heparin, Roche and 0.25 % $NaNO_2$) followed by 4 % formaldehyde (FA) in 0.1 M PB (Sigma-Aldrich, pH 7.4) containing 5 % sucrose. The brain and spinal cord were extracted, post-fixed in 4 % FA for 16 h later and transferred to a 30 % sucrose in PBS for 3 days for cryoprotection. The samples were embedded in TissueTek® O.C.T. TM compound at -20 °C. Serial sections were cut coronally at 40 µm on a cryostat and were kept at -20 °C until further processing. Spinal cord samples were stained on-slide using the nickel-enhanced DAB (3,3'-diaminobenzidine) protocol (VectaStain ABC Elite Kit, Vector Laboratories; 1:100 in Tris-buffered saline plus TritonTM X-100) for anatomical assessment of sprouting. For the transcriptomic study, animals were deeply anesthetized using pentobarbital (i.p., 0.2 ml, Streuli Pharma AG) and transcardially perfused with ice cold Ringer's solution (105 IU/L heparin, Roche and 0.25 % $NaNO_2$). All procedures were done rapidly to minimize time between animal sacrifice and tissue collection to preserve RNA quality. Brains and spinal cords were rapidly dissected and intermediate layer of spinal level C5 were extracted by crude trimming. The spinal samples were snap frozen in liquid nitrogen and stored at -80 °C until further processing. Spinal tissue bordering spinal level C5 was embedded in TissueTek® O.C.T. TM compound and analyzed for anatomy to ensure specificity of spinal level in the processed samples. The brains were immersed in 4 % FA overnight and transferred to a 30 % sucrose in PBS for 3 days for cryoprotection. The samples were embedded in TissueTek O.C.T. TM compound at -20 °C. Serial sections were cut coronally at 40 µm on a cryostat and were kept at -20 °C until further processing.

Analysis of lesion completeness.
For the accurate analysis of lesion size, brain cross-sections were bathed on-slide in Cresyl violet solution for 1 min, dehydrated in a series of increasing ethanol concentrations and washed in Xylol before coverslipping with Eukitt. Brain sections at four defined landmarks (1.98 mm, 0.98 mm, -0.22 mm, -1.34 mm, in relation to Bregma) were analyzed for stroke volume and depth of cortical lesion. Transverse spinal sections were cut and immunostained with an antibody against the γ-subunit of protein kinase C (PKC) (PKC-γ). Sections were incubated with rabbit anti-PKC-γ (Santa Cruz Biotechnology, 1:200) overnight, followed by goat anti-rabbit Alexa 488 (Molecular Probes, Invitrogen, 1:200) for 1 h, then coverslipped in VectaShield mounting medium (Vector Laboratories) and visualized under a Zeiss fluorescence microscope. Lesion completeness on the spinal level was analyzed as percentage of signal in the stroked dorsal funiculus in relation to the healthy dorsal funiculus.

Stereological quantification of axonal sprouting.
The spatial distribution of labeled CST fibers within the denervated hemicord was assessed. Cross sections of cervical spinal cord were digitalized (Zeiss Axio Scan.Z1, x 100, adapted aperture - three sections/spinal level/animal). Using the moving least squares deformation in FIJI (ImageJ) (Schaefer et al., 2006) with five defined landmarks within the spinal cord, all sections were fitted into a template of the grey matter. This alignment allowed for correction of potential tissue shrinkage as well as stretching due to tissue processing. An intermediate step of manual drawing of the fibers using Photoshop

(Photoshop CS6) by an experienced observer blind to the treatment schedule of each animal was necessary to decrease noise levels of the staining background. A defined grid (20x20 panels) was aligned to the grey matter-white matter boundaries of the ventral horn on one side and the midline on the other. The fiber density was analyzed as grey-scale densitometry along each vertical and horizontal grid line using FIJI (ImageJ). Further analysis with a custom-made automated algorithm in MATLAB (R2015b) determined extended minima of the grey values, where each intersection of a fiber with a grid line resulted in a local minimum and was counted as a fiber. Fiber counts were normalized with an animal-specific normalization factor calculated as the mean grey value of the labeled dorsal CST area of all analyzed sections of the animal divided by the mean grey value of the CST area of all animals in the experiment. False color coded heat-maps for all groups were created with each panel representing the fiber counts at the medial and ventral grid point (Schmitz and Hof, 2005). Using this analysis, midline crossing CST fibers were automatically counted approx. each 100 µm and summed up to approx. 200 µm bins. Difference maps were created as stroked group maps minus sham group map. For a more detailed analysis, cross sections of cervical spinal cord were digitalized (Zeiss Axio Scan.Z1, x 100, Z-Stack over 20 µm with 10 slices, adapted aperture - three sections/animal) and CST fibers crossing the border between dorsal funiculus to grey matter, midline crossing fibers and crossing fibers at 4 further virtual lines spaced regularly between the midline and the outer border of the ventral horn were counted at cervical levels C5/C6. Counts of fibers were summed according to lamina predefined in the spinal cord atlas (Watson et al., 2009).

RNA purification and RNA-Sequencing.
Total RNA was prepared from all samples simultaneously by using the NucleoSpin® RNA XS Kit (Macherey-Nagel,Germany) under the protocols of the manufacturer with an additional TRI-Zol/chloroform extraction step (Thermo Fisher Scientific, USA) due to the high lipid content of the spinal cord. The quality of the isolated RNA was determined with a Qubit® 1.0 Fluorometer (Life Technologies, California, USA) and a Bioanalyzer 2100 (Agilent, Waldbronn, Germany). Only samples with a 260 nm/280 nm ratio between 1.8-2.1 and a 28 S/18 S ratio within 1.5-2 were further processed. The TruSeq RNA Sample Prep Kit v2 (Illumina, Inc, California, USA) was used in the succeeding steps. Briefly, total RNA samples (100 ng) were ribo-depleted using Ribo Zero Gold (Epicentre®, USA) and then fragmented. The fragmented samples were reversed transcribed to cDNA, end-repaired and polyadenylated before ligation of TruSeq adapters containing the index for multiplexing Fragments containing TruSeq adapters on both ends were selectively enriched with PCR. The quality and quantity of the enriched libraries were validated using Qubit® 1.0 Fluorometer and the Caliper GX LabChip® GX (Caliper Life Sciences, Inc., USA). The product is a smear with an average fragment size of approximately 260 bp. The libraries were normalized to 10 nM in Tris-Cl 10 mM, pH 8.5 with 0.1 % Tween 20. The TruSeq SR Cluster Kit v4-cBot-HS (Illumina, Inc, California, USA) was used for cluster generation using 10 pM of pooled normalized libraries on the cBOT. Sequencing were performed on the Illumina HiSeq 4000 single end 125 bp using the TruSeq SBS Kit v4-HS (Illumina, Inc, California, USA).

Sequencing data analysis.
All statistical analysis was performed using the R environment for statistical computing (R version 3.4.1 (2017-06-30)) with Bioconductor (Huber et al., 2015) and dedicated packages. Mapping of reads to the mouse reference genome (mm10) was performed using SALMON (v0.8.1), followed by gene quantification with Tximport (v1.0.3) using standard settings (Soneson et al., 2015). Expression level estimation was reported as counts per million mapped fragments (CPM) together with confidence intervals for each sample, while genes with an expression level of ≤ 5 were excluded to avoid technical artifacts. As a result, 16995 transcripts were included in the analysis. Raw gene counts were normalized using *edgeR* (Robinson et al., 2010) and differential expression was expressed as the average CPM of one group divided by the average CPM of the sham operated

animals, significantly differentially expressed gene (SDEG) were defined by a false discovery rate (FDR) < 0.05 and a log-fold change > 1.5. Hierarchical clustering was conducted in R (*pheatmap*, euclidean distance) using the identified SDEGs. Competitive gene set analysis was conducted by NEXUS Personalized Health Technologies (ETH Zurich, Switzerland) using gene set libraries GO, Reactome and Motiv of the MSigDB gene set collection C2 (Subramanian et al., 2005) and further analyzed using *limma* functions (Ritchie et al., 2015) with a FDR < 0.05. Gene set network clustering was performed as described (Prummer et al., unpublished, manuscript in preparation), disregarding overly large sets with more than 200 genes. Briefly, for each comparison, a network of gene sets as nodes was constructed in which a pair of nodes is connected by an edge if the proportion of shared genes between them (Jaccard index) exceeds 0.2. Such a candidate network was then pruned by removing isolated singletons and doublets and by splitting larger networks into smaller clusters ("communities") based on the edge betweenness property. Automated labeling of these clusters is achieved by splitting gene set names into individual words, excluding a predefined list of trivial words, and using the four most frequent of the remaining terms as labels. These clusters with the according labels were used on for an investigator supervised interrogation method to describe main events for each network analysis.

Spinal cord extracts.
Rats were decapitated and the spinal cords were dissected, followed by immediate homogenization in ice-cold extraction buffer (3.7 % CHAPS and 5 mM EDTA in PBS) containing 1x HALT TM protease and phosphatase inhibitor (Thermo Fisher Scientific). The homogenates were incubated for 30 min on ice, and centrifuged 4 times for 15 min at 13'000 g at 4°C. The supernatants were aliquoted, snap-frozen in liquid nitrogen, and stored at -80°C until further usage.

Neurite outgrowth assay.
N1E-115 cells were obtained from ATCC and maintained at 37 °C in a humidified atmosphere with 5 % CO_2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 2 % L-Glutamine and 1 % Penicillin-Streptamycin. Neuron like differentiation was induced by switching the medium to Neurobasal supplemented with 2 % L-Glutamine and 1 % PenStrep. Cells were plated in differentiation medium at a density of 10.000 cells/cm^2. After 24 h the cells were treated with spinal cord extract and molecular factors and left to grow for another 24 h. The assays were stopped by the addition of 4 % FA at room temperature for 15 min. The cells were then counterstained with Coomassie solution (0.25 % Coomassie Brilliant Blue R250, 50 % MeOH, 10 % HoAC) for five minutes, followed by two consecutive washes with PBS and stored at 4 °C. Randomized images were acquired on a ScanR HCS microscope (Olympus) equipped with an UPLSAPO 10x objective. Mean neurite outgrowth was quantified in ImageJ by applying a grid to the pictures and counting intersections of neurites with the grid lines and total cell bodies and calculating the ratio thereof (Ronn et al., 2000). Each experiment was conducted in three technical replicates. To test the efficacy of the spinal cord extract, serial dilutions of rat Nogo-A-Δ20 and spinal cord extract were transferred onto a 0.45 µm PVDF membrane. Subsequently, membranes were blocked for 30 min with 5 % milk powder (Migros, Switzerland) in TBS-T (10 mM Tris, 150 mM NaCl, 0.01 % Tween-20, pH 7.5) and probed 1 h with 11C7 1:40.000 at RT. The membranes were washed 3 times in TBS-T, probed with secondary HRP-coupled antibody for 1 h at RT and washed again 3 times in TBS-T. Detection was performed using SuperSignal TM West PICO (Thermo Scientific, USA) or WesternBright TM Sirius (Advansta, USA) chemi-luminescent substrates and images were acquired on the Gel Doc TM imager (Biorad, USA). Densitometry analysis was performed with ImageJ.

In situ hybridization.
Spinal tissue was collected as described above for RNA-Sequencing, but was directly snap-frozen in TissueTek® O.C.T. TM compound for sectioning and stored at -80 °C until further processing. Sections of 16 µm thickness were cut on a cryostat at -20 °C and stored again at -80 °C. Sections were further processed using either the

RNAScope assay (Advanced Cell Diagnostics, RNAscope® 2.5 HD Assay-RED) or the viewRNA assay (Thermofisher Scientific, QVT 1-Plex Assay Kit) according to the manufacturer's protocol. Briefly, in both kits, slices were fixed for 20 min before being hybridized to the probe (RNAScope: TGF-β1 - catalogue #407751; viewRNA: Sema6a - catalogue #VB6-3116963-VCP, Ntng2 - catalogue #VB1-3046339-VT), which were further amplified using branched DNA amplification methods. The probe was detected using a Fast Red detection kit, which results in both a chromogenic as well as a fluorescent signal. Slices were counterstained with DAPI and digitalized (Zeiss Axio Scan.Z1, x 200). Quantification of fluorescent signal was done in two ways. First, densitometric analysis as the mean grey value of four defined regions (corticospinal tract in the dorsal funiculus, ventral horn, dorsal horn and intermediate layers) of the denervated hemicord was performed after applying a gene-specific threshold which was defined for 5 images and averaged. Additionally, for a more thorough analysis, particle analysis (ImageJ) was performed to obtain the coordinates of the single mRNA molecules, which were then transformed using the Moving Least Squares plugin (Schaefer et al., 2006) in MATLAB (R2015b) to fit into a custom-made template of the cervical spinal cord. This again allowed for correction of potential tissue shrinkage as well as stretching due to tissue processing. As particles would sometimes cluster, resulting in an under-representation of the mRNA count, number of particles were multiplied by the area size and divided by the average area of one single particle. A false color coded heat map was created, allowing to visualize the difference between stroked groups to control groups (fold change) over the whole spinal level.

Statistics.
Statistical analysis was performed with Prism 7.0 (GraphPad Software Inc.) and R. For statistical tests within groups over time, one-way ANOVA with repeated measured followed by Dunnett correction for multiple comparison tests or linear mixed models were used. The Student's t-test was used to test for differences between 2 groups (unpaired), or 2 time points within one group (paired). To detect differences between groups and within groups over time, and for comparison of more than two groups over time, two-way ANOVA with repeated measures followed by Bonferroni correction for multiple comparisons was used. The threshold for significance for all experiments was set at *P = 0.05. Smaller P-values were represented as **P = 0.01 and ***P = 0.001. Other symbols might be used to indicate two comparisons in one graph. In bar graphs, all data are plotted as mean ± standard error of the mean (SEM), while data were normalized to solvent controls in the neurite outgrowth assays. In box plot graphs, data are represented as median ± 25th percentile (box) and min/max (whiskers). Dots represent individual animals or technical replicates of experiments.

3.3 | Results

3.3.1 | Histological Assessment of Lesion Size

We assessed the sprouting of contralesional CSMNs at time points representing key time windows of structural plasticity, e.g. branching, pathfinding, target interaction and synapse refinement (7 dpi, 14 dpi, 28 dpi and 42 dpi). Photothrombotic strokes with intraperitoneal

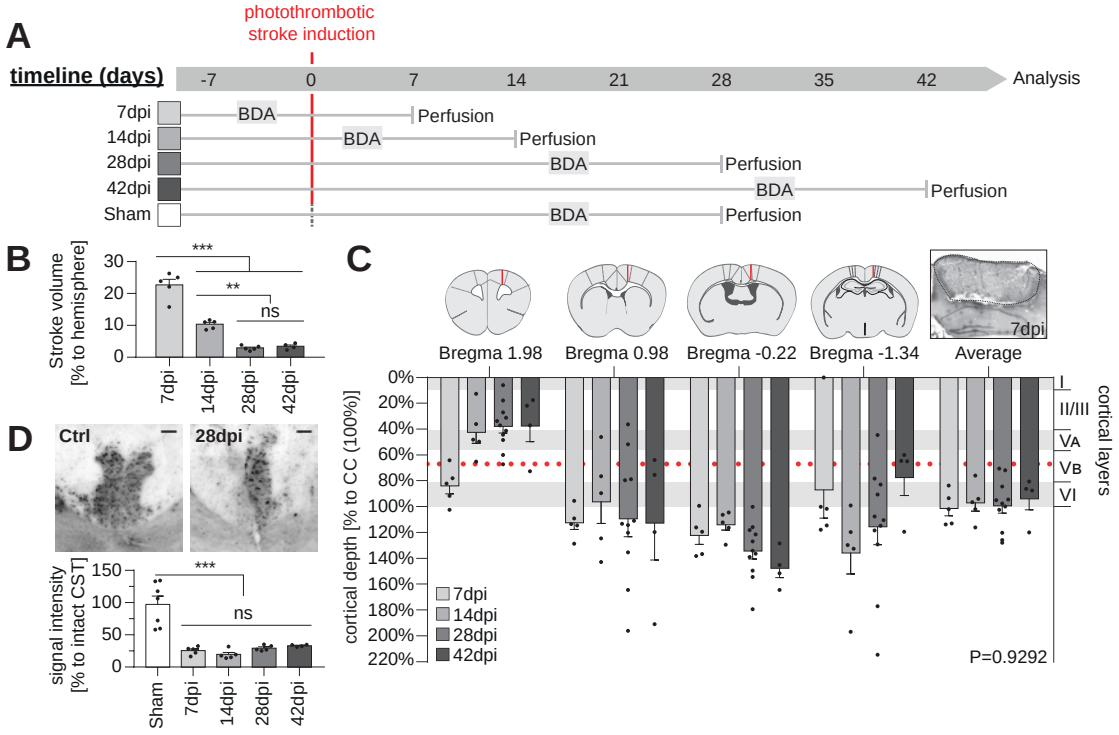


Figure 3.1 – Lesion size and depth after unilateral cortical stroke. **(A)** Experimental timeline of the study. Adult C57BL/6 mice (n = 30) received a large unilateral cortical stroke. Anterograde tracing of the CST was performed 10 days prior perfusion and sprouting was analyzed at 7 dpi, 14 dpi, 28 dpi and 42 dpi. Control animals received a sham operation. **(B)** Analysis of stroke lesion volume showed a reduction in volume over time. **(C)** Stroke depth at four selected distances in relation to Bregma showed no significant differences among the experimental groups with strokes reaching into deep cortical layers to the corpus callosum (CC). Location of CSMNs in layer V is indicated by a red dotted line. Representative coronal section of a brain at 7 days after stroke shows the extent of the lesion within the cortex. **(D)** Immunoreactivity for the CST marker PKC-γ in the cervical spinal cord. Representative pictures show the intact CST on both sides of a sham animal and an absence of signal on the left (lesioned) side in a stroke animal (scale bar = 50 µm). Quantification of signal strength in relation to the intact CST shows no difference between groups.

injections of Rose Bengal and skull illumination over the sensorimotor cortex unilaterally produced large consistent lesions in the right sensorimotor cortex of all mice. The lesion volume decreased over time (Figure 3.1B), reflecting wound contraction and shrinkage of the stroke scar tissue. The lesion reached into deep cortical layers (Figure 3.1C), with no significant differences between groups. On the level of the cervical spinal cord, a crucial target of the CST, PKC-γ immunoreactivity as a marker of the CST showed a large decrease unilaterally (contralateral to the stroke side), demonstrating a successful ablation of the sensorimotor cortex. No difference between experimental groups was seen (Figure 3.1D).

3.3.2 | The Contralesional Corticospinal Tract Re-innervates the Denervated Hemicord after Stroke

In mice, a significant degree of spontaneous re-innervation of the stroke-denervated cervical spinal cord by sprouting of contralesional corticospinal fibers can be detected 28 days after lesion (Bachmann et al., 2014, Ueno et al., 2012). To assess the spatial distribution of the contralesional corticospinal input to the stroke-denervated hemicord grey matter after 28 dpi, the anterograde tracer BDA was injected into the intact, contralesional forelimb motor cortex of stroked and sham-operated animals (Figure 3.2A). The fiber density of contralesional CSMNs increased on all cervical spinal levels within the denervated hemicord in injured compared to sham-operated animals (Figure 3.2B, C) with a marked increase in fiber counts especially on cervical levels C3 and C6 (Figure 3.2C, D). Interestingly, new projections targeted primarily laminae IV-V and VI-VII on C3 and C6, respectively (Figure 3.2E, F). This finding confirms previous observations that contralesional corticospinal input is mainly increased on cervical level C6 within intermediate laminae in chronically recovered animals after stroke (Bachmann et al., 2014, Lindau et al., 2014, Ueno et al., 2012, Wahl et al., 2014).

Increased innervation by the contralesional CST might be established by increasing terminal arbors of the pre-existing crossed projections that can be found in mice (Figure 3.2B, left) or by formation of new projections from the intact side that grow across the midline (Lindau et al., 2014, Weidner et al., 2001). To investigate the origin of midline crossing fibers within the intact hemicord, fibers were counted on spinal levels C5/C6 at the border between the grey matter and the CST in the dorsal funiculus, at the spinal cord midline, as well as on four vertical lines along the medio-lateral axis (Figure 3.2G). An increased number of fibers was found to branch off the CST into the grey matter as well as at the midline, with a concomitant increase in fiber counts at a defined region within the grey matter of the stroke-denervated side (Figure 3.2H, line D2). These results suggest an increase in new projections arising in the CST of the intact hemicord with extensive branching in the appropriate layers of the denervated hemicord. This confirms previous findings that the contralesional CST sends projections over the midline to re-innervate the denervated hemicord (Omoto et al., 2011, Ueno et al., 2012). Furthermore, these data suggest that CSMNs could change their segmental termination field to re-innervate target areas of another spinal segment, while it remains an open question whether this contributes to functional recovery.

