Title

Targeting pro-growth extracellular matrix proteins in the post-stroke brain

Author

Bridges, Samuel

Publication Date

2022

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Targeting pro-growth extracellular matrix proteins in the post-stroke brain

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy in Neuroscience

by

Samuel Patrick Bridges

2022

ABSTRACT OF THE DISSERTATION

Targeting pro-growth extracellular matrix proteins in the post-stroke brain

by

Samuel Patrick Bridges
Doctor of Philosophy in Neuroscience
University of California, Los Angeles, 2022
Professor S. Thomas Carmichael, Chair

Stroke is a devastating neurological condition and the leading cause of lasting motor, sensory and cognitive deficits. These disabilities occur due to the central nervous system's limited ability to repair itself after injury. Following cortical ischemic stroke, adjacent surviving tissue initiates a limited process of repair characterized in part by axonal sprouting and cortical remodeling. Neurons that undergo axonal sprouting have a unique transcriptional profile, differentially regulating genes in a time and age-specific manner. Among these genes are a number of differentially regulated extracellular matrix proteins, including those that are important in peripheral nervous system regeneration.

The extracellular matrix is comprised of a network of proteins that provide support to cells throughout the body. In the central nervous system, the extracellular matrix is implicated in development, ensuring developing axons are guided to the correct destination, modulating signaling pathways that coordinate proliferating cells, and providing a general environment of

support. In the mature brain, the extracellular matrix plays key roles in plasticity and support, and undergoes dramatic changes in composition and function following injury. This thesis investigates two differentially regulated extracellular matrix proteins for their ability to induce axonal sprouting and functional recovery following stroke. Matrilin-2 (Matn2) is an adapter protein important for peripheral nerve regeneration but paradoxically is one of the most down-regulated genes in sprouting neurons in young animals. Unique cartilage matrix associated protein (Ucma) is a novel extracellular matrix protein that is one of the most upregulated genes in young animals, but whose expression is almost totally abolished in aged animals following stroke, potentially contributing to the lack of recovery seen as a result of age. Both proteins increased neurite outgrowth in primary neurons, and rescued neurite outgrowth in a stroke-like growth inhibitory environment. Viral overexpression of Matn2 and Ucma revealed that both enhanced axonal sprouting in the post-stroke brain. When assessing ability to enhance functional recovery, Matn2 overexpression accelerated return of motor function to baseline, while Ucma overexpression initially accelerated recovery but only partially restored motor function. Further studies to test translatability of these proteins revealed that restriction of Matn2 adjacent to the injury site still enhanced axonal sprouting, but Ucma overexpression in this context was detrimental to axonal sprouting.

In summary, these studies identify novel pro-growth extracellular matrix proteins that enhance axonal sprouting and functional recovery after stroke and identify the pro-growth extracellular matrix as a new target for neural repair after injury.

The dissertation of Samuel Patrick Bridges is approved.

Samantha Butler

Jason Hinman

Alvaro Sagasti

S. Thomas Carmichael, Committee Chair

University of California, Los Angeles

2022

Table of Contents

List of Figures

Page

Acknowledgements

The work described throughout this dissertation would not have been possible without the assistance, mentorship, and support given to me by a number of people during the course of my studies. First, I must acknowledge my mentor, Tom Carmichael. Tom provided an ideal learning environment, supporting and pushing me towards doing the best science possible. His enthusiasm for this work was ever present, and kept me encouraged during the good times and bad.

The Carmichael lab has proved to be an excellent environment for learning and growing, and I am so fortunate to have worked with such talented and giving individuals. My fellow graduate students, Catherine, David, Thanh, Kiro, Natalie and Elle have commiserated with me during hard times and cheered me on during good, all while we traveled along the same path of graduate school. A heartfelt thanks to Catherine for telling me Tom was recruiting graduate students during my first year, and starting me down this path. Post-docs in the lab have served as mentors and sounding boards, among them Amy, Irene, Teena and Alec (to name a few). To the technicians and project scientists in the lab, Jenni, Jose, Nora, Tiffani and so many others, thank you for keeping everything sane and moving forward. Altogether, the Carmichael lab has served as a scientific family, without whom this work would not have been possible.

I have been fortunate enough to work with some of the talented undergraduate students at UCLA. To Tim, Luigi and Charlie, thank you for giving your time and energy to help with these studies, and for letting me practice my own style of mentorship. I know you will all do excellent things in life, and I look forward to seeing where all of you go in life.

Thank you to my committee members, Samantha Butler, Jason Hinman and Alvaro Sagasti, for your feedback throughout this work and enthusiasm for the results shown. I thank you especially for reminding me to be excited about positive data. My thanks to the Broad Stem Cell Research Center Microscopy Core and Ken Yamauchi for assistance with super resolution microscopy.

The Neuroscience Interdisciplinary Program at UCLA has helped me grow as a scientist through multiple opportunities, bringing in top tier scientific talent from all over the work and providing a supportive network. My thanks in particular to Felix Schweizer for his leadership and desire to see all students grow while still having fun, and Jenny Lee for being a constant smiling presence and veritable fount of knowledge of all things administrative.

Finally, my thanks to my family, close and far away. You have all been so enthusiastic about the work I am doing, and fostered my growth throughout this process. To Thomas, Brita, and my nephew Jack, you inspired me to continue doing good work, and gave me candid pictures to look at during stressful times. To my mother, Penny, your delight in the images taken throughout helped keep me excited about what came next, and your reminders to relax sometimes were much appreciated. Finally, to my two cats, Hobbes and Loki, you helped keep me sane during pandemic lockdowns, and provided welcome distractions throughout the years.

The work described here was supported by the National Institute of Neurological Disorders and Stroke, the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation, and UCLA Department of Neurology.

Vita

Education

Doctor of Philosophy, Neuroscience — anticipated 2022
University of California — Los Angeles, CA

Bachelor of Science, Neuroscience — August 2015
George Mason University — Fairfax, VA
Honors: Summa Cum Laude

Bachelor of Science, Health Sciences — August 2009
James Madison University — Harrisonburg, VA

Experience

Graduate Student Researcher — June 2016-Present
S. Thomas Carmichael Lab — University of California Los Angeles

Research Assistant — March 2014 - June 2015
N. Kabbani Lab — George Mason University

Publications and Posters

King, J. R., Nordman, J. C., **Bridges, S. P.**, Lin, M.-K., & Kabbani, N. (2015). Identification and Characterization of a G Protein-binding Cluster in α7 Nicotinic Acetylcholine Receptors. *The Journal of Biological Chemistry*, *290*(33), 20060–70.

Sozmen, E. G., DiTullio, D. J., Rosenzweig, S., Hinman, J. D., **Bridges, S. P.**, Marin, M. A., Kawaguchi, R., Coppola, G., & Carmichael, S. T. (2019, November 20). White matter stroke induces a unique oligo-astrocyte niche that inhibits recovery. Journal of Neuroscience.

Society for Neuroscience 2014

A chilling connection; menthol's effect on nicotinic acetylcholine receptor expression and synaptic structure in the rodent brain. S.P. Bridges et. al.

Virginia Youth Tobacco Forum January 2015

Data Blitz – *Synaptic Biomarkers of Nicotine and Menthol Addiction in the Brain*

Society for Neuroscience 2017

Matrilin-2: a pro-growth extracellular matrix protein in the post-stroke brain. S.P Bridges, S.T. Carmichael

American Society for Matrix Biology 