To study the time course of increased contralesional CST input into the denervated hemicord, the anterograde tracer BDA was injected as before and CST fibers in the cervical

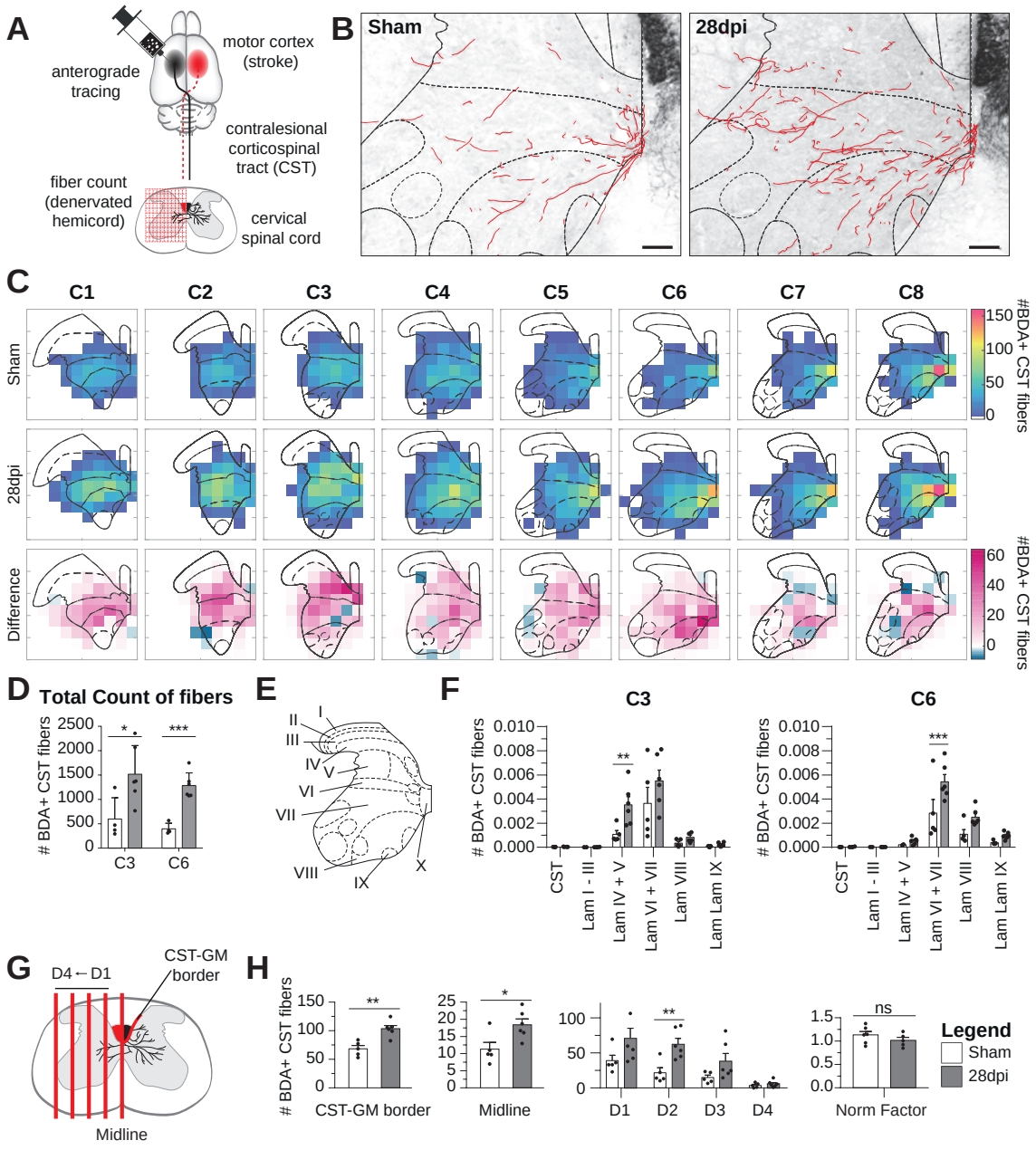


Figure 3.2 – Contralesional corticospinal fibers cross the midline on all cervical levels 28 days after large unilateral stroke. **(A)** Schematic illustration of contralesional CST rewiring into the denervated hemicord after stroke as seen by anterograde tracing. Large cortical stroke of the sensorimotor cortex leads to disruption of CST (red dotted line), while sprouting of the contralesional cortex can be observed on spinal levels (black) **(B)** Transverse sections of the cervical spinal cord with highlighted CST fibers show increased fiber density originated in BDA-labeled contralesional CST 28 days after stroke compared to sham animal (scale bar = 100 µm). **(C)** Average false color coded heat maps of BDA-labeled fiber counts on cervical levels C1-C8 of sham animals (upper panel row, n= 5) and stroked animals at 28 dpi (medium panel row, n= 6). Difference map (lower panel row) shows an increase of labeled fibers compared to sham animals (red) or a decrease respectively (blue) (average of stroke - average of sham). **(D)** Total fiber density over the whole grey matter further showed a significant increase in projections of the contralesional CST into the denervated hemicord at cervical levels C3 and C6. *Figure legend continues.*

Figure 3.2 – *(← Figure legend continued.)* **(E)** Schematic representation of the Rexed laminae, upon which laminar analysis in (F) was based. **(F)** Laminar analysis on two spinal levels, C3 and C6, shows increased fiber density especially in laminae IV+V and VI+VII, respectively. **(G)** Fiber density in (H) was determined by stereological intersection counting using the lines D1-D4 for the grey matter, as well as the spinal cord midline and the CST-grey matter border. **(H)** Detailed analysis of crossing fibers on spinal level C5/C6 at the border of the CST to the grey matter within the intact hemicord, at the spinal cord midline as well as on four regularly spaced lines along the medio-lateral axis of the denervated hemicord. Normalization factor shows no difference among groups.

spinal cord were analyzed at 7 dpi, 14 dpi, 28 dpi, 42 dpi (Figure 3.1A). A gradual increase in fiber counts over time compared to sham-operated controls was observed on cervical level C5/C6, with a peak of fiber density at 28 dpi (Figure 3.3A), in line with previous observations (Ueno et al., 2012). Laminar analysis showed a similar trend with increasing projections of the contralesional CST within laminae IV+V and VI+VII, but due to low sample numbers, resulting in large variations, these results did not reach statistical significance (Figure 3.3B). After six weeks (42 dpi), contralesional midline crossing fiber levels reached a plateau, but as opposed to previous studies we did not find a decrease of sprouting fibers at chronic stages (Ueno et al., 2012). These divergent findings could potentially reflect differences in the lesion models used in the studies.

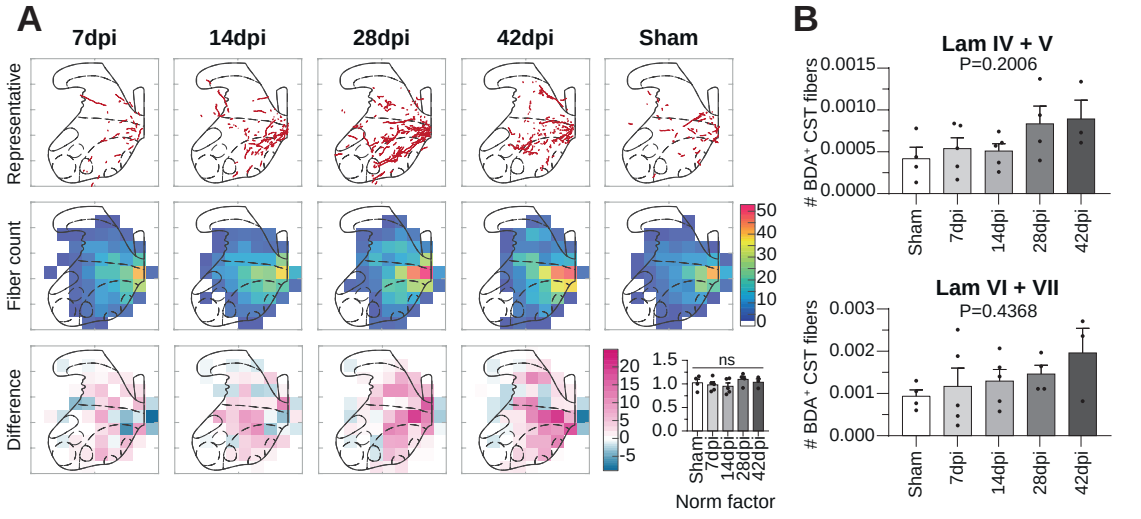


Figure 3.3 – Contralesional corticospinal input into the denervated side is increased over the course of four weeks. **(A)** Average false color coded heat maps of BDA-labeled fiber counts on cervical level C5/C6 after 7 dpi (n = 4), 14 dpi (n = 4), 28 dpi (n = 5), 42 dpi (n = 3) and after sham operation (n= 4). Upper panel row: representative drawings of fiber innervation. Medium panel row: Average false color coded heat map of BDA-labeled fiber counts reveal a gradual increase in re-innervation of the denervated hemicord by the contralesional CSMNs. Lower panel row: Difference map shows a gradual increase of BDA-labeled fibers compared to sham animals. Increase of fiber count is indicated by red colors, decreased counts by blue colors. Normalization factor shows no difference among groups. **(B)** Laminar analysis shows a trend in increased fiber density across groups.

3.3.3 | The Affected Spinal Hemicord Grey Matter Shows Differential Gene Expression after Stroke

The spinal target area of the midline crossing CSTs fibers was analyzed to identify the transcriptional profile of the tissue encountered by the sprouting fibers at selected, critical time points (Figure 3.4A). Control samples were collected right after sham operation. Cortical stroke depth was measured for all animals and revealed no significant differences between groups; almost all animals showed lesions reaching into deep cortical layer (Figure 3.4B). To assess impact of lesion severity on functional performance, functional impairment was measured using the horizontal ladder test. Four days after injury, all stroked animals showed a strong deficit in limb placement and ladder rung targeting as detected by a reduction in success score and an increase in the number of forelimb foot faults (compared to baseline levels). Thereafter, the performance improved gradually over the course of the next weeks and reached a plateau after 28 dpi, showing that animals are well recovered after four weeks (Figure 3.4C). Based on these observations, the time points 4 dpi, 7 dpi, 14 dpi, 28 dpi and 42 dpi were selected for a transcriptomic analysis of the microdissected lamina IV-VII of the stroke-denervated grey matter of the C5/C6 cervical spinal cord (n = 8/group). Quality of the extracted RNA and correct anatomical dissection (Figure S3.1) allowed for a selection of five samples for each time points for RNA-Sequencing on the Illumina 4000 HiSeq platform.

Genes with significantly different expression (SDEGs) after stroke were identified. Alignment of reads to the mouse transcriptome showed a successful mapping to the genome ($\sim$ 70 %, Table S3.1) comparable to previous studies on spinal tissue (Shi et al., 2017). Using a false discovery rate (FDR) of < 0.05 and a minimum log_2-fold change of 1.5, a total of 955 SDEGs were identified with the most pronounced differential regulation (891 SDEGs) seen at 28 dpi (Figure 3.5A). This late dynamic phase is in line with recent findings of growth-related transcriptome changes in sprouting CSMNs at 28 days after pyramidotomy (Fink et al., 2017). Interestingly, only few SDEGs were shared between time points as seen in the UpSet plot, a novel visualization technique for overlapping genes replacing the Venn diagram (Lex et al., 2014). This suggests that transcriptomic profiles are highly specific for each time point, probably reflecting different biological processes of growth induction, fiber elongation and network formation (Figure 3.5B).

Two independent approaches were used for prioritization of the observed gene expression changes: Hierarchical clustering of the 955 SDEGs followed by gene ontology (GO) term analysis allowed for an identification of genes with a similar temporal expression change and biological function. A time-point specific analysis was opted for by gene set enrichment analysis (GSEA) of each group (4 dpi, 7 dpi, 14 dpi, 28 dpi and 42 dpi), followed

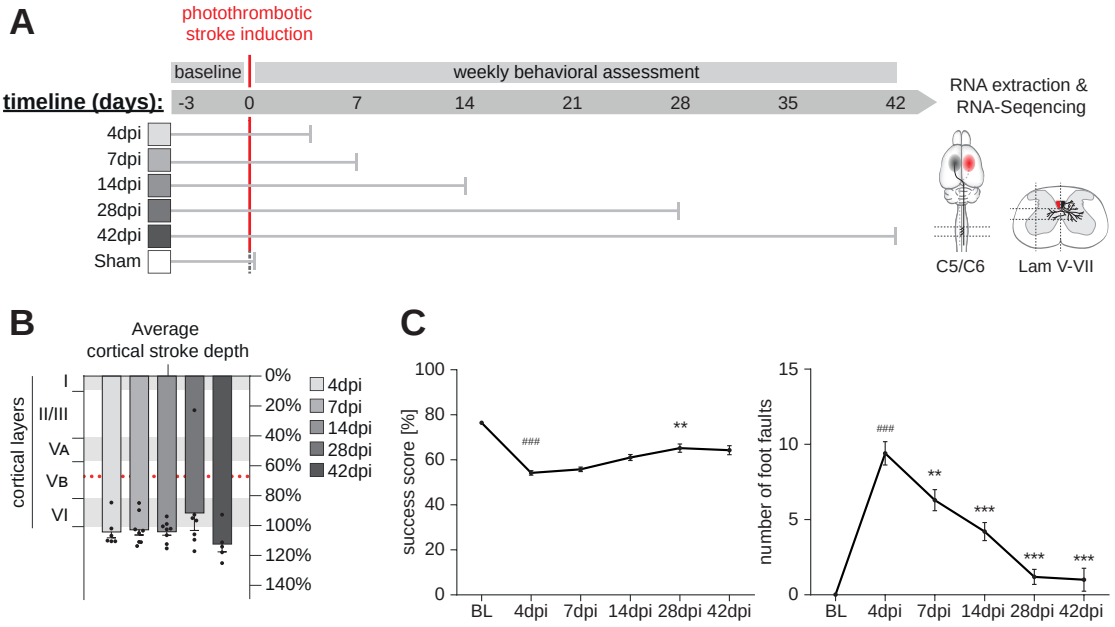


Figure 3.4 – Large unilateral cortical strokes lead to motor function impairments that recover over time. (**A**) Experimental time line of the study. Animals received a large unilateral cortical stroke and tissue samples were collected at 4 dpi, 7 dpi, 14 dpi, 28 dpi and 42 dpi (n= 8/group). Control animals received a sham operation. (**B**) Analysis of stroke core depth showed no significant difference among groups and successfully ablated the CST unilaterally. Location of targeted CSMNs in layer V is represented by a red dotted line. n = 1 animal was excluded from further analysis. (**C**) Forelimb motor impairment was seen in the horizontal ladder task at 4 dpi with a subsequent recovery up to 42 dpi in two measured parameters, success score and number of forelimb foot faults. Data are represented as mean ± SEM **P = 0.001, *P = 0.01 compared to 4 days after the injury, ## P = 0.001 compared to baseline levels (linear mixed effect estimation followed by Dunnett adjustment).

by network enrichment analysis (NEA), which allows for a more detailed description for differentially regulated biological functions at specific time points after stroke.

Unbiased hierarchical clustering of all SDEGs identified four expression patterns (Figure 3.6A, B). Additional GO term analysis for all genes of each cluster was performed using investigator supervised interrogation methods. Cluster 1 included a mere 19 SDEGs that were down-regulated upon stroke induction and stayed at lower levels persistently (Table 3.1). Cluster 2 comprised 644 SDEGs that were upregulated at late time points and included genes involved in developmental processes such as immune related pathways (Cxcl15, Cr2, CD27, Oas1B, IL20RB), anatomical structure development (Sema6A, Notch3, Syngap1, TGFβR3, Itga3, LIF, GDF7, PlxnA1) and morphogenesis of a branching structure (TGF-β1, Sox9, Pax2, GDF7, PlxnA1). On the other hand, 253 SDEGs were down-regulated at late time points (cluster 3) and were associated with metabolic processes such as the potentially neurotoxic oxidative phosphorylation pathway (Tseng et al., 2012) or generation of precursor metabolites (Banf1, NdufBs, ATP5s, Psmb8) and cell proliferation (Rgcc, Rps6, Rpl24, Ran). Finally, cluster 4 included 39 genes that were upregulated tran-

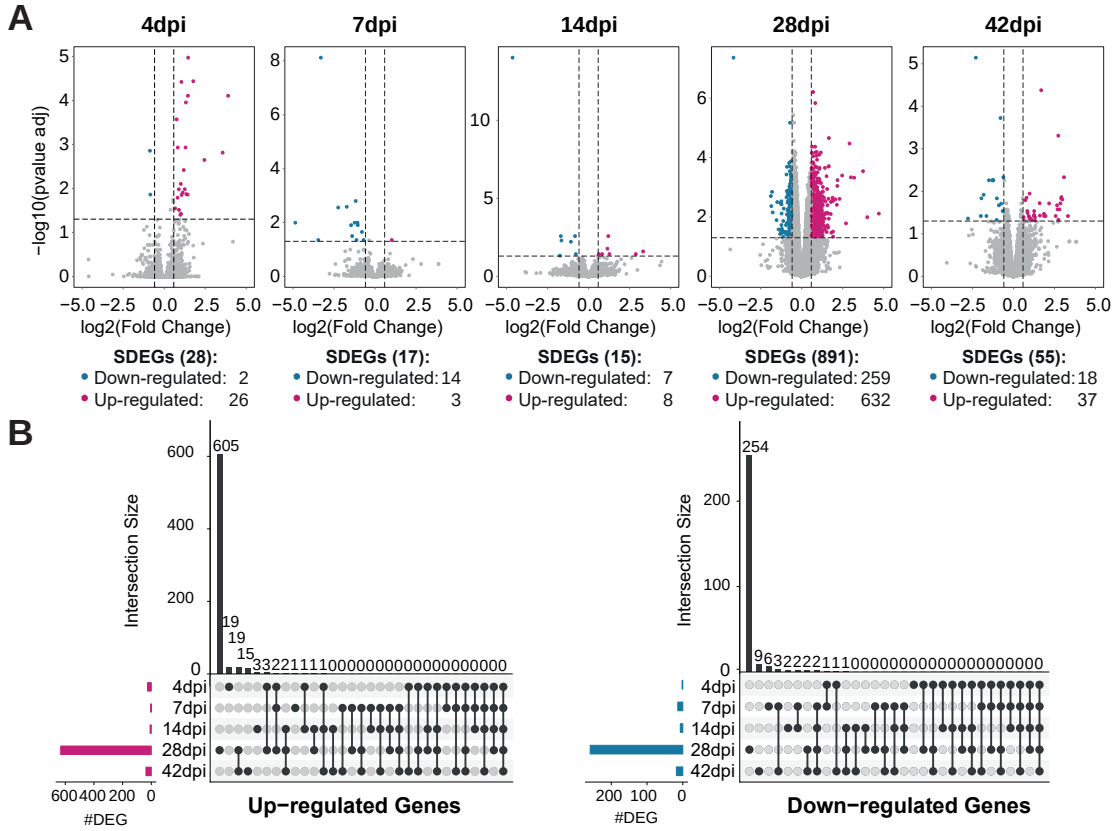


Figure 3.5 – Comparison of gene expression between stroke-denervated and sham spinal cords at 4, 7, 14, 28 and 42 days after injury reveals injury related changes in lamina IV-VII on spinal levels C5/C6. **(A)** Volcano plots display the log_2 fold change versus $-log_{10}$ of the adjusted p value for each differentially expressed gene after comparison of the respective time point to the sham group. Down-regulated SDEGs are indicated in blue, upregulated SDEGs in red. **(B)** UpSet Plots for upregulated genes (left) and down-regulated genes (right) indicate that there is little overlap of SDEGs between groups. The vertical bar plot shows number of genes differentially regulated for the time point(s) indicated by dots in the combination matrix in the lower part of the figure. Each column of the combination matrix represents an exclusive intersection set of time points that share differentially regulated genes. The horizontal bar plot depicts total number of genes of the respective group.

siently at early time points and down-regulated to baseline levels thereafter. These genes were primarily associated with inflammatory pathways (C1qa - C1qc, C4b, CSF1R, Fcgr2, Trem2).

To gain further insight into the biological function of the SDEGs at each selected time point, gene set enrichment analysis (GSEA) was used to identify differentially regulated pathways of the REACTOME, MOTIV and GO databases. This allowed for identification of gene classes that are over-represented in our data set and have an association to the established pathway databases of various pre-defined disease phenotypes (Figure 3.7F). This GSEA was further analyzed using NEA, a powerful method to integrate the detected gene sets with the information on the relationship between shared genes. These methods allowed

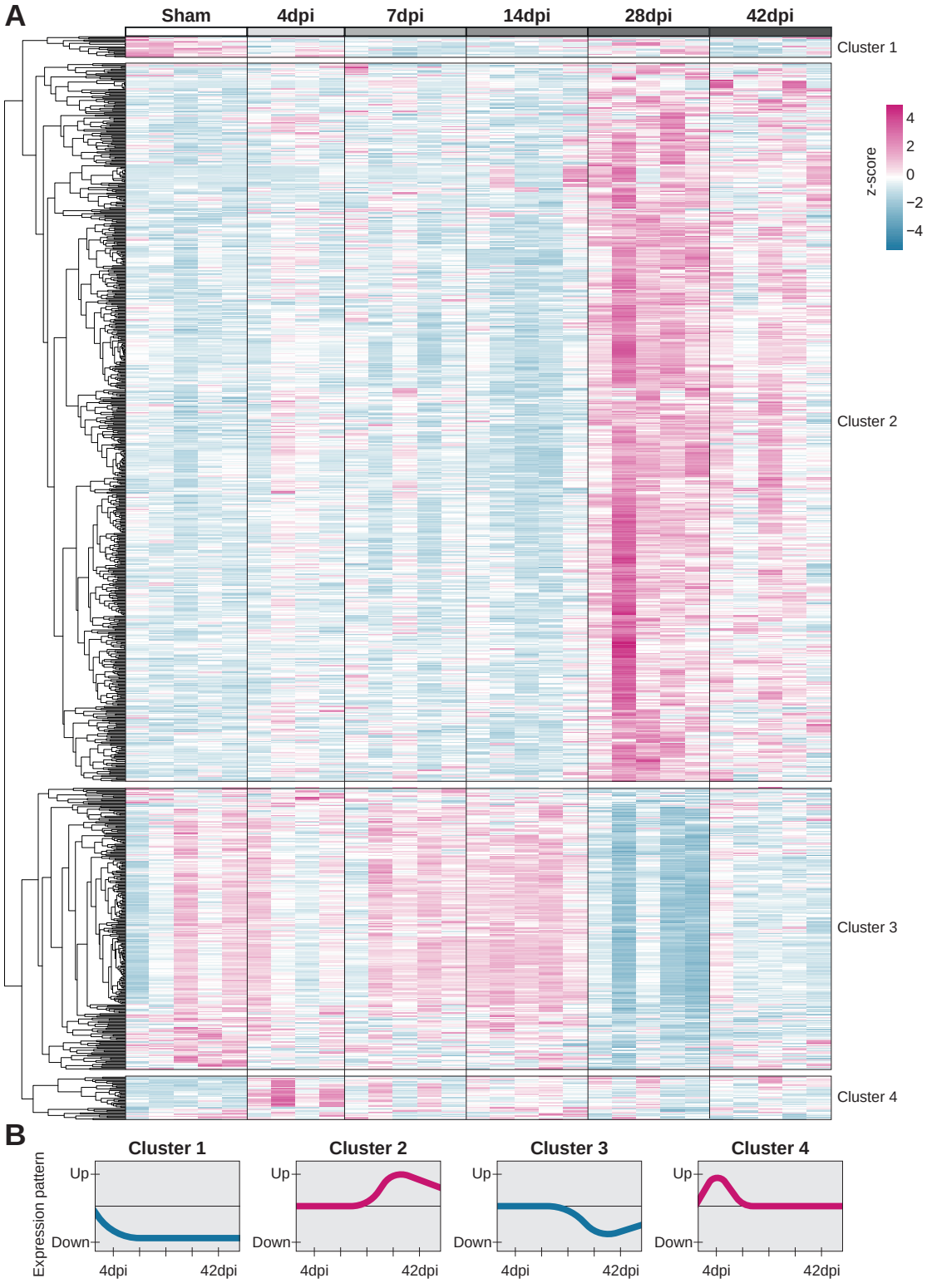


Figure 3.6 – Heatmap of genes differentially expressed between post-stroke and sham animals. **(A)** Scaled expression values of all 955 differentially expressed genes are shown for each group (Sham, 4 dpi, 7 dpi, 14 dpi, 28 dpi and 42 dpi, FDR < 0.05) with red values indicating an upregulation of the gene while blue values indicate a down-regulation. Columns represent single samples (n = 5 per condition), rows represent single genes. Ontologic hierarchical clustering among rows identified four clusters employing correlation distance measure. **(B)** Clusters show four patterns of gene expression after stroke: persistently down-regulated genes (cluster 1), late upregulated genes (cluster 2), late down-regulated genes (cluster 3) and early upregulated genes (cluster 4).

Table 3.1 – Top 10 significantly differentially expressed genes (SDEGs) of each cluster defined by hierarchical clustering (ordered by p-value corresponding to time-point of peak of the down-/upregulation).

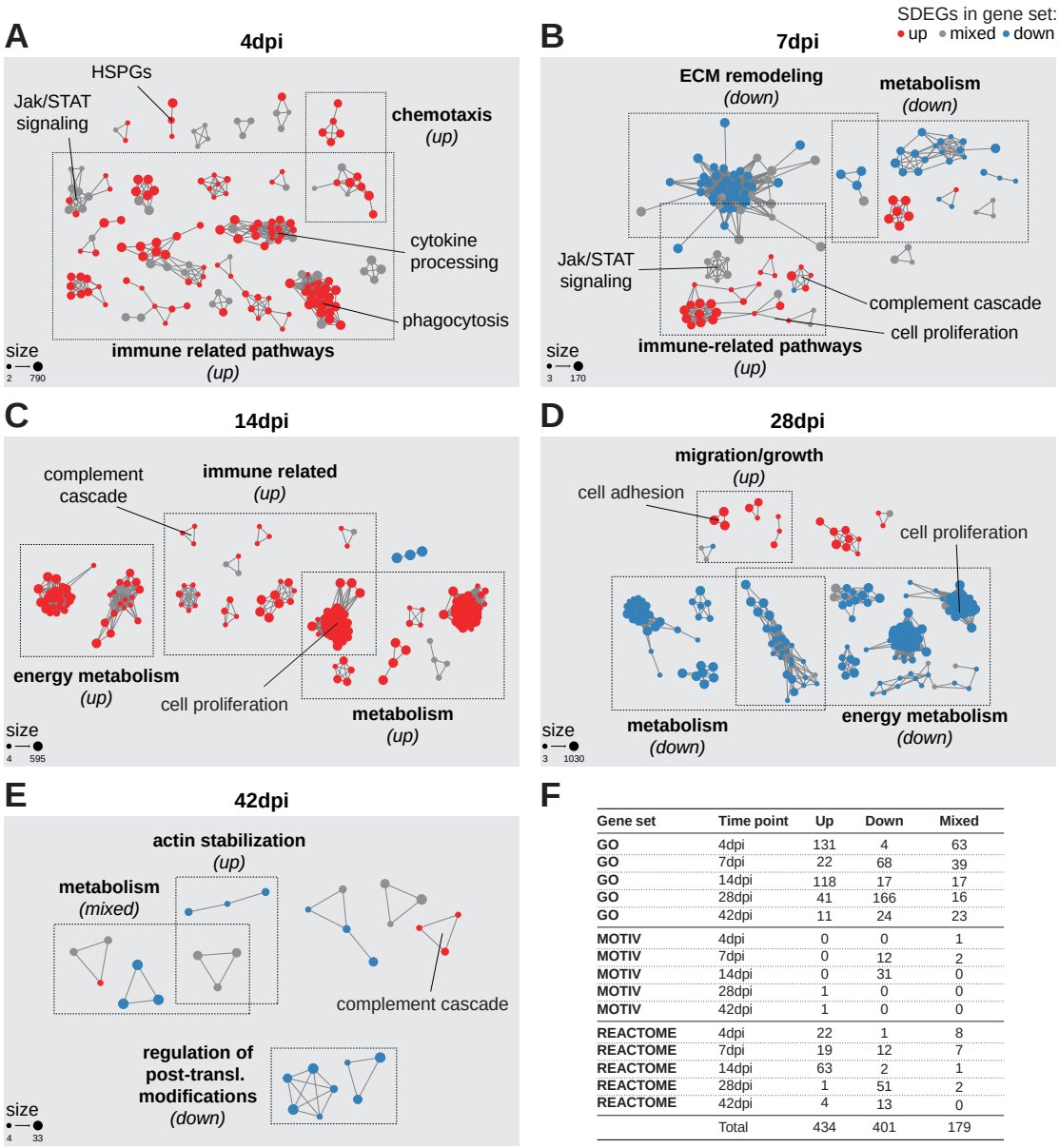
		logFC to Sham group				
Expression pattern	**Gene ID**	**4dpi**	**7dpi**	**14dpi**	**28dpi**	**42dpi**
Cluster 1: persistently down	Gm4294	-8.73	-5.79	-0.76	-6.43	0.45
	Tdrd6	-1.37	-1.23	-1.75	-1.10	-1.65
	Rny3	-1.32	-2.25	-1.42	-0.99	-1.95
	Gm7276	-1.06	-0.32	-0.47	-0.75	-0.69
	Ipcef1	-0.89	-1.06	-0.75	-0.45	-0.74
	Vgf	-0.87	-0.63	-0.51	-0.29	-0.66
	Gm22154	-0.79	-1.13	-1.02	-0.30	-1.49
	Rmrp	-0.57	-1.07	-0.60	-0.28	-0.79
	Gm24245	-0.51	-0.76	-0.89	-0.09	-1.23
	Gm24270	-0.51	-0.76	-0.89	-0.09	-1.23
Cluster 2: late up	Prl	6.29	7.31	5.36	5.83	3.81
	Gh	5.69	5.93	4.36	4.70	3.84
	AY036118	0.50	0.94	3.32	3.74	2.74
	Rn18s-rs5	0.26	0.72	2.88	3.20	2.57
	Gm20594	0.28	0.15	2.19	3.00	2.66
	Mir6240	0.06	0.39	1.61	2.92	2.73
	Gm10722	1.01	2.81	1.17	2.70	3.32
	Lars2	0.05	0.65	1.98	2.46	2.17
	Gdf7	0.38	0.80	1.53	2.31	1.65
	Gm15662	-0.07	0.49	1.74	2.18	1.87
Cluster 3: late down	Cbx3-ps6	-0.22	-1.44	-3.19	-7.15	-1.43
	Tmem265	0.70	0.50	-1.50	-6.29	0.41
	Gm43079	-0.53	-0.10	0.22	-4.16	-0.43
	Gm8206	0.01	0.02	0.44	-1.88	-0.89
	Pkn3	-0.72	-1.40	-0.71	-1.86	-0.86
	Rab40b	-1.17	-1.51	-0.99	-1.81	-1.82
	Gm10062	-0.94	-0.38	-1.03	-1.77	-0.90
	Hist1h2ae	0.15	0.13	-0.20	-1.54	-0.51
	Gm16062	-0.64	-0.42	-0.27	-1.49	-0.38
Cluster 4: transiently up	Itgax	4.19	3.87	4.45	4.00	3.40
	Clec7a	3.90	2.31	2.62	1.91	2.43
	Cd52	2.45	1.49	1.36	0.52	0.74
	Ptpn6	1.46	0.60	0.80	1.18	1.32
	Fcgr3	1.46	0.80	0.68	-0.04	0.32
	Mpeg1	1.45	0.91	0.81	0.70	0.83
	Ifi30	1.43	0.99	0.91	0.36	0.84
	C4b	1.41	0.93	1.13	1.33	1.46
	Ly86	1.32	0.76	0.81	-0.03	0.18
	Fcgr2b	1.30	0.83	1.15	0.56	0.74

for a more detailed description of biological events within the stroke-denervated hemicord at selected time point after stroke (Figure 3.7).

At early time points, 4 dpi and 7 dpi, immune related gene sets, such as the complement cascade, phagocytosis and antigen presentation, governed the enhanced pathways in the network analysis (Figure 3.7A, B). At 7 dpi, the network analysis revealed a down-regulation of metabolic processes, such as mitogen-activated protein kinase (MAPK) pathway activation. These regulations were not seen at the earlier time point, suggesting highly dynamic processes to take place within the early days after stroke. Strikingly, at 7 dpi, extracellular matrix (ECM) remodeling related gene sets were over-represented in comparison to the sham group, while the genes associated with these gene sets were primarily down-regulated in the target area after stroke at this time point. At both early time points, Jak/STAT related gene sets were enriched, highlighting an active environment in terms of transcription.

At 14 dpi, NEA showed an over-representation of positively regulated immune- and metabolism related gene sets, including cell proliferation pathways. Energy-metabolism related gene sets were found enriched and genes within showed an upregulation at 14 days post-stroke. Interestingly, similar over-represented gene sets were found at 28 dpi, however, genes of these networks were down-regulated compared to sham conditions. This was the case for cell proliferation, metabolism- and energy metabolism-related gene sets, suggesting a silencing of the target area in terms of metabolic processes at 28 dpi. On the other hand, an enrichment of gene sets related to growth and migration concomitant with an upregulation of the genes within was observed, suggesting that a subset of the SDEGs at 28 dpi are involved in tissue regeneration in this phase after stroke. At the latest measured time points, 42 dpi, an enrichment of immune- and metabolism related gene sets could be observed. Notably, several actin related gene sets were found that regulate filament stabilization, possibly reflecting synapse and dendritic arbor stabilization (Konietzny et al., 2017). Additionally, we found an upregulation of gene sets related to the complement cascade, which, in light of studies linking the complement cascade to synaptic remodeling (Stevens et al., 2007), might contribute to structural reorganization at 42 dpi.

In conclusion, both the hierarchical clustering as well as the NEA suggest that the transcriptome of the spinal grey matter intermediate and pre-motor layers undergoes stroke-related changes and that at least two distinct phases can be defined: an early phase within the first week after stroke, which is mainly associated with inflammatory responses, and a later phase at four to six weeks after stroke, in which a highly dynamic process of SDEGs shape the tissue environment for re-innervating CSMNs.



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Gene set	Time point	Up	Down	Mixed
GO	4dpi	131	4	63
GO	7dpi	22	68	39
GO	14dpi	118	17	17
GO	28dpi	41	166	16
GO	42dpi	11	24	23
MOTIV	4dpi	0	0	1
MOTIV	7dpi	0	12	2
MOTIV	14dpi	0	31	0
MOTIV	28dpi	1	0	0
MOTIV	42dpi	1	0	0
REACTOME	4dpi	22	1	8
REACTOME	7dpi	19	12	7
REACTOME	14dpi	63	2	1
REACTOME	28dpi	1	51	2
REACTOME	42dpi	4	13	0
	Total	434	401	179

Figure 3.7 – Network enrichment analysis (NEA) at selected time point reveals specific overall changes within the spinal cord grey matter after stroke. **(A-E)** Network visualization of GO/REACTOME/MOTIV term enrichment analysis for 4 dpi **(A)**, 7 dpi **(B)**, 14 dpi **(C)**, 28 dpi **(D)** and 42 dpi **(E)**. The nodes represent gene sets, while the size indicates the genes included in the set in the database of GO/REACTOME/MOTIV. Notably, they do not reflect the number of genes of our data set represented in this gene set. The colors of the nodes reflect the differential regulations of genes of our data set that are represented by the node (see legend upper right corner). Network edges represent mutual overlap between gene sets. **(F)** Gene set enrichment analysis result summary table. Number of gene sets with FDR < 0.05 of mixed, consistently up or down regulated genes.

3.3.4 | The Spinal Grey Matter Represents a Growth-Enhancing Environment at Four Weeks after Stroke

The number of SDEGs was highest at 28 dpi, a time where circuit formation by the CST sprouts can be expected in the cervical spinal grey matter (Fink et al., 2017). We found several genes to be differentially regulated at this stage that have previously been associated with negative regulation of axonal path finding but also with branching. We observed an upregulation of two Slit-ROBO pathway related genes, Plexin A1 (PlxnA1) and Slit-ROBO Rho GTPase Activating Protein 1 (Srgap1) (Delloye-Bourgeois et al., 2015, Nawabi et al., 2010). Upregulation was also observed for SRY-Box 9 (Sox9), which has recently been identified as an astrocytic inhibitor of recovery after stroke and spinal cord injury (SCI) (McKillop et al., 2013, 2016, Sun et al., 2017). In another study, an acute knockdown of Sox9 increased structural plasticity of the contralesional CSMNs and resulted in a better functional outcome (Xu et al., 2018). Furthermore, we found several growth-promoters to be down-regulated in the target area at 28 dpi. These include brain derived neurotrophic factor (BDNF), in line with a previous report showing a down-regulation at chronic stages after stroke (Sist et al., 2014), as well as two metallothioneins (I and II), which have been shown to increase neurite outgrowth *in vitro* (Chung et al., 2013). These findings might reflect a switch from pro-growth molecules towards a stable plateau phase as indicated by the observed peak of sprouting fibers after 28 dpi in this and previous studies (Ueno et al., 2012).

Upregulated SDEGs at 28 dpi also included several genes involved in axonal guidance and sprouting, as seen in the NEA and hierarchical clustering. Integrin subunit alpha 3 (Itga3) as a proposed receptor for Netrin-1 (Nikolopoulos and Giancotti, 2005) might facilitate and enhance cell-contact-dependent axon guidance. Leukemia inhibitory factor (LIF), a cytokine modulating glial and neuronal function during development, has been implicated in axonal regeneration after spinal cord and optic nerve injury (Blesch et al., 1999, Leibinger et al., 2009). Administration of LIF *in vitro* leads to increased neurite outgrowth and promotes elongation but not arborization (Cafferty et al., 2001). *In vivo*, LIF administration leads to increased levels of neurotrophins such as NT-3 (Blesch et al., 1999), potentially caused by activation of glial cells as well as peripheral inflammatory cells after injury (Holmberg and Patterson, 2006, Ostasov et al., 2015). Thus, in addition to the growth-inhibitory effects described above, growth-promoting molecules can be found within the target area of sprouting CSMNs at 28 days after stroke.

Based on several criteria including the ones outlined above, four candidates were chosen for further analysis of their growth-regulating potential in *in vitro* neurite outgrowth assays:

Semaphorin 6A (Sema6a), Netrin G2 (Ntng2) as well as two members of the transforming growth factor (TGF) superfamily, growth differentiation factor (GDF) 7 and TGF-β 1. Additionally, the tissue localization of these candidates was assessed using *in situ* hybridization.

3.3.5 | The Candidate Genes Semaphorin 6A and Netrin-G2 Rescue Neurite Outgrowth *in Vitro*

In our screen, Sema6a was found to be upregulated 1.5-fold 28 days after stroke, comparable to previous studies of the peri-infarct or contralesional cortex (Buga et al., 2012, Krüger et al., 2006). Sema6a, a member of the axon guidance molecule family of Semaphorins, has been shown to inhibit axonal growth and migration of neurons during development (Kerjan et al., 2005, Mauti et al., 2007, Xu et al., 2000). This negative regulation of axonal growth has been mainly attributed to interactions with the receptors PlexinA4 and PlexinA2 (Haklai-Topper et al., 2010, Shim et al., 2012, Suto et al., 2005), however, binding to other Plexin receptors is possible and might lead to differential signaling (Janssen et al., 2013, Mauti et al., 2007). Sema6a *null* mice show pronounced deficits in the CST formation during development (Faulkner et al., 2008, Rünker et al., 2008), with axons being misguided at several choice points along the neuraxis, resulting in hypoplasia of the CST. While previous reports have demonstrated an inhibitory effect of Sema6a on neurite outgrowth of dorsal root ganglion (DRG) neurons *in vitro* (Shim et al., 2012), a growth-promoting effect on PC12 cells was recently found that was just as effective as that of neurotrophins (Tian et al., 2017). Cortical upregulation of Sema6a has been linked to functional recovery in combination with rehabilitative training *in vivo* (Rogalewski et al., 2010). These findings make Sema6a an interesting candidate as a modulator — inhibitory or growth-enhancing — for stroke-induced structural plasticity.

To assess a potential growth promoting role of Sema6a in an inhibitory environment, we used a well-defined brain derived neuronal cell line (N1E-115). After induction of differentiation, N1E-115 cells extend neurite-like processes. Addition of spinal cord extract (SCE) inhibits this process of extension in a dose dependent manner (Figure S3.2). While Sema6a did not have an neurite inducing effect on its own, combined treatment with spinal cord extract (SCE) and Sema6a resulted in a partial rescue of neurite outgrowth (Figure 3.8A, B). *In situ* hybridization showed ubiquitous increase in expression of Sema6a throughout the entire grey matter of the denervated hemicord, with slightly lower levels within the white matter (CST, Figure 3.8E-G). This might hint towards a growth-promoting role of Sema6a

for sprouting neurons in the denervated spinal cord. Interestingly, we found an enrichment of Sema6a especially in the ventral horn and in motoneurons (MNs) (Figure 3.8F). Based on these results, we propose that Sema6a positively regulates the growth of CSMN sprouts into the stroke-denervated spinal target area.

We found Ntng2 to be upregulated 1.8-fold in the spinal target area of sprouting CSMNs in our transcriptomic screen. Ntng2, a vertebrate-specific member of the UNC-6/netrin family, is a membrane-anchored protein that has been identified as a pre-synaptic adhesion molecule, steering the localization of its ligand netrin-G ligand 2 (NGL-2) in a lamina-specific manner in cortical structures (Nishimura-Akiyoshi et al., 2007, Soto et al., 2013). While disruption of Ntng2 signaling was associated with several mental disorders (Aoki-Suzuki et al., 2005, Eastwood and Harrison, 2008, Pan et al., 2010), knockout of this gene did not influence axonal development, as there were no notable differences in outgrowth, trajectory and specificity of axonal terminations (Nishimura-Akiyoshi et al., 2007, Zhang et al., 2008). Nonetheless, a growth-promoting effect for primary cortical neurons on Ntng2 was observed (Nakashiba et al., 2002). Furthermore, Ntng2 has recently been linked to encoding of sensorimotor behavior, as Ntng2 *null* mice show pronounced motor deficits in several behavioral tasks such as optokinetic reflex, rotarod or the hanging wire test (Zhang et al., 2016). Furthermore, the ligand NGL-2 has been shown to be reduced in MN after sciatic nerve crush and was subsequently upregulated at the time of synaptic reformation, linking Ntng2 signaling to recovery in this lesion paradigm (Berg et al., 2010). These findings suggest a possible role of Ntng2 in motor connectivity and a potential role in CST rewiring after stroke.

In the N1E-115 neurite outgrowth assay, Ntng2 had no direct effect on outgrowth but partially rescued outgrowth in the presence of growth-inhibitory spinal cord extract (SCE) (Figure 3.8C, D). Interestingly, Ntng2 seems to be expressed primarily in MNs in the ventral horn at 28 dpi (Figure 3.8E-G), potentially highlighting an interaction with its motoneuronally expressed ligand NGL-2 also in this injury type (Berg et al., 2010). This ventral horn specific expression might reflect a role for Ntng2 as an attractive guidance cue for sprouting CST fibers, steering their path into the ventral, motor quadrant of the spinal grey matter. These results provide evidence that Ntng2 can increase neurite outgrowth after injury and propose it as a relevant candidate factor for post-stroke recovery.

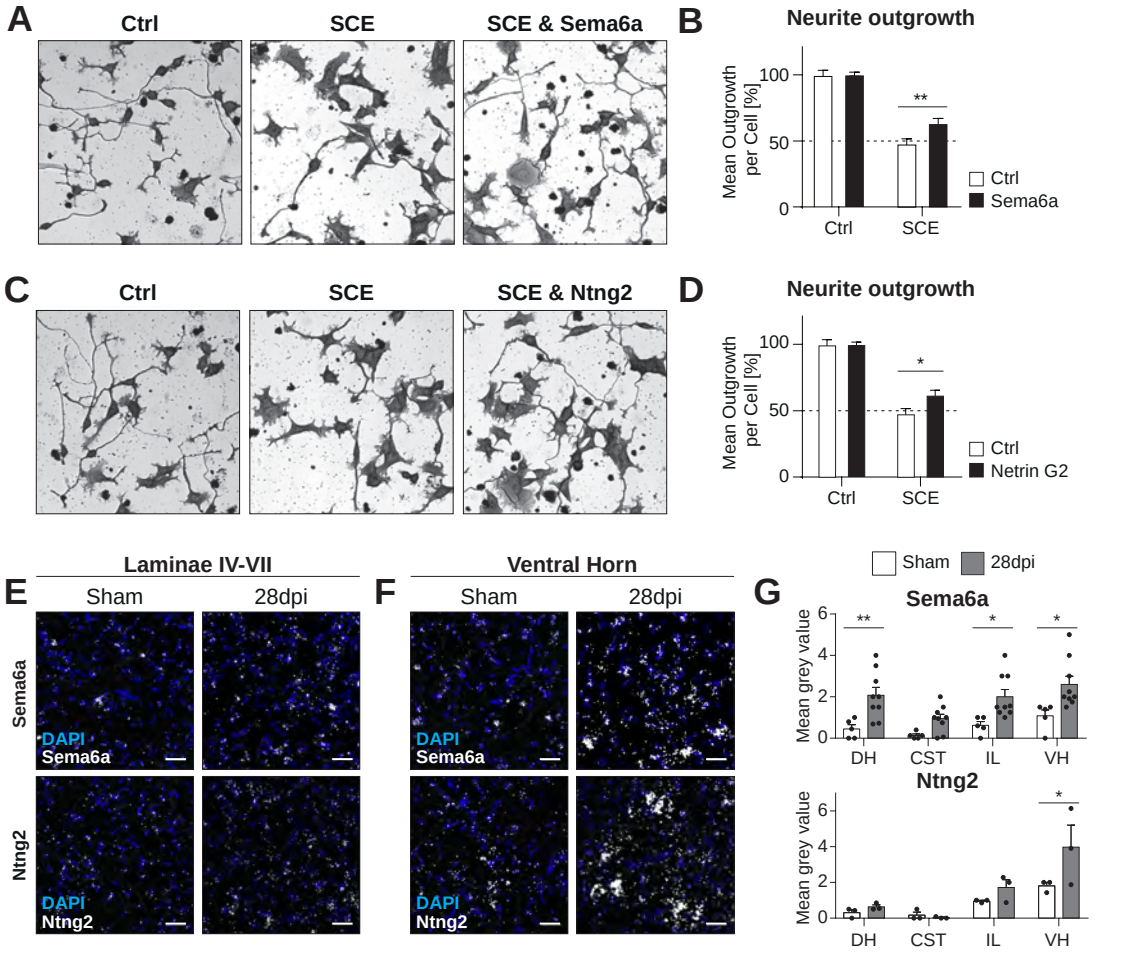


Figure 3.8 – Semaphorin 6a and Netrin G2 rescue neurite outgrowth partly and are expressed in the grey matter and in the ventral horn after stroke, respectively. **(A,C)** Representative pictures of N1E-115 cells 24h after addition of control medium (Ctrl), spinal cord extract or the combination of spinal cord extract and Sema6a or Ntng2, respectively. **(B, D)** Quantification of mean neurite outgrowth per cell normalized to medium control condition. Both Sema6a and Ntng2 had no effect under control conditions, but partially rescue neurite outgrowth in the presence of inhibitory spinal cord extract. **(E, F)** Representative pictures of *in situ* hybridization show expression of Sema6a and Ntng2 mRNA in spinal cord grey matter (E) as well as in the ventral horn (F). **(G)** Quantification of mRNA signals as assessed by *in situ* hybridization reveals an ubiquitous increase in expression of Sema6a within the spinal cord and a selective expression change of Ntng2 within the ventral horn at 28 dpi.

3.3.6 | Candidate Gene TGF-β, but not GDF7, Rescues Neurite Outgrowth from Inhibition by Spinal Cord Extract *in Vitro*

The bone morphogenic protein (BMP)/TGF-β superfamily has recently been implicated in post-stroke structural plasticity in a study by Li et al. (2015). GDF10 induced axonal outgrowth *in vitro*, while *in vivo* gain- or loss-of-function studies showed a positive correlation of GDF10 expression with structural plasticity of the peri-infarct cortex concomitant with

improved motor behavior. This regulation of axonal sprouting is mediated by binding to a complex formed by TGF-β receptors I and II. For TGF-β, which also signals through this receptor complex, a controversial effect on neurite outgrowth has been described in the literature. Paradoxically, both TGF-β administration as well as inhibition of Smad2, a molecule downstream to and directly activated by the TGF-β receptor complex, were shown to increase axonal outgrowth (Knöferle et al., 2010, Stegmüller et al., 2008, Yamada et al., 1994). This differential effect of TGF-β-signaling might be caused by the context-specific actions of TGF-β as previously described in the context of cancer (Lenferink et al., 2010, Li et al., 2015). Thus, in the spinal cord, TGF-β signaling might have a differential outcome on neurite outgrowth compared to the cortex as the tissue environment differs, e.g. in the respective inflammatory responses (Olson, 2010, Schnell et al., 1999).

Two members of the TGF-β superfamily were found to be upregulated in the spinal grey matter 28 days after stroke: GDF7 and TGF-β1. While TGF-β1 was shown to increase re-elongation of injured hippocampal neurons as well as primary cortical neurons (Abe et al., 1996, Li et al., 2015) and thus presented an excellent candidate for further analysis, less is known about GDF7. In the primate, GDF7 is primarily expressed in the primary motor cortex (Watakabe et al., 2001), however, it's role there is still elusive. By formation of a heterodimer with BMP7, GDF7 leads to repulsion of commissural axons during spinal cord early development (Butler and Dodd, 2003).

In our neurite outgrowth assay, we could neither detect a direct growth promoting effect of GDF7, nor did we find an influence of GDF7 on the growth-inhibition exerted by the spinal cord extract (Figure 3.9A, B). On the other hand, TGF-β1, which again did not influence neurite outgrowth by itself, showed a strong antagonizing, growth enhancing effect in the presence of growth-inhibitory spinal cord extract (Figure 3.9C, D). *In situ* hybridization confirmed the 1.5-fold increase of mRNA levels for TGF-β1 within the intermediate layers of the cervical spinal cord (Figure 3.9E). An even more pronounced upregulation of TGF-β1 was seen in the stroke-affected CST in the dorsal funiculus that persisted until 42 dpi (Figure 3.9, E-G). These results make TGF-β1 a prime candidate for growth induction and sprouting in the stroke-denervated spinal cord.

3.4 | Discussion

We used a unilateral large stroke to investigate the transcriptome of the stroke-denervated spinal cord from 4 days to 6 weeks after injury, a time window during which fibers of the

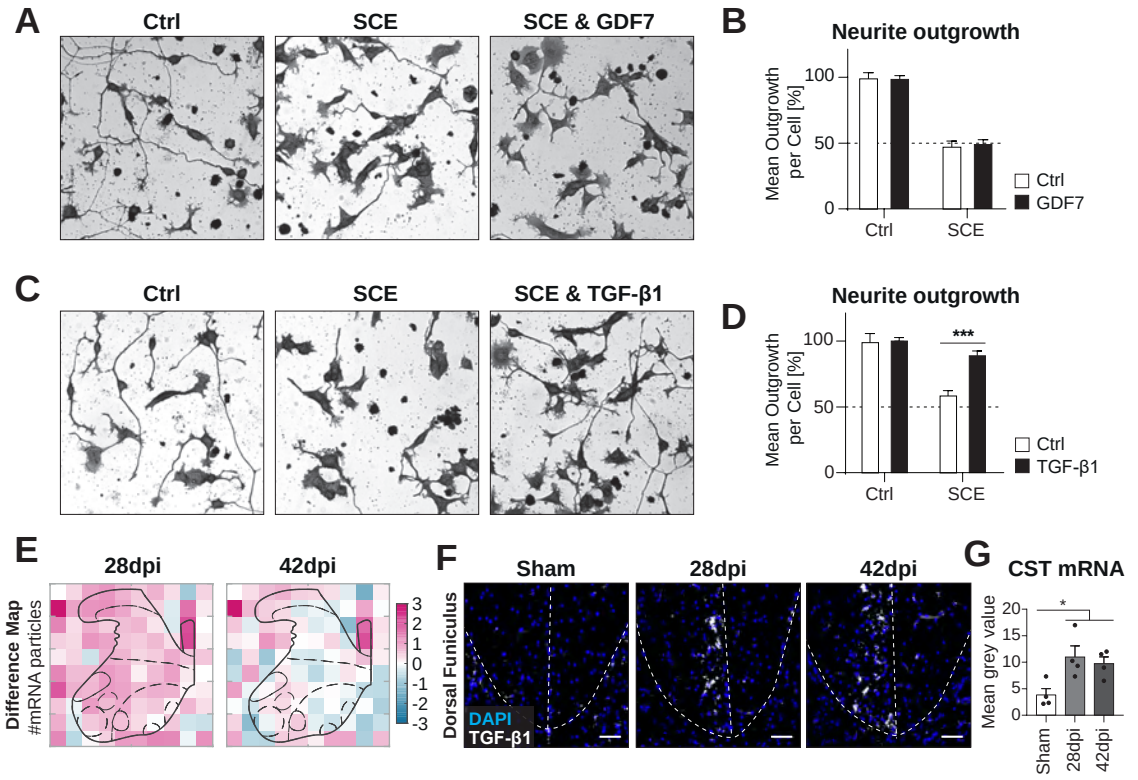


Figure 3.9 – TGF-β1, but not GDF7, rescues neurite outgrowth in the presence of growth-inhibitory spinal cord extract and is highly upregulated in the dorsal funiculus of the denervated hemicord after stroke. **(A,C)** Representative pictures of N1E-115 cells 24h after addition of control medium (Ctrl), spinal cord extract or the combination of spinal cord extract and **TGF**-β1 or GDF7, respectively. **(B, D)** Quantification of mean neurite outgrowth per cell normalized to medium control condition. Both **TGF**-β1 and GDF7 had no effect under control conditions. TGF-β1, but not GDF7, rescued neurite outgrowth in the presence of inhibitory spinal cord extract. **(E)** False color coded heatmaps of the counts of mRNA dots confirm upregulation of TGF-β1 in the intermediate laminae at 28 dpi, which is reduced to baseline levels at 42 dpi, and a persistent upregulation in the main CST in the dorsal funiculus. **(F)** Representative pictures of *in situ* hybridization for TGF-β1 in sham-operated animals and 28 and 42 days after stroke in the dorsal funiculus. **(G)** Quantification of the mRNA expression of TGF-β1 in the main CST as assessed by densitometry shows increase that persists to 42 dpi.

contralesional CST initiate sprouting, grow across the spinal midline and re-innervate the stroke-denervated spinal hemicord, mainly laminae IV-VII in the mouse. Transcriptomic profiling of the target area of newly innervating CSMNs revealed stroke-induced differential expression of a total of 955 genes, with the most pronounced changes seen at day 28 after stroke. Network analysis showed an enrichment of inflammation-related pathways in the early phases after stroke, whereas migration and growth-related pathways were highlighted at later stages. For three upregulated genes, Sema6a, Ntng2 and TGF-β1, their potential to overcome spinal cord extract mediated growth-inhibition was shown by *in vitro* neurite outgrowth assays.

Lesion size and location are important determinants of which areas participate in the observed structural plasticity in rodents, monkeys as well as humans (Cramer, 2008, Dijkhuizen et al., 2003, Murphy and Corbett, 2009, Rehme et al., 2011). Unilateral ablation of all motor related areas induces sprouting of the contralesional cortex on several levels such as the brainstem and the spinal cord in mice (Bachmann et al., 2014, Omoto et al., 2011, Ueno et al., 2012) and, potentially to a lesser extent, in rats (Dijkhuizen et al., 2003, Lindau et al., 2014, Weidner et al., 2001) and non-human primates (Buetefisch, 2015, Morecraft et al., 2016, Murata et al., 2015). Such a rewiring was also proposed for stroke patients, based on e.g. transcranial magnetic stimulation measurements, providing an indirect measure of circuitry reformation (Buetefisch, 2015, Chollet et al., 1991, Feydy et al., 2002, Rehme et al., 2012). Increased projections establish within the first four weeks after stroke in rodents (Murphy and Corbett, 2009, Ueno et al., 2012), during which plasticity can be further increased by rehabilitative training (Biernaskie and Corbett, 2001). We confirmed previous studies that contralesional CSMNs input after stroke is increased over the course of four weeks, with intermediate laminae IV-VII representing the main target zone for reinnervating fibers (Brosamle and Schwab, 1997, Lindau et al., 2014, Ueno et al., 2012, Wahl et al., 2014). This is comparable to innervation patterns observed during development (Asante and Martin, 2013, Canty and Murphy, 2008). Innervation was highest at spinal levels C3 and C6, where MNs primarily contribute to shoulder and upper arm muscle movements (Bachmann et al., 2014).

The contralesional input into the denervated hemicord is mainly due to midline crossing fibers as shown by fiber counting. A contribution by ipsilateral projections as described by Lindau et al. (2014) and Weidner et al. (2001) in rats cannot be excluded. CST fibers normally do not cross the spinal cord midline. For sprouts to reach the denervated hemicord, they thus are probably attracted by a potent growth-promoting factor gradient comparable to developmental innervation processes (Joosten et al., 1994, Kuang and Kalil, 1994), they must overcome the inhibitory cues present at the midline (Omoto et al., 2011), and then follow cues leading to the target area (Joosten et al., 1994, 1991). Interestingly, we also found an increase in fibers/collaterals exiting the intact CST, i.e. crossing the border between the CST in the dorsal funiculus and the grey matter. This suggests that not only segmental CST arbors sprout and switch their body side, but that CSMNs previously projecting to lower spinal levels switch their segmental target.

Soluble, membrane and extracellular matrix factors attracting and guiding sprouting fibers in the stroke-denervated spinal cord are mostly unknown at present but should be reflected in the transcriptomic profile of the target tissue. Several transcriptomic *in vivo* studies focused on early time points (Hori et al., 2012, Lu et al., 2003, Rickhag et al., 2006,

Sarabi et al., 2008, Zhao et al., 2017) in a quest to find candidates to boost neuroprotection and rescue neurons of the penumbra (Dirnagl et al., 1999). Studies of later time points (Buga et al., 2014, 2012, Carmichael, 2005, Keyvani et al., 2002, Krüger et al., 2006) were also restricted to the stroke penumbra. Due to the close vicinity to the lesion site, the extensive data sets produced in these studies are difficult to interpret as factors influencing neuronal sprouting responses might also be involved in other processes like inflammation, neurogenesis or angiogenesis. Here, we studied the transcriptomic profile of the CST target area, the spinal cord, which is far remote from the acute processes around the cortical lesion size, with the goal to obtain insight into the target-derived factors initiating and steering axon growth and pathfinding as well as the formation of new synapses and functional connections.

Using two network analysis methods, hierarchical clustering and NEA, we identified two major classes of stroke-induced mRNA changes in the spinal cord: a group of early, mostly inflammation related genes (4 dpi — 7 dpi) followed by changes related to growth and migration at 28 dpi — 42 dpi. In the early inflammatory phase, an enrichment of immune related gene sets was found in the NEA. These included phagocytosis and antigen presentation related gene sets, suggesting an activation of microglia and an infiltration of other myeloid cells, potentially related to clearance of debris within the spinal target area, e.g. as part of Wallerian degeneration (WD) (Thomalla et al., 2005, Weishaupt et al., 2010). This hypothesis is strengthened by the finding of an early upregulation of triggering receptor expressed on myeloid cells 2 (TREM-2), a pattern receptor specific to microglia and myeloid cells that is activated by tissue debris and leads to phagocytic responses (Neumann et al., 2009).

In the last decade, the functional role of inflammatory responses after CNS injury has been investigated more thoroughly, with both detrimental and beneficial effects on tissue repair being revealed (David and Kroner, 2011, Ekdahl et al., 2009, Neumann et al., 2009, Sierra et al., 2013). Immune cells, i.e. microglia and infiltrating myeloid cells, contribute to tissue damage by enhancing the inflammatory response through the release of pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ); these factors can act directly on neurons or activate neurotoxic astrocytes (Aloisi, 2001, Kingham et al., 1999, Liddelow et al., 2017). Additionally, anti-inflammatory cytokines such as interleukin (IL)-10 are released in sub-acute phases after stroke, subsequently leading to a decreased inflammation state and reduced lesion volumes (Dugue et al., 2017, Spera et al., 1998). Strikingly, both of these actions, promotion of axonal regeneration and neurotoxicity, can occur simultaneously in the same tissue by the same cell type, as was shown for DRG neurons implanted into an immune-activated spinal environment (Gensel

et al., 2009). The effect of macrophages as growth-promoter or on neuronal cell death, however, was dependent on the stimulus by which they were activated, calling attention to the variety of phenotypes microglia/macrophages can portray.

Interestingly, factors that participate in the detrimental pro-inflammatory response early after stroke, such as TNF-α and IL-6, have also been implicated as beneficial players in the structural plasticity after CNS injury. TNF-α *null* mice do not show locomotor recovery after cortical traumatic brain injury, nor do they show axonal sprouting in response to the injury (Oshima et al., 2009). Similarly, IL-6 has been established as a mediator of inflammation, but can also stimulate neurite outgrowth (Parish et al., 2002, Suzuki et al., 2009). These data show that inflammatory responses, while on one hand being detrimental to neuronal survival, can have a positive effect on structural plasticity. Most notably, the complement cascade has been recently highlighted as a potential trigger of structural plasticity with administration of complement component 3 (C3) a starting from 7 dpi after stroke resulting in an improved functional outcome and increased structural plasticity (Stokowska et al., 2016). Our data set may provide a tool to gain a deeper insight into potential inflammatory signaling mechanisms for sprouting and rewiring neurons.

The most pronounced gene expression changes were found at 28 dpi. This observation is similar to results in the contralesional cortex, where the most pronounced expression changes were also found at later stages (21 dpi) (Krüger et al., 2006). Most SDEGs were found to be upregulated in our study, which also compares well to the contralesional cortex (Krüger et al., 2006). Several potential modulators of growth were identified using DEA. Among the many biological processes differentially regulated within the spinal CST target area at 28 days after stroke, growth-related events were found to be enriched. For three established growth-modulators, which we found to be upregulated — Sema6a, Ntng2 and TGF-β1 — *in vitro* results showed that their presence antagonizes the neurite growth inhibitory effect of spinal cord extract. Several additional genes have been found to be differentially regulated in this screen, that may provide novel candidates as modulators of the different phases and processes leading to stroke recovery.

Li et al. (2010) identified the mRNA expression changes of horizontal cortical sprouting neurons of the penumbra, which have previously been linked to structural plasticity and functional recovery after stroke (Carmichael, 2003). The authors report that sprouting neurons switch to a growth-promoting transcriptome early after stroke (7 dpi), with differentially regulated key processes including extracellular signaling molecules, transcription factors, and epigenetic control molecules. At 21 dpi, they observed an upregulation of cytoskeletal and synaptic proteins, which are needed for axon elongation and arborization, complementing our findings of a growth-promoting environment at 28 days after stroke.

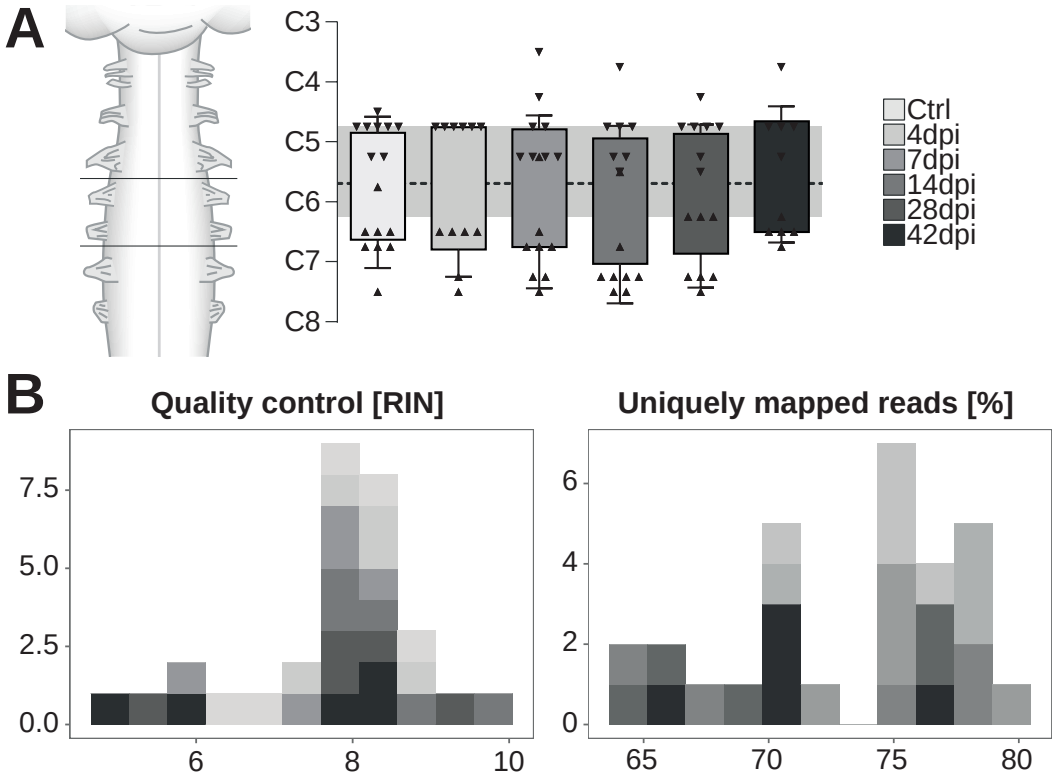
This is further confirmed by the study of Fink et al. (2017), where regulations of pathways that facilitate axon growth in contralesional CSMNs were found 28 days after transection of the CST axons in the pyramid in NgR1 *null* mice, a condition with enhanced compensatory sprouting on spinal levels. These as well as present data highlight the interplay of extrinsic tissue-derived and intrinsic neuronal factors that shape post-stroke structural plasticity.

In summary, the data from the present screen provide a tool to study several key questions regarding stroke recovery, including: *(1)* What are the inflammatory processes early after stroke on the spinal level and how do they influence stroke recovery, e.g. by altering sprouting and directed collateral growth? *(2)* How do spinal interneurons react to stroke-induced denervation and how do they rearrange their inputs over the course of stroke recovery? *(3)* How does the tissue environment including microglia and astrocytes influence the sprouting of contralesional CSMNs but also of other areas that increase projections into the cervical spinal cord upon stroke, such as several brainstem nuclei (Bachmann et al., 2014)? *(4)* How do subtypes of cells influence axonal pathfinding, target interaction and synapse formation? *(5)* How is synaptic stabilization/elimination in the spinal cord regulated after stroke and what is the role of training and rehabilitation on the underlying molecular mechanisms?

3.5 | Conclusion

Here, we provide a data set consisting of differentially expressed genes in the intermediate laminae IV-VII of the cervical C5/C6 spinal cord after large cortical strokes over the time course of six weeks. We identified two major phases that shape the spinal environment after stroke: An early, mostly inflammatory phase is followed by processes of structural plasticity including neurite growth and tissue remodeling, with the most pronounced changes observed at 28 dpi. Three of the found growth modulators were shown to induce neurite outgrowth in the growth-inhibitory environment of spinal cord extract *in vitro*. We propose that the stroke-denervated spinal grey matter, in particular its intermediate laminae, express growth factors and represent a growth-promoting environment for sprouting CST fibers originating from the contralesional motor cortex. Future studies may highlight additional candidates and pathways that influence the post-stroke recovery processes and may lead to novel neuroregenerative treatment options for stroke patients.

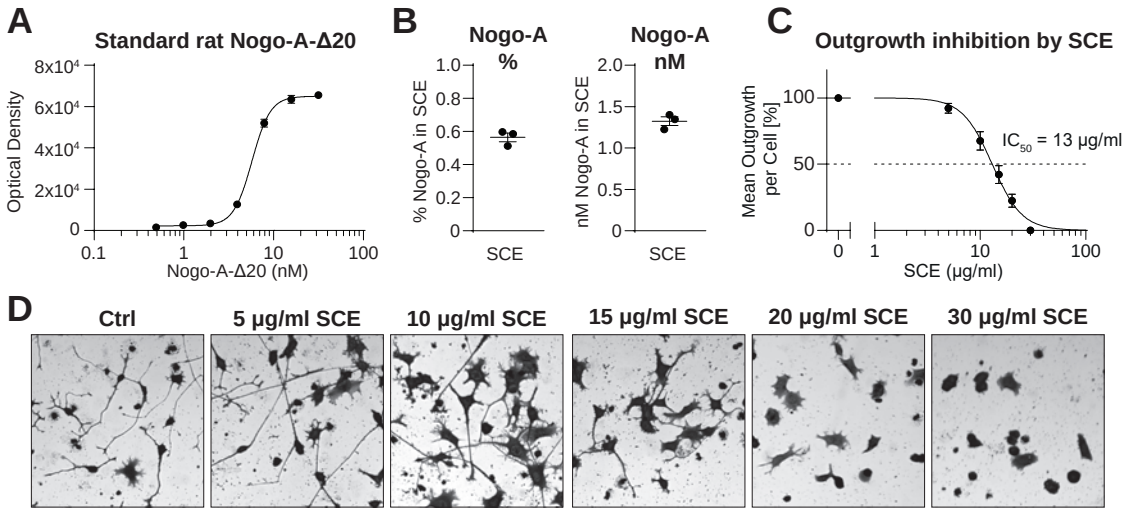
3.6 | Supplementary Material



Supplementary Figure S3.1 – Quality controls for RNA-Sequencing samples. **(A)** Anatomical assessment confirmed successful dissection of cervical layers C5/C6. **(B)** Quality and Quantity control for RNA levels showed adequate results for further analysis.

Supplementary Table S3.1 – Uniquely mapped reads for all given groups (average of n=5 samples of each group)

Uniquely mapped reads	Sham	4dpi	7dpi	14dpi	28dpi	42dpi
number	23.811.448,60	23.501.240,60	24.050.536,20	25.454.729,60	24.064.961,80	19.177.461,00
%	74,29	71,60	75,33	72,16	68,32	70,97



Supplementary Figure S3.2 – Spinal cord extract contains Nogo-A and inhibits neurite outgrowth in a dose dependent manner. (**A-C**) Representative standard curve of Nogo-A-Δ20 (**A**) used to calculate the percentage of Nogo-A (**B**) and nM concentration of Nogo-A (**C**) in the spinal cord extracts. (**D**) SCE inhibits outgrowth of N1E-115 cells in a dose dependent manner. Mean IC_{50} of three independent spinal cord extractions was calculated at 13 µg/ml. (**E**) Representative pictures of neurite inhibition on N1E-115 cells by increasing SCE concentrations

3.7 | References

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4

The Spinal Immune Response and its Potential Impact on Structural Plasticity and Recovery after a Large Cortical Stroke

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4.1 | Introduction

The central nervous system (CNS) has long been regarded as immune-privileged due to the restrictions posed by the blood brain barrier (BBB) (Fumagalli et al., 2015, Medawar, 1948). This concept, however, has been revisited and while it is still acknowledged that the CNS is immunologically unique, several members of the peripheral immune system have been described to participate in homeostasis as well as pathological conditions of the CNS (Louveau et al., 2015, Prinz and Priller, 2017). Resident immune cells, microglia, were first described by Del Rio-Hortega (1932). Microglia account for 10-15 % of the total CNS mass and are broadly distributed throughout the brain and spinal cord (Lawson et al., 1990). As part of the innate immune system, microglia have been attributed various functions in healthy conditions and in CNS disease (Hanisch and Kettenmann, 2007). Under physiological conditions, microglia portray a ramified morphology and constantly screen their environment through dynamic reorganization of their dendritic structures (Davalos et al., 2005, Kettenmann et al., 2011, Nimmerjahn et al., 2005). They have been appreciated for their role in neuronal proliferation and differentiation as well as in maturation and elimination of synaptic connections during development (Gemma and Bachstetter, 2013, Graeber, 2010, Schafer et al., 2012, Stevens et al., 2007, Tremblay et al., 2010). Furthermore, after pathological stimuli, peripheral immune cells are recruited to the lesion site, where they enter the CNS simultaneously with BBB breakdown (Prinz and Priller, 2017). These might include cells of the adaptive and innate immune system, including monocytes, neutrophils, T cells and B cells.

In response to pathological stimuli, the CNS resident microglia and infiltrating macrophages are rapidly activated (Hanisch and Kettenmann, 2007, Khan et al., 2017, Nimmerjahn et al., 2005), and are classified into two types: microglia/macrophages of type M1, which release inflammatory mediators like reactive oxygen species (ROS), matrix metalloprotease 9 (MMP9) and pro-inflammatory cytokines and chemokines (Mayer et al., 2016, Mosser and Edwards, 2008). In contrast, M2 microglia/macrophages produce protective anti-inflammatory cytokines, such as interleukin (IL)-4, IL-10 and transforming growth factor (TGF)-β, which participate in tissue repair and wound healing (David and Kroner, 2011, Mayer et al., 2016). Emerging evidence, however, indicates that these two stages of polarization represent extremes on a scale and that the transition between

them is highly dynamic rather than representing a switch from one to the other (Hanisch and Kettenmann, 2007). For many pathological conditions of the CNS, the role(s) of the immune response in the pathophysiological process remains controversial, as both detrimental and beneficial roles have been described and may occur simultaneously or in sequence (Gensel et al., 2009, Kabba et al., 2018, Sierra et al., 2013).

After stroke, microglia are rapidly mobilized to the lesion site due to ”danger signals” from damaged or necrotic neurons (Aloisi, 2001, Kono and Rock, 2008, Schilling et al., 2005). Here, together with infiltrating macrophages and other myeloid cells, they contribute to neuronal cell death and tissue damage through pro-inflammatory factors like tumor necrosis factor α (TNF-α) and ROS (Dugue et al., 2017). These are directly neurotoxic and also lead to further activation of other glial cells, such as astrocytes (Aloisi, 2001, Block et al., 2007, Kingham et al., 1999, Liddelow et al., 2017). Furthermore, the pro-inflammatory factors alter the permeability of the BBB and attract more peripheral leukocytes to enter the CNS, further exacerbating inflammation and brain damage (Iadecola and Anrather, 2012, Konsman et al., 2007). Microglia/macrophages also clear cellular and myelin debris, and release anti-inflammatory cytokines such as IL-10 and TGF-β, thereby aiding tissue repair (Iadecola and Anrather, 2012, Kettenmann et al., 2011, Neumann et al., 2009, Patel et al., 2013). Critical factors that may shape microglia/macrophage function and polarization are time and distance to lesion site (Hanisch and Kettenmann, 2007, Perego et al., 2011). At the lesion site after stroke and spinal cord injury (SCI), neurotoxic M1 microglia/macrophages govern over growth-promoting M2 macrophages, suggesting that polarizing the phenotype towards an M2 phenotype might enhance functional outcome after CNS insult (Gensel et al., 2009, Hu et al., 2012, Kigerl et al., 2009).

Intervening with microglial function by systemic depletion of microglia has been shown to result in discrepant outcomes after stroke, with one study reporting larger lesion volumes after selective depletion of microglia (Szalay et al., 2016), while others found reduced infarct volumes using the same stroke model of transient middle cerebral artery occlusion (MCAO) (Frieler et al., 2011). While highlighting the complex and diverse response of microglia to ischemic insults, both studies confirm a role of microglia in acute neuroprotection, primarily through the release of cytokines and neurotrophins (Glanzer et al., 2007, Neumann et al., 2006, Streit, 2002). Additionally, microglia and macrophages have been implicated in the sub-acute and chronic stages of stroke recovery. Neurogenesis can be influenced both positively and negatively by M2 or M1 microglial phenotypes, respectively (Butovsky et al., 2006). Macrophages, which are in many studies indistinguishable from microglia due to similar morphology and expression of genetic markers, exhibit a strong proangiogenic function in vascular repair during wound healing through releasing VEGF and IL-8

(Medina and O'Neill, 2011, Willenborg et al., 2012), and the activation state of microglia has been associated with endothelial cell proliferation, which subsequently leads to BBB repair and angiogenesis (Welser et al., 2010). Not surprisingly, M1 polarized macrophages have been shown to hinder axonal regeneration after CNS trauma (Horn et al., 2008, Kitayama et al., 2011, Popovich et al., 1999). Conversely, M2 macrophages may convey their positive influence on axonal regeneration after SCI or optic nerve crush by secreting protective molecules such as IL-10 and oncomodulin (Shechter et al., 2009, Yin et al., 2006). Transgenic knockdown of CX3CR1, the fractalkine receptor that is expressed by various immune cell populations (Harrison et al., 1998, Jung et al., 2000, Nishiyori et al., 1998), led to improved recovery after SCI (Donnelly et al., 2011), and this improvement was associated with increased axonal sprouting of serotonergic axons and increased synaptogenesis (Freria et al., 2017). These findings suggest a relation of microglia and macrophages to structural plasticity, a key element of functional recovery after stroke (Carmichael, 2003, Carmichael et al., 2017, Wahl et al., 2014).

Following a stroke, the CNS undergoes structural changes to compensate for lost functions. Intact brain regions with a functional relation to the lesioned tissue (Murphy and Corbett, 2009, Ward, 2004) project new collaterals into the denervated areas. This is the case for the peri-infarct cortex forming horizontal projections (Carmichael and Chesselet, 2002) or, in the case of larger strokes, the contralesional cortex sending projections across the midline on brain stem and spinal levels (Bachmann et al., 2014, Lindau et al., 2014, Raineteau and Schwab, 2001, Wahl et al., 2014). This compensatory rewiring has been linked to functional recovery, as selective silencing of spinally sprouting contralesional corticospinal motor neuron (CSMN) led to re-appearance of initial deficits (Wahl et al., 2014). Interestingly, microglial activation has been observed on spinal levels after cortical ischemia in several studies (Schmitt et al., 1998, Wu and Ling, 1998). Thus, while microglia are primarily involved in clearance of debris and phagocytosis of necrotic neurons in many pathological conditions (Neumann et al., 2009), they may also be a high priority candidate in the initiation and maintenance of structural plasticity.

Here, we studied the potential involvement of spinal microglial cells in post-stroke recovery of the corticospinal tract (CST). We investigated the spatial distribution of activated microglia/macrophages on spinal levels after stroke in correlation to the ablated CST. While we found an increase in the number of Iba1$^+$ microglia or macrophages as early as four days after initial injury in the dorsal funiculus, overlapping with the degenerating CST, we did not see infiltration of macrophages in the termination area of the CST projection area (CPA). Activation levels of Iba1$^+$ cells, as measured through morphological assessment, were increased both in the dorsal funiculus as well as the CPA starting from four

days after stroke. In the dorsal funiculus, microglia/macrophages continued to portray an alerted morphology, while spinal microglia/macrophages of the CPA returned to their ramified morphology after seven days post injury (dpi). This suggests a selective activation of microglia in the spinal gray matter of the stroke-denervated hemicord. We further characterized the CX3CR1 transgenic mouse line for functional outcome after stroke. Heterozygous CX3CR1$^{+/GFP}$ mice show no difference in lesion size after 24 h, suggesting that reduction of CX3CR1 does not alter neuronal survival after stroke induction. However, CX3CR1$^{+/GFP}$ mice displayed a slower recovery rate in the horizontal ladder task, a specific task for cortically controlled fine motor movements. This suggests that the neuronal-microglial communication via fractalkine signaling is involved in post-stroke circuit reformation. The possibility to deplete microglia locally and specifically was explored using spinal injections of the phagocyte toxin clodronate, which successfully ablated CX3CR1^{+} cells in the immediate area of injection 5 days after administration. These findings allow for a more detailed description of the role of microglia/macrophages in structural plasticity associated with functional recovery after stroke in the future.

4.2 | Methods

Animals.

A total of 30 adult C57BL/6J mice (2-3 month, 20-25 g, female, Charles River) were used in this study. Breeding pairs of CX3CR1$^{GFP/GFP}$ were graciously provided by Prof. Dr. Melanie Greter (Institute of Experimental Immunology, UZH Zurich). In this mouse line, the second exon of the CX3CR1 gene is replaced with the enhanced green fluorescent protein (eGFP) reporter gene (Jung et al., 2000). Backcrossing to a C57BL/6J background resulted in heterozygous CX3CR1$^{+/GFP}$ mice which were used in this study (n = 12). Animals were housed in groups of four to five under a constant 12 h light/dark cycle with food and water ad libitum. All experimental procedures were approved by the veterinary office of the canton of Zurich, Switzerland.

Photothrombotic stroke.

For all surgeries, mice were anesthetized using 3-4 % isoflurane and transferred in a stereotactic frame (David Kopf Instruments, USA). Anesthesia was maintained at 1-2 % isoflurane throughout the surgery. Body temperature was maintained at 37 °C on a heating pad. All animals received a unilateral photothrombotic stroke to lesion the sensorimotor cortex as previously described (Bachmann et al., 2014, Watson et al., 1984). Briefly, the skull was exposed by a midline incision of the scalp, blood and periosteum was cleaned off to ensure a homogenous light application. An opaque template with a defined opening (3x5 mm) was aligned to the midline so that the right motor and pre-motor cortex was exposed (i.e., -2 to +3 mm a/p | 0 to 3 mm m/l in relation to Bregma)(Tennant et al., 2011). Five minutes

after intraperitoneal injection of 0.1 ml Rose Bengal (10 mg/ml in 0.9 % NaCl, Sigma-Aldrich), the skull was illuminated with a cold light source (Olympus KL1500LDC, 150W, 3000K) placed firmly on top of the skull for 10.5 minutes at maximal output. Control animals received a sham operation according to this protocol without illumination of the skull. Post-operative care included recovery on a heat mat, sustained analgesia provided via drinking water (Novalgin, 2 mg/ml with 5 % sucrose) and antibiotic treatment where necessary for 3 days.

Behavioral testing.

Behavioral tests were performed prior surgery (baseline) as well as 4, 7, 14 and 28 days after photothrombotic stroke of the right motor cortex. The horizontal ladder test was used as a sensitive assessment of skilled limb placement (Metz and Whishaw, 2002). Three trials on a 40 cm long stretch on the irregularly spaced rungs of 1 cm distance were recorded on each testing day (Panasonic HDC-SD800 High Definition Camcorder) and evaluated using frame-by-frame analysis (VLC media player, VideoLAN). Blinding was not possible due to obvious fine motor deficits induced by the photothrombotic stroke. Forepaw placement was scored as previously described (Maier et al., 2008). Briefly, a full success (score 4, 1 point) was counted when all four digits were placed correctly in front of the rung. Misplacement of one or more digits or of the palm of the paw was counted as partial success (score 2 resp. 3, 0.5 points), while a complete misplacement and subsequent slip was counted as no success (score 1, 0 points). The success score was expressed as percentage of success (sum of success points) in relation to maximal possible outcome (total amount of steps taken). The success rate and complete misses represents the number of correct paw placements (score of 4) or misplacements (score of 1), respectively, divided by the total amount of steps taken.

Local depletion of microglia.

Injections were performed unilateral within the medial prefrontal cortex or spinal cord to establish time course of depletion in brain and spinal tissue. Mice were deeply anesthetized using intramuscular injection of a combination of Medetomidin (Domitor, 0.105 mg/kg body weight, Provet AG), Midazolam (Dormicum, 1.4 mg/kg body weight, Roche) and Fentanyl (0.007 mg/kg body weight, Kantonsapotheke Zurich) and subsequently transferred in a stereotactic frame. Injections of PBS or clodronate disodium salt (10-100 mg/ml, Calbiochem) were given at stereotactic coordinates (+2 mm a/p | +0.3 mm m/l in relation to Bregma) or between spinal vertebra C3 and C4 to target spinal segment C5 (+1 mm from midline, dura was locally punctured before inserting the needle). Single injections of 100 nl, 300 nl or 1 µl were given with a 35 g needle on a 10 µl Nanofil syringe (Hamilton, Switzerland) driven by an electrical pump with a constant flow rate of 6 nl/s (World Precision Instruments, Germany) at 1.5 mm or 1 mm depth for brain and spinal cord, respectively. To avoid backflow of the tracer, the needle was kept in position for 5 min after each injection. Post-operative care included recovery on a heat mat, sustained analgesia provided via drinking water (Novalgin, 2 mg/ml with 5 % sucrose) and antibiotic treatment where necessary for 3 days.

Perfusion and tissue procession.

Animals were deeply anesthetized using pentobarbital (i.p., 0.2 ml, Streuli Pharma AG) and

transcardially perfused with ice cold Ringer's solution (105 IU/L heparin, Roche and 0.25 % $NaNO_2$). Brains and spinal cords were dissected and immersed in 4 % formaldehyde (FA) in 0.1 M PB overnight. Thereafter samples were transferred to 30 % sucrose/PBS and kept at 4 °C until further use. Brains and spinal cords were embedded in TissueTek® O.C.T. TM compound and serial sections were cut at 40 µm. Brain samples were collected on slide and further used for lesion completeness assessment. Spinal cord sections were collected free-floating in 0.1 M PB, transferred to anti-freeze solution (15 % sucrose, 30 % ethylenglycol in 50 mM PB) 24 h later and stored at -20 °C until further use.

Analysis of lesion completeness.

For the accurate analysis of lesion size, brain cross-sections were bathed on-slide in Cresyl violet solution for 1 min, dehydrated in a series of increasing ethanol concentrations and washed in xylol before coverslipping with Eukitt. Brain sections at four defined landmarks (1.98 mm, 0.98 mm, -0.22 mm, -1.34 mm, in relation to Bregma) were analyzed for stroke volume and depth of cortical lesion. To evaluate lesion size in acute slices, mouse brains were acutely perfused with ice cold Ringer's solution and cut into coronal slices of 1 mm thickness on ice. Subsequently, the slices were immersed in 1% 2,3,5-triphenytetrazolium chloride (TTC) and imaged directly. Infarct area was measured using the Swanson method (Swanson et al., 1990) to correct for cortical swelling. Lesion volume was calculated indirectly as the difference of the size of the contralesional hemisphere minus the non-infarct volume of the ipsilesional hemisphere / contralesional hemisphere.

Immunohistochemistry.

Free-floating sections of spinal tissue were treated with peroxide (6 % in 70 % methanol) to quench endogenous peroxidase to reduce background staining. Sections were then permeabilized for 2 h in blocking buffer (10 % normal goat serum, 3 % bovine serum albumine, 1 % Triton in 0.05 M TBS at pH 7.4) and directly incubated with the primary antibody (rb α Iba1, 1:1000, WAKO, USA) in blocking buffer overnight. The sections were then washed and incubated in secondary antibody (biotinylated gt α rb, 1:200, Jackson ImmunoResearch Laboratories, USA) in blocking buffer for 90 min. After one more washing step, sections were incubated with ABC complex (VectaStain ABC Elite Kit, Vector Laboratories) for 45 min. They were then washed once more before being incubated in DAB (3,3'-diaminobenzidine, 1 mg/2ml 0.05M Tris HCl pH 8.0). After 10 min, the staining was precipitated by adding peroxide (0.01 %) and further incubation for 10 min. Sections were thoroughly washed, mounted on slides and air dried overnight. Before coverslipping, sections were dehydrated using increasing ethanol concentrations and xylol incubation. For fluorescent staining, free-floating sections were permeabilized with TNB (0.5 % TopBlock (LuBioScience, Switzerland) in 0.1 M Tris) containing 5 % normal goat serum and 0.4 % Triton X-100 for 60 min. Primary antibody was delivered in the same solution (rb α GFAP, 1:1000, DAKO Agilent, USA; ms α NeuN, 1:300, Chemicon, USA) for 16 h. Sections were then washed and incubated with the secondary antibody (Cy3 gt α rb, 1:200, Life Technologies, USA; AlexaFluor 647 gt α ms, 1:200, Life Technologies, USA) for 60 min before being washed, mounted on slides and air dried overnight. Optionally, sections were incubated

in DAPI (1:1000, Molecular Probes D3571) for 5 min before mounting. On the next day, slides were coverslipped with fluorescence mounting medium (Mowiol, Merck, Switzerland).

Activation index of Iba1$^+$ cells.
Activation index was measured on Iba1 immunostained slices. Slides were digitalized (Zeiss Axio Scan.Z1, x 100, three sections/spinal level/animal, z-stack of 5 images/slice with 2 µm distance). Cells were counted by an experienced observer; Blinding was not possible due to obvious microglial activation. To assess microglial activation, we used a FIJI-based algorithm to select 20 cells out of all selected cells in a semi-random manner that allowed an even distribution throughout the grey matter. We then measured the soma diameter and the longest process (selected by eye). Activation index was calculated as the longest process of the cell divided by the diameter of the soma; thus, smaller values represent a more activated state of the cell.

Statistics.
Statistical analysis was performed with Prism 7.0 (GraphPad Software Inc.) and R. For statistical tests within groups over time, one-way ANOVA with repeated measured followed by Dunnett correction for multiple comparison tests. The Student's t-test was used to test for differences between 2 groups (unpaired), or 2 time points within one group (paired). To detect differences between groups and within groups over time, and for comparison of more than two groups over time, two-way ANOVA with repeated measures followed by Bonferroni correction for multiple comparisons was used. To evaluate the three-way interaction of the activation index over time, spinal level and region, measurements were examined by a linear mixed effects model using *lme4* in R. Multiple comparisons were corrected for multiplicity by simultaneous tests based on a multivariate t distribution (lmer formula = activity index $\sim$ 1 + Group + Level + Region + Group:Level + Group:Region + Level:Region + Group:Level:Region + (1|Animal) + (1|Slice)). The threshold for significance for all experiments was set at *P = 0.05. Smaller P-values were represented as **P = 0.01 and ***P = 0.001. In bar graphs, all data are plotted as mean $\pm$ standard error of the mean (SEM). In box plot graphs, data are represented as median $\pm$ 25th percentile (box) and min/max (whiskers). Dots represent individual animals.

4.3 | Results

4.3.1 | Iba1$^+$ Cells are Transiently Activated within the Former Corticospinal Tract Projection Area

To address microglial activation on spinal levels, the activation marker ionized calcium-binding adapter molecule 1 (Iba-1), which labels microglia and macrophages (Greter et

al., 2015), was used. The morphology of Iba1$^+$ cells was evaluated at selected time points (2 dpi, 4 dpi, 7 dpi, 28 dpi) after stroke (Figure 4.1A). An activation index was defined as the longest process divided by the soma diameter, utilizing the morphological change of microglia to retract their processes upon activation concomitant with an increase in soma diameter (Davis et al., 1994, Stence et al., 2001) (Figure 4.1B). Based on this criterion, activation of Iba1$^+$ cells was observed after 4 dpi in the dorsal funiculus in the area of the degenerating CST, and persisted until 28 dpi (Figure 4.1C-E). In the CST projection area (CPA), defined as the termination area of fibers of the contralateral CST in the healthy condition, the activation of Iba1$^+$ cells was only observed transiently, with cells portraying an alerted morphology at 4 dpi. After 7 dpi, the activation index was returning towards normal levels and was comparable to sham levels at 28 dpi. Infiltration of blood born monocytes was assessed by the number of Iba1$^+$ cells, and showed an increase of Iba1$^+$ cells in the CST area of the dorsal funiculus after 4 dpi. These levels remained increased until 28 dpi. Infiltration was not observed in the ventral and dorsal horn of the spinal grey matter or in the CPA at any of the measured time points. This infiltration and activation pattern holds true for cervical levels C2-C6 with no statistical difference between the segments (Figure S4.1). In summary, microglia/macrophages are activated early and transiently in the CPA, in addition to an infiltration and persistent activation in the CST area, suggesting a role for microglia/macrophages in phagocytosis of cellular and myelin debris related to the degradation of the CSTs and its terminals.

4.3.2 | CX3CR1$^{+/GFP}$ Mice Show Slower Behavioral Recovery Rate after Stroke but no Difference in Lesion Size

We investigated the spontaneous functional recovery of heterozygous CX3CR1$^{+/GFP}$ mice, in which the fractalkine receptor gene was replaced by eGFP. Under physiological conditions, the constant release of fraktalkine keeps resident microglia quiescent, while an acute insult may interrupt this release, thereby activating microglia (Kierdorf and Prinz, 2013, Lauro et al., 2006). Additionally, the fraktalkine:CX3CR1 interaction is implicated in the capability of monocytes to infiltrate the CNS. Characterization of this transgenic mouse line showed no overt phenotype; monocyte infiltration, dendritic cell migration/differentiation and microglial activation in selected lesion models show no alteration in response to the reduction of fractalkine (Jung et al., 2000). Following an ischemic insult, a protective inflammatory environment was observed in CX3CR1 *null* mice, while susceptibility to brain ischemia in CX3CR1$^{+/GFP}$ compared with wild-type animals did not differ (Fumagalli et al., 2013). To evaluate whether this neuron-microglia communication is involved in func-

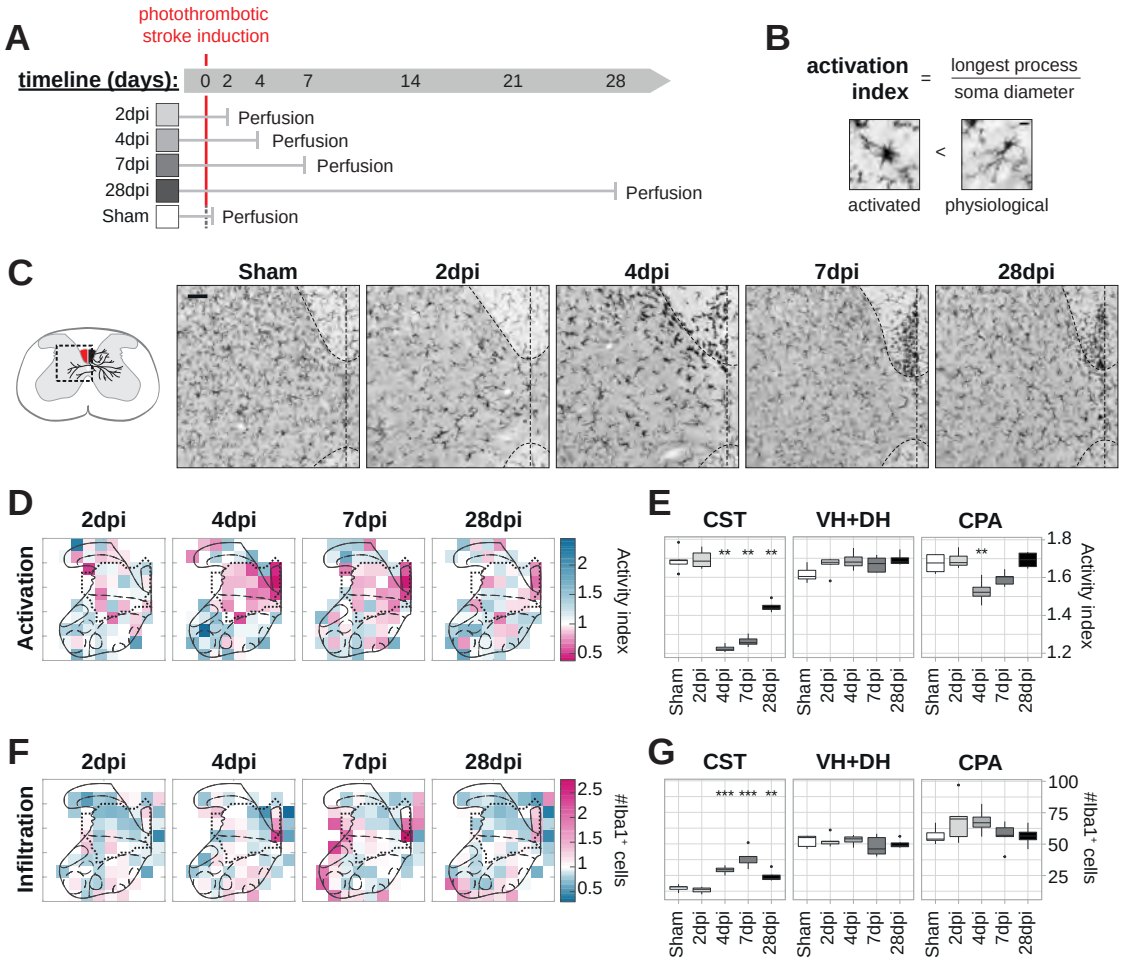


Figure 4.1 – Microglia/macrophage (Iba1$^+$ cells) activation and infiltration on spinal level C5 at 2, 4, 7 and 28 days after stroke. **(A)** Timeline of the study. Animals received a photothrombotic stroke and were sacrificed 2 dpi, 4 dpi, 7 dpi, 28 dpi thereafter. Control animals received a sham operation (n = 5/group). **(B)** Activation index was calculated as the longest process of the cell divided by the soma diameter, thus lower values represent an activated morphology and higher values a morphology reflecting the resting, tissue surveying function. **(C)** Representative pictures of Iba1 immunostaining of spinal cord grey matter (boxed area) in Sham and stroked animals at 2 dpi, 4 dpi, 7 dpi, 28 dpi. **(D)** Difference map of activation index of Iba1$^+$ cells at selected time points (fold change to Sham group). Red values depict more cells with a tendency towards activated morphology, while blue values depict more cells with a ramified, resting morphology. **(E)** Higher activity stages of Iba1$^+$ cells was seen after 4 dpi in the CST as well as the former CST projection area (CPA), while Iba1$^+$ cells showed only a transient activated morphology in the CPA. **(F)** Difference map of number of Iba1$^+$ cells at selected time points (fold change to Sham group). Increase in cell number is depicted in red, reduction in blue colors. **(G)** Increased cell numbers are observed after 4-28 dpi within the degenerating CST but not in the grey matter (VH+DH; CPA).

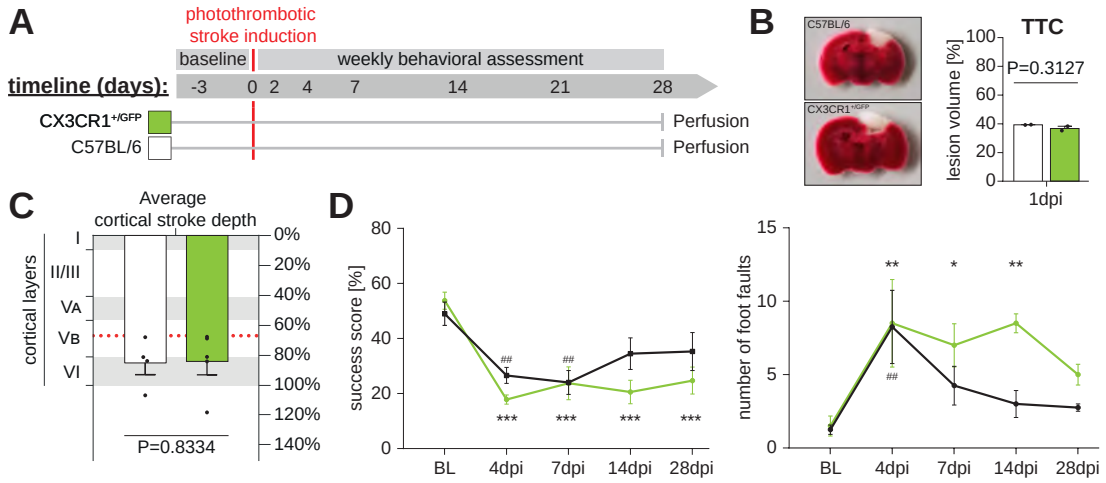


Figure 4.2 – CX3CR1$^{+/GFP}$ show no difference in neuroprotection but display slower recovery rates after stroke. **(A)** Timeline of the study. CX3CR1$^{+/GFP}$ (n = 4) or C57BL/6 WT (n = 4) mice received a photothrombotic stroke after handling and baseline recording. Behavior was assessed weekly until 28 dpi. **(B)** Representative pictures of lesion size 24 hours after stroke induction as assessed by TTC staining. No difference between groups was observed (n = 2/group). **(C)** Analysis of stroke core depth showed no significant difference among groups and successfully ablated the CST unilaterally. Location of targeted CSMNs in layer V is represented by a red dotted line. **(D)** Two parameters were assessed in the horizontal ladder task. Success score and number of foot faults revealed a deficit at 4 dpi for CX3CR1$^{+/GFP}$ and C57BL/6 mice, while CX3CR1$^{+/GFP}$ showed a slower recovery rate. Statistical analysis (multiple comparison to group-specific baseline level) is depicted as # for C57BL/6 mice (black line) or * for CX3CR1$^{+/GFP}$ (green line).

tional recovery, heterozygous CX3CR1$^{+/GFP}$ mice were trained in the horizontal ladder task before induction of a stroke and their motor behavior was subsequently monitored weekly (Figure 4.2A). First, we aimed to define the impact of heterozygous reduction of CX3CR1 on lesion size; variable stroke sizes would lead to differing levels of motor recovery. We found no difference in lesion volume 24 h after photothrombotic stroke (Figure 4.2B), in line with previous findings that only a full deficiency of CX3CR1 leads to decreased lesion volumes at early time points after stroke (Fumagalli et al., 2013). Also after 28 dpi, lesion volumes were comparable between CX3CR1$^{+/GFP}$ and wild-type C57BL/6 mice (Figure 4.2C), suggesting that the progression of wound healing is unaltered in the knock-down animals. CX3CR1$^{+/GFP}$ animals showed a similar functional deficit as wild-type stroke mice on the horizontal irregular ladder (number of foot faults; overall success score) at 4 dpi. However, the mutants recovered at a slower rate as compared to wild-type C57BL/6 mice up to 28 dpi (Figure 4.2D). These findings suggest that the neuron-microglia communication via fractalkine signaling is involved in functional recovery after stroke.

4.3.3 | Clodronate Depletes Microglia/Macrophages Specifically and Locally

To evaluate the role of spinal microglia/macrophages in recovery after stroke, we sought to locally deplete microglia in the spinal cord without affecting their role in tissue damage control in the ischemic area of the cortex. In a first series of pilot studies, we established clodronate-dependent depletion of microglia/macrophages in the brain due to better technical feasibility. We found that clodronate exerts its toxic effect between 5 to 7 days post injection (dpi) at a concentration of 50 mg/ml (1 µl injections) (Figure 4.3A, B, C). At effective concentrations and time points, clodronate may deplete microglia/macrophages by $\sim$ 70 % (Figure 4.3C). In the spinal cord, CX3CR1^{+} cells were reduced in the vicinity of the injection site (Figure 4.3D). Notably, due to the surgical setup, injections often led to increased inflammation in the injection area (Figure 4.3B (PBS injection); Figure 4.3D (spinal injection)), warranting an optimization of the surgical procedure and administrated concentrations. This is especially the case for spinal injections, where puncturing of the tissue often leads to enhanced bleeding. We further tested the effect of clodronate on other cell types to ensure a microglia/macrophage specific depletion. Increasing concentrations of clodronate did not detectably affect GFAP^{+} astrocytes or NeuN^{+} neurons, while CX3CR1^{+} cells were effectively reduced (Figure 4.4).

4.4 | Discussion

The inflammatory responses that occur in the CNS after lesion are complex and not well understood; however, they have repeatedly been linked to repair processes (Hanisch and Kettenmann, 2007, Hu et al., 2014, Jerry Silver, Martin E. Schwab and Popovich, 2015, Jin et al., 2010). Here, we show a transient activation of microglia/macrophages in the stroke-denervated spinal grey matter at early stages. This finding suggests activated microglia/macrophages as potential candidates to trigger growth-promoting processes within this area. Structural plasticity, in particular of contralesional CSMNs, is a key element of post-stroke recovery. Reduction of the fractalkine signaling led to a slower behavioral recovery of skilled forelimb movements as assessed in the irregular horizontal ladder, while no effect on lesion size was seen. Future experiments making use of the here established clodronate-dependent depletion of microglia in a local and specific manner might allow for a deeper insight into these local immune cell functions in the stroke denervated spinal cord,

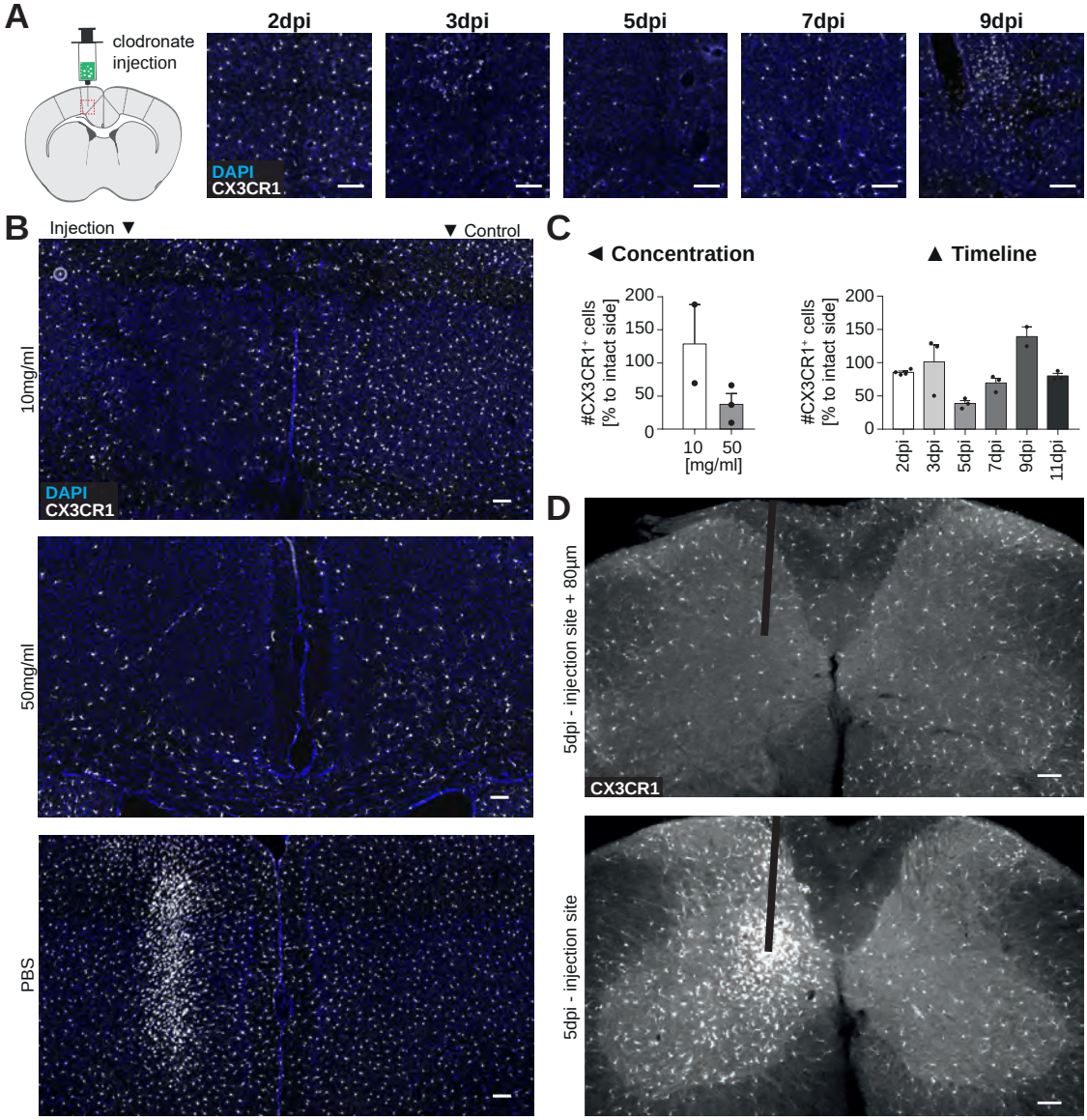


Figure 4.3 – Local injection of clodronate depletes microglia/macrophages locally with a peak at 5 days after administration. **(A)** Unilateral clodronate injections (1 µl, 50+mg/ml) into the cortex of adult CX3CR1$^{+/GFP}$ mice reduced the number of CX3CR1^{+} cells between 3 days post injection (dpi) and 7 dpi. **(B)** Reduction of CX3CR1^{+} cells is more pronounced at 50 mg/ml clodronate compared to 10 mg/ml clodronate. PBS injections served as a control. **(C)** Quantification of data shown in (A) and (B) confirms effective reduction in CX3CR1^{+} after injection of 50 mg/ml clodronate (left) at 5 dpi (right). **(D)** Spinal injection of 50 mg/ml clodronate (100 nl) led to depletion of microglia in the immediate surrounding area to the injection site (+80µm caudal). However, the injection itself led to an increased inflammation at the injection site (increase in CX3CR1^{+} cells) that was not resolved by clodronate injection, likely due to insufficient clodronate concentration. Injection trajectory is indicated by a black line. Scale bar = 100 µm.

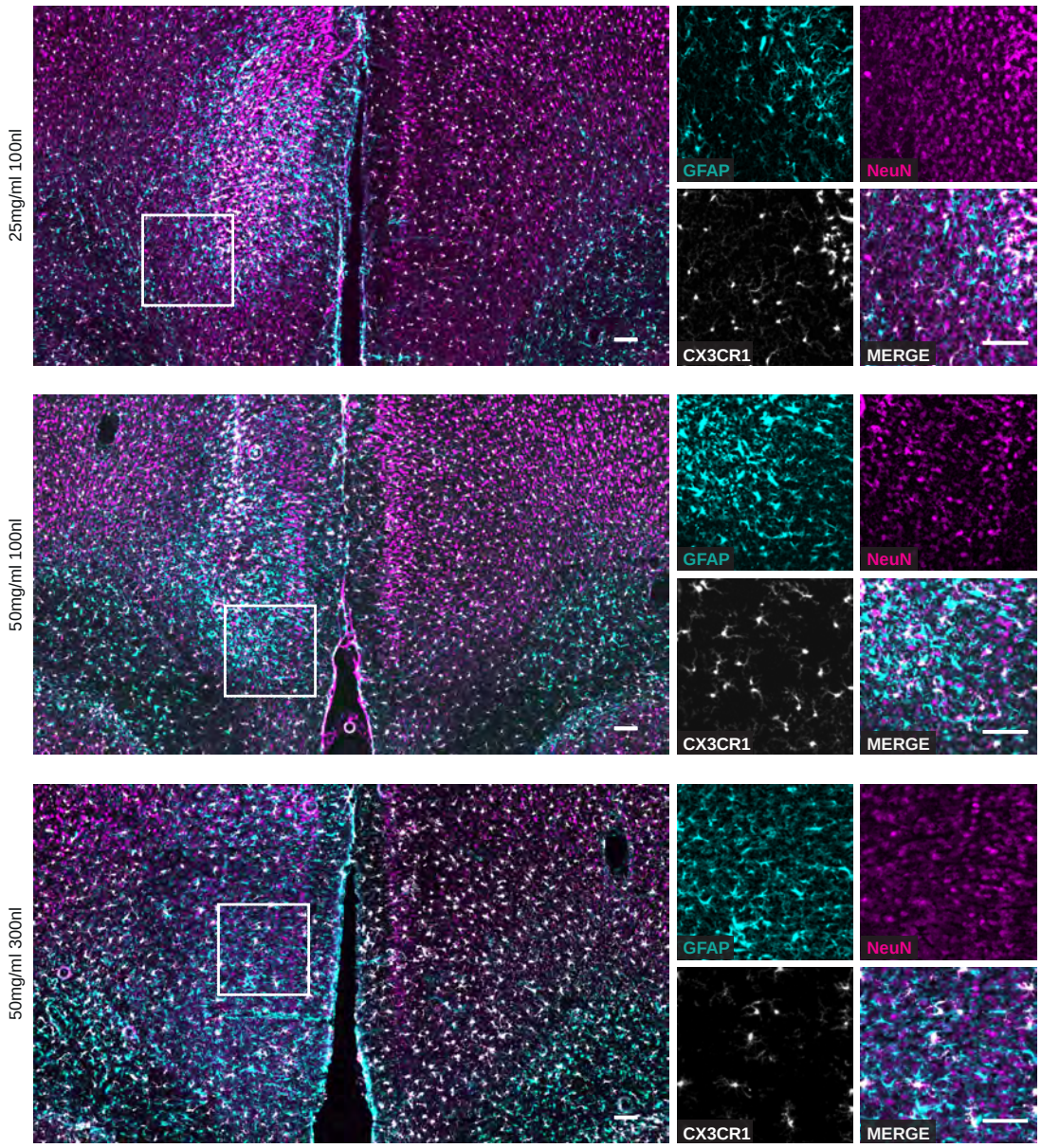


Figure 4.4 – Local injection of clodronate depletes microglia/macrophages specifically. Increasing concentrations of clodronate showed no effect on numbers of GFAP$^+$ astrocytes and NeuN$^+$ neurons, while CX3CR1$^+$ cells decreased with increasing concentrations. Scale bar = 100 µm.

their molecular repertoire and how this might influence structural plasticity after traumatic insults to the CNS.

Previous studies showed that microglia activation can be seen following focal cerebral ischemia at remote places such as the frontoparietal cortex, thalamic nuclei, internal capsule as well as the grey matter of lumbar spinal cord after 3 dpi (Morioka et al., 1993, Wu and Ling, 1998). Here, we provide evidence of microglia/macrophage activation in the cervical spinal cord, with transiently increased levels of an activation-related morphology in the intermediate layers of the grey matter, which overlapped with the former projection area of the CST at 4 dpi. Increased but persistent activation levels were also observed in the white matter, specifically within the corticospinal tract itself in the dorsal column. This is in line with findings in *post-mortem* tissue of stroke patients, where an early and transient increase of CD68 immunoreactivity (4-14 dpi), a marker associated with macrophage infiltration and activation, was observed at low levels in the intermediate grey matter, and at persistently higher levels in the CST in dorsal funiculus (up to 5 month after stroke) (Schmitt et al., 1998).

The difference in activation between grey and white matter is best explained by the amount of degenerating fibers and myelination in the respective areas. In the grey matter, the phagocytosis of fibers and synapses may be resolved within one week, while in the white matter, a continuing activation and increased infiltration up to 28 days after stroke, as observed in the present work, may indicate that here, clearance of cellular and myelin debris is a laborious process that requires recruitment of macrophages. This is strengthened by the finding that also after nerve injury, phagocytosis of myelin and cellular debris is a substantially slow process taking months to years in humans (Griffin et al., 1992, Vargas and Barres, 2007). Future studies should address the mechanism by which microglia get activated.

In many conditions, reactive microglia cannot be distinguished from activated macrophages. This distinction, however, might be crucial, as microglia and monocytes may differ in their function after CNS insults such as SCI (London et al., 2013, Shechter et al., 2009). We suggest that the subpopulation of activated Iba1$^+$ cells in the intermediate spinal laminae primarily comprises microglia as no increase in Iba1$^+$ cells was observed here. This is furthermore underlined by the finding that post-ischemia microglia precede and predominate the inflammatory response in the ischemic core (Khan et al., 2017, Li et al., 2013, Schilling et al., 2005). Using recently developed microglia-specific staining markers (Bennett et al., 2016) or microglia-specific transgenic reporter mice (Buttgereit et al., 2016), further knowledge about the subtype of immune cell that is activated specifically in this area may be obtained in the future.

Several mechanisms by which spinal immune cells could influence functional recovery after stroke are conceivable. Activated spinal microglia/macrophages are involved in phagocytosis of CST synapses, and of cellular and myelin debris (Avellino et al., 1995, Neumann et al., 2009). The overlap of immune cell activation with the former CST projection area observed in this study indicates a direct relation to phagocytosis of axonal debris or dendritic stripping of pre-injury target cells (Blinzinger and Kreutzberg, 1968). Insufficient clearance of debris has been shown to negatively influence regeneration processes (Neumann et al., 2009), thus providing one possibility for activated spinal microglia/macrophages to influence post-stroke recovery. Additionally, recent work has highlighted their role in the triggering of other cell types such as reactive astrocytes (Liddelow et al., 2017), which in turn can influence post-stroke recovery. Like microglia/macrophages, reactive astrocytes can have neurotoxic or neuroprotective roles (Becerra-Calixto and Cardona-Gómez, 2017, Liddelow et al., 2017). Astrocyte-derived factors such as ciliary neurotrophic factor (CNTF) have been shown to positively influence axonal regeneration (Müller et al., 2007). Thus, depending on the stimulus conveyed from microglia to astrocytes, a differential reaction of the astrocyte might occur. Moreover, factors released by microglia/macrophages can also have a direct effect on axonal regeneration. This was shown e.g. in the optic nerve crush model, where administration of macrophage-derived oncomodulin led to increased axonal regeneration (Yin et al., 2009, 2003). Based on this, we hypothesize that the subpopulation of activated microglia/macrophages observed in the intermediate laminae of the cervical spinal cord may play a crucial role in shaping a favorable tissue environment for axonal sprouting and growth of the contralesional CSMNs after stroke.

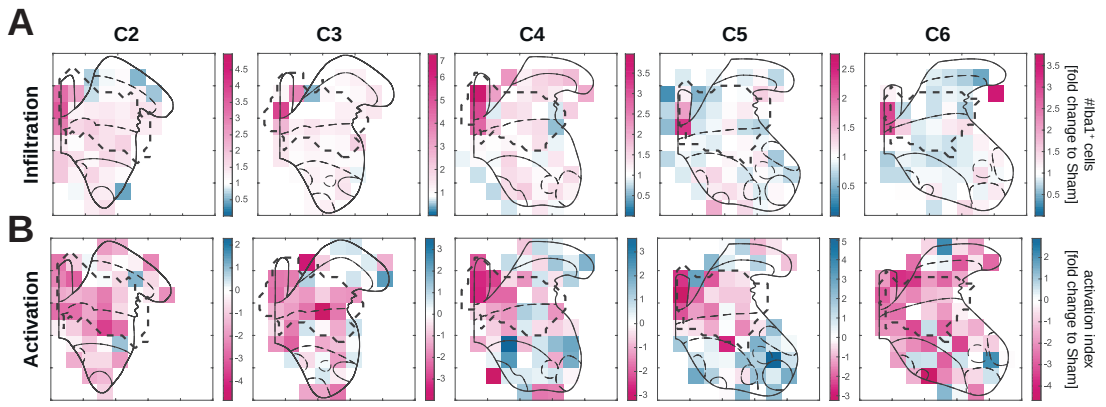
The reduction of the fractalkine receptor in the CX3CR1$^{+/GFP}$ mouse did not lead to differences in lesion volume, comparable to previous reports (Fumagalli et al., 2013, Van Der Maten et al., 2017). This was further confirmed by functional assessment in the horizontal ladder task, as we found no difference between CX3CR1$^{+/GFP}$ and wild-type C57BL/6 mice in the initial deficit after stroke at 4 dpi. In the recovery process, however, CX3CR1$^{+/GFP}$ mice showed a slower recovery rate in two parameters measured in this skilled movement task: total success score and absolute number of foot faults. Fractalkine signaling has been associated with neuron-microglia communication, and CX3CR1 deficient microglia were shown to be highly neurotoxic (Cardona et al., 2006). While it seems conceivable that CX3CR1$^{+/GFP}$ microglia/macrophages may influence survival of neurons in the penumbra in an extent that is not detectable with the methods used in this study, neuronal cell death of post-injury targets for sprouting contralesional CSMN might explain the observed functional recovery deficit. Other changes in microglia function in the CX3CR1$^{+/GFP}$ mice including reduced secretion of pro-regenerative factors cannot be excluded, however.

To further investigate the role of microglia in the stroke-denervated spinal cord, we established a tool to selectively and locally deplete microglia. Depletion of microglia in the cortex was successful after injection of 100 nl of 50 mg/ml clodronate, a substance that is pinocytozed by immune cells and leads to toxicity and death within 5 dpi (Frith et al., 1997, Torres et al., 2016). We found a selective effect of clodronate on CX3CR1^{+} cells, while astrocyte and neuron numbers were unchanged, confirming previous findings that clodronate does not affect neurons or astrocytes *ex vivo* or *in vitro* (Kohl et al., 2003, Kumamaru et al., 2012). However, an indirect effect of microglial depletion on neuronal survival in later time points cannot be excluded. In the future, an optimized injection setup as well as a clearly defined time schedule of microglial depletion will enable us to study in more detail the role of the subpopulation of activated spinal microglia on sprouting of contralesional CSMNs after stroke.

4.5 | Conclusion

In the present study, we show an activation of microglia/macrophages in the stroke-denervated spinal cord. This activation was transient in the intermediate laminae of the grey matter but persisted over much longer times in the CST in the dorsal column. A beneficial role of microglia in shaping a growth-permissive environment is highlighted by the observed functional deficit of CX3CR1$^{+/GFP}$ animals in the horizontal ladder skilled motor task. Using a newly established tool for selective and local depletion of microglia, ongoing studies are aiming to evaluate the functional roles of subpopulations of activated spinal microglia in structural plasticity after stroke.

4.6 | Supplementary Material



Supplementary Figure S4.1 – Infiltration and Activation of Iba1$^+$ cells along the cervical spinal cord (C2-C6) at 4 dpi. **(A)** Difference maps for infiltration as assessed by count of Iba1$^+$ cells in 4 dpi mice as compared to Sham group (fold change). **(B)** Difference maps for activation index calculated as longest process / soma diameter of Iba1$^+$ cells at 4 dpi compared to Sham group (fold change). No statistical differences were found between the segments and spinal levels.

4.7 | References

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5

On to the Future: Concluding Remarks and Perspectives

The aim of this work was to identify molecular factors that influence the structural plasticity underlying stroke recovery in mice. To address this, we first established the target area and time course of sprouting contralesional corticospinal motor neurons (CSMNs) that re-innervate the stroke-denervated spinal hemicord after large insults. In the course of six weeks after injury, contralesional CSMNs initiated collaterals that crossed the midline to innervate target cells in the premotor intermediate laminae IV-VII of cervical levels C3 and C6. RNA-Sequencing of this spinal target area revealed a highly time specific regulation of gene expression with the most pronounced changes observed at 28 days post injury (dpi). Through further analysis of the transcriptomic data set using gene set enrichment analysis and network analysis, two major phases were identified that might influence structural plasticity. First, in an early inflammatory response within the first week after stroke, genes related to infiltration of immune cells and phagocytosis were predominant in the gene set analysis. This was further strengthened by the histological observation of a transient activation of microglia/macrophages within the target area of the sprouting contralesional CSMNs 4 days after stroke. Reduction of fractalkine signaling in knock-out animals led to a decreased behavioral recovery rate after stroke in a skilled motor task, suggesting that microglia and neuron-microglia communication via fractalkine signaling positively affect stroke recovery. Thus, an early inflammatory phase might promote a growth-friendly tissue environment in the stroke-denervated spinal hemicord. Secondly, we found an upregulation of many growth-related genes four to six weeks after stroke. Three of the upregulated factors were confirmed as positive growth-modulators that promote growth in presence of inhibitory spinal cord extract *in vitro*. Additional differentially regulated genes of this screen may provide further candidates as modulators of different phases of structural plasticity. Taken together, these results suggest that the stroke-denervated spinal grey matter, in particular its intermediate laminae, express factors and represent a growth-promoting environment for sprouting corticospinal tract (CST) fibers originating from the contralesional motor cortex. While these findings contribute important new aspects on the molecular mechanisms of post-stroke recovery, they also raise new questions.

5.1 | On the Notion of Post-Stroke Circuitry Reformation

The formation of new circuits within the brain is one of the fundamental elements of post-stroke recovery that can be observed across several species, including rodents, primates and humans (Buetefisch, 2015, Carmichael et al., 2017, Morecraft et al., 2016, Murata et al., 2015, Ward, 2017). Which areas participate in this post-stroke rewiring seems to primarily depend on size and location of the ischemic insult (Dijkhuizen et al., 2003, Hsu and Jones, 2006, Zhu et al., 2010). After considerable tissue loss, several lines of evidence suggest that sprouting of the contralesional cortex is crucial for behavioral recovery (Bachmann et al., 2014, Benowitz and Carmichael, 2010, Kartje-Tillotson et al., 1985, Lindau et al., 2014, Morecraft et al., 2015, Ueno et al., 2012, Wahl et al., 2014). We investigated this post-stroke circuitry reformation after large unilateral stroke to the mouse motor cortex and confirmed the major termination area of these sprouting contralesional CSMNs as the intermediate laminae of spinal segments C3 and C6. Remarkably, we found that sprouting contralesional CSMNs not only switch their projection from one body side to the other within one segment, but we also observed an increase in fibers crossing the border between grey matter and the CST in the dorsal funiculus, a finding that indicates that CSMNs additionally are able to switch segments. During development of the CST, overshooting of the target innervation level can be observed with subsequent collateral branching into the appropriate target segment and elimination of the overshooting distal axon parts (Kuang et al., 1994, Kuang and Kalil, 1994). The segment switching of sprouting CSMNs after stroke may follow similar steps to re-innervate a segment rostral to the pre-stroke target. These findings raise several questions concerning post-stroke circuitry reformation.

It will be of high interest to investigate whether the segmentally switching contralesional CSMNs previously projected to a lower cervical level or whether hindlimb-designated CSMNs rewire onto cervical levels, as has been observed in case of stroke lesions which selectively eliminated forelimb sensory motor cortex (Starkey et al., 2012). This question could be analyzed using a double tracing technique by injection of a retrograde tracer into the ipsilateral hemicord before lesion at lumbar levels to label hindlimb-projecting contralateral CSMNs, or at lower cervical levels (C7-C8) to label contralateral CSMNs of other segments. After lesion induction, a second retrograde tracer may be applied to the denervated hemicord, thus labeling re-innervating CSMNs. The number of double-labeled cells will reveal whether contralesional CSMNs are able to switch their target segment to participate in forelimb recovery after stroke. Alternatively, recent studies established genetic markers for hindlimb and forelimb projecting CSMNs (Sahni et al., 2017), opening

new possibilities to address this proposed segment switch. These markers could also allow for selective silencing of subpopulations of midline crossing CSMNs using chemogenetic approaches (Alexander et al., 2009). This would enable us to address the question of the functional importance of segment switching CST fibers in post-stroke recovery. Additionally, this finding of segment-switching sprouting CSMNs should be transferred to other species, as there might be differences in the motor circuitry pre- as well as post-stroke.

As a next step, it is crucial to identify the neuronal subpopulations targeted by re-innervating contralesional CSMNs. As monosynaptic connections of CSMNs onto spinal motoneurons seem to be rare or lacking in rodents or have so far not been proven to be functional (Alstermark, 2004, Babalian et al., 1993, Bannister and Porter, 1967, Bareyre et al., 2005, Terashima, 1995), premotor interneurons are the candidates for polysynaptic connectivity to restore motor behavior. Indeed, Ueno et al. (2012) suggested two specific types of interneurons that are targeted specifically by re-innervating contralesional CSMNs in the grey matter of the stroke-denervated hemicord: propriospinal neurons (PNs) and segmental interneurons (sINs). In monkeys, the relay of cortical control via PNs has recently been associated with recovery of precision grip after CST specific lesions (Tohyama et al., 2017). Here, PN transmission may relay feedback of environmental changes to re-coordinate movements. On the other hand, sINs may relay cortical control directly onto motoneurons (MNs) of the same segment. On spinal levels C3 and C6, where increased fiber density of the contralesional CST was particularly high in our study, MNs innervate shoulder muscles of upper arm and forearm muscles, respectively. Increased innervation on these spinal levels may reflect a recovery of specific movement types such as grasping and reaching (Alstermark and Isa, 2012). However, there are several subpopulations of interneurons within the grey matter that play individual roles in the highly complex coordination of movement (Alaynick et al., 2011, Lu et al., 2015). Identification of the specific subtype of interneurons targeted by contralesional CSMNs after stroke might allow further conclusions about functional recovery of specific compensatory movements (Lai et al., 2014, Whishaw, 2000). Ideally, a mono-synaptical transneuronal anterograde tracer injected into the contralesional cortex would specifically label these target cells which could then be further analyzed for their identity and roles in movement control. Unfortunately, while several trans-synaptic anterograde tracing techniques have been proposed in recent years (Beier et al., 2016, Libbrecht et al., 2016, Zeng et al., 2017), they have not proven successful in experimental settings like the CST thus far. A promising start for developing further tools is the development of dual component synapse detectors such as mGRASP (Kim et al., 2012, Lee et al., 2016). Here, two components tethered to the pre- and post-synaptic membrane reconstitute as a

GFP signal only when neurons engage in a synapse. Further engineering of this system, potentially by binding-triggered release of *Cre* leading to translation of GFP, might allow for a labeling of the pre- and post-synapse instead of just the synaptic cleft in the future.

It is unknown what signal underlies the enhanced re-innervation of the two specific cervical levels C3 and C6 compared to other cervical levels. This signal might stem from the targets within the spinal segments C3 and C6. A differential transcriptomic profiling of the specific segments might allow for identification of these target-derived signals but would depend on knowledge of when this trigger might be expressed at its highest level. Alternatively, as shoulders and forearm muscles are used by rodents for daily living tasks such as grooming and food intake, activity of the muscles might convey a signal to the sprouting contralesional CSMNs. Indeed, rehabilitative training increases sprouting of contralesional CSMNs (Maier et al., 2008, Okabe et al., 2016, Zai et al., 2011, Zhao et al., 2013), most likely by inducing growth-promoting and reducing growth-inhibiting factors within the spinal grey matter of the stroke-denervated hemicord (Choi et al., 2016, Himi et al., 2016, Ishida et al., 2015, Li et al., 2015, Zhao et al., 2013), suggesting that muscle activity in itself can increase rewiring of the cortex. This might be tested by long-term stimulation of specific muscles or by silencing muscles using e.g. botulinum toxin with subsequent analysis of the sprouting response of the contralesional cortex after stroke.

The time frame of contralesional CSMN sprouting within the critical period after stroke was established in the mouse; it correlated well with published data in other species (Biernaskie et al., 2005, Murphy and Corbett, 2009). Increased fiber density of the contralesional CST on spinal levels C5/C6 was observed over the course of four weeks after stroke. This time frame matches the sprouting response observed in horizontally projecting cortical neurons, which increase fiber density locally in the peri-infarct cortex (Carmichael, 2003). We found a stable plateau phase of fiber density of contralesional CSMNs within the stroke-denervated hemicord from 28 days after stroke onwards, in contrast to a previous study reporting a reduction of fiber density at 42 dpi (Ueno et al., 2012). This discrepancy may stem from the lesion model applied. After non-ischemic brain lesions such as aspiration of the motor cortex, a lack of synchronous activity waves associated with triggering of axonal sprouting has been observed (Carmichael and Chesselet, 2002). This might influence the strength of contralesional motor cortex activation, resulting in an altered sprouting response. Thus, future studies should focus on defining the time frame carefully depending on the lesion model, especially when investigating potential growth-enhancing therapeutics, as they might miss the most efficacious time window for the treatment.

In future experiments, a focus should be placed on circuitry refinement in the late stages of structural plasticity and how this might follow the rules of Hebbian synaptic plasticity

(Hebb, 1949, Murphy and Corbett, 2009). Considering the beneficial effect of rehabilitation training on synaptogenesis, dendritic branching and neuronal sprouting, refinement of connections seems to be a crucial part of post-stroke recovery. These studies might make use of recently developed *ex vivo* spinal cord cultures (Fernandez-Zafra et al., 2017, Zhang et al., 2010), *in vivo* optogenetic approaches with spinal fiber implantation (Montgomery et al., 2016) or two-photon live imaging of the spinal cord target region in freely moving animals (Sekiguchi et al., 2016) concomitant with stimulation of the contralesional CST.

5.2 | On the Notion of Spinal Growth-Promotion for Structural Plasticity

We addressed gene expression changes within the stroke-denervated spinal grey matter. We found that stroke recovery is orchestrated by distinct phases of molecular changes in the denervated spinal hemicord. These results resemble the changes observed in the peri-infarct cortical region after small stroke lesions (Carmichael et al., 2005). In the spinal grey matter, an early inflammatory response is followed by highly dynamic gene expression changes at 28 to 42 days after stroke. Gene expression changes may reflect several key elements of stroke recovery, e.g. inflammation, angiogenesis and in particular circuitry repair including neurite growth, cell adhesion, synaptogenesis, synaptic refinement and myelination.

Several upregulated genes in our data set were linked to gene sets describing growth and migratory pathways at 28 dpi. These include soluble, membrane-bound and extracellular matrix linked factors which could be involved in initiation and guidance of sprouting fibers of the contralesional CST, but also of other supraspinal command centers re-wiring on a spinal level such as the brainstem nuclei (Bachmann et al., 2014). A caveat of the presented data is that information about the specific cell types that differentially regulate the proposed candidates is missing. Candidates should thus be tested for co-localization with established cell type markers. Given that the extrinsic factors may only steer growing axons when these possess the machinery to react to those signals (Cai et al., 2001), future studies should address the interaction between extrinsic factors found in our screen and intrinsic factors of sprouting neurons as established e.g. by Li et al. (2010) for horizontally projecting cortical neurons and Fink et al. (2017) for sprouting contralesional CSMNs. A highly specific "interactome" achieved by comparison of this study to intrinsic properties of sprouting neurons might feature pairs of extrinsic factors and intrinsic receptors thereof,

and provide highly specific candidates as modulators of structural plasticity after injury. These could in the future serve to develop the basis of novel neuroregenerative treatment options for stroke patients.

One open question regarding the molecular machinery of structural plasticity is whether transcriptional changes within the soma of cortical neurons reflect the machinery that reacts to extracellular stimuli at distant places, such as the spinal cord. In contrast, axonal autonomy attained by the presence of an axonal translational machinery within the growth cone might allow the growing axons to respond appropriately to guidance cues and other stimuli in their vicinity (Brittis et al., 2002, Lin and Holt, 2008, Rishal and Fainzilber, 2014). Interestingly, many of the molecules proposed to influence CST rewiring, including BDNF, Netrin-1 and Sema3a, rely on axonal translation in invertebrates or in *in vitro* vertebrate systems (Campbell et al., 2001, Jung et al., 2012, Leung et al., 2006, Yao et al., 2006). Thus, the neuronal receptors to extracellular guidance cues might not be altered within the soma but within the growth cone or branch tip, complicating the interpretation of the thus far obtained data of sprouting neurons. Currently applied technology to isolate growth cones from developmental tissue might, in the future, allow for a better understanding of the contribution of the local translation machinery to circuitry reformation (Hatch et al., 2017).

We highlighted three established growth-modulators, Semaphorin 6A (Sema6a), Netrin G2 (Ntng2) and Transforming growth factor (TGF)-β1, with the latter strongly antagonizing the neurite outgrowth inhibition of spinal cord extract *in vitro*. Future studies should therefore focus on the role of TGF-β1 *in vivo* and its influence on structural plasticity and stroke recovery. Two studies along these lines are currently ongoing in our laboratory. First, we will virally administer TGF-β1 cDNA to the spinal cord, inducing an overexpression of the protein starting from 7 dpi (to exclude direct neuroprotective effects of TGF-β1 during stroke) (Brionne et al., 2003, Dobolyi et al., 2012, Makwana et al., 2007). Using our established sprouting analysis, this study will allow us to investigate whether TGF-β1 in the spinal cord enhances sprouting of contralesional CSMNs after stroke. We hypothesize that increased expression of TGF-β1 will lead to increased structural plasticity concomitant with functional improvements of motor behavior. However, one limitation is that indirect effects of TGF-β1, e.g. by evoking inflammatory responses within the spinal cord target area from glial cells such as reactive astrocytes (Liddelow et al., 2017), cannot be excluded. Therefore, a second study will target the signaling of TGF-β1 on a more specific level by knockdown of the co-receptor TGF-βRII in contralesional CSMNs using transgenic TGF-βRII$^{flox/flox}$ mice in combination with cortical administration of a *Cre*-virus. Given the involvement of the contralesional cortex in functional recovery in this lesion model

(Bachmann et al., 2014, Wahl et al., 2014), we hypothesize that knockdown of TGF-βRII in contralesional CSMNs will decrease their fiber density by blocking their TGF-β1 response and thus functional recovery after stroke.

5.3 | On the Notion of the Spinal Inflammatory Response in Post-Stroke Recovery

The immune response to an ischemic insult is a major factor in stroke recovery and is triggered within minutes. Neuronal cell death within the immediate lesion site results in a sudden increase of extracellular ATP as well as neurotransmitters (Dávalos et al., 2000, Melani et al., 2005), leading to the activation of microglia and the infiltration of other immune cells (Iadecola and Anrather, 2012, Kono and Rock, 2008). Microglia/macrophage activation within the ischemic core can been seen within the first day (Ito et al., 2001), while a slower reaction is seen in the peri-infarct region, where microglia/macrophage activation peaks between 4 and 7 days after initial insult. This activation can also be observed at remote locations, such as the spinal cord, as shown here on a molecular basis and by morphological analysis. We describe a spatial and temporal activation profile of spinal microglia/macrophages after cortical stroke that matches the time frame of peri-infarct region activation with a peak after 4 days in the grey matter of the stroke-denervated hemicord. In the CST area within the dorsal funiculus, an increase and long-lasting activation was observed, suggesting an activation of microglia/macrophages due to Wallerian degeneration (WD) and myelin degradation.

More importantly, as to the question of how spinal microglia/macrophages are activated, is the question of why. Current literature describes both detrimental as well as beneficial roles for microglia/macrophages after central nervous system (CNS) injury (Block et al., 2007, Streit, 2005, Yong and Rivest, 2009), while the versatile microglia/macrophage response might be stimulus-dependent (Hanisch and Kettenmann, 2007). After pathological stimuli, microglia are known to phagocytose cellular and myelin debris, which may directly affect the success of regeneration (Neumann et al., 2009). Furthermore, microglial activation might also prime pre-injury targets that have lost their input: the concept of synaptic stripping describes phagocytosis of "vacant" dendrites which could clear up space for re-innervating fibers to synapse onto the pre-injury targets (Blinzinger and Kreutzberg, 1968, Trapp et al., 2007). An experiment to investigate dendritic stripping would include labeling of the pre-injury target cells of CSMN by injection of monosynaptic anterograde

tracers from the motor cortex. As discussed above in section 5.1, these tracers are under investigation in many labs but are so far not working reliably. Once they are, one might further engineer them to encode different fluorophores according to viral brainbow techniques (Kobiler et al., 2010, Lichtman and Sanes, 2008) or to label the infected cells only sparsely (Lu and Yang, 2017) to allow for distinction of selected target cells. After stroke induction, this specific labeling might allow for assessment of microglial engulfment of synaptic elements of the pre-injury target cells using microglia-specific antibodies (Bennett et al., 2016) or reporter mice (Buttgereit et al., 2016, Jung et al., 2000). This is, however, driven by the hypothesis that re-innervating contralesional CSMN indeed establish connections to pre-injury targets.

One crucial factor limiting our understanding of microglial function after activation is that microglial morphology does not necessarily reflect function (Hirbec et al., 2017). As activated microglia may release both pro- and anti-inflammatory cytokines, knowledge about the microglial transcriptomic profile is pivotal for each pathological condition. This has been addressed for several CNS diseases thus far, such as amyothropic lateral sclerosis, spinal cord injury or multiple sclerosis (Lewis et al., 2014, Noristani et al., 2015, Zhu et al., 2017). However, our study investigates a narrow subpopulation of activated spinal microglia in the intermediate laminae of the cervical spinal cord. Currently applied sorting techniques such as fluorescence-activated cell sorting or laser-capture microdissection would result in an insufficient yield for a thorough transcriptomic profiling. Using "Patch-Seq", a combination of patch-clamping cells in an electrophysiological setup and single cell RNA-sequencing (Cadwell et al., 2016, Fuzik et al., 2016), would allow for a specific selection of activated microglia within the intermediate laminae of the stroke-denervated hemicord to evaluate their responses and reaction profile at the different post-injury time points.

Apart from the early response of microglia/macrophages to cortical stroke observed, we have also shown a role of microglia 42 days after stroke, a time window in which refinement of the circuitry is assumed (Carmichael et al., 2017, Ueno et al., 2012). We have found an upregulation of complement cascade related gene sets in our whole-tissue transcriptomic study of the stroke-denervated grey matter. The complement cascade has been linked to synaptic remodeling (Stevens et al., 2007) and interestingly enough, microglia in their surveillance state seem to be able to sense synaptic activity (Kettenmann et al., 2013). This might allow them to participate in circuit reformation and refinement after stroke, thus turning the spotlight on microglia/macrophages as effectors of several phases of stroke recovery.

5.4 | Conclusion

The work presented in this thesis provides insights into the endogenous molecular machinery of an important target of the motor cortex, the cervical spinal cord, during spontaneous recovery after stroke in adult mice. We provide evidence that the stroke-denervated spinal grey matter regulates its gene expression in a highly specific temporal manner. We propose that an early inflammatory phase triggers a growth-friendly environment for re-innervating contralesional CSMNs. Several growth-promoting genes of secreted factors were upregulated and we confirmed their potential to enhance neurite outgrowth in a growth inhibitory CNS extract environment *in vitro*. Our data set provides a solid starting point for future studies addressing key elements of the post-stroke recovery process, with the goal to improve neuroregenerative treatment options for stroke patients.

5.5 | References

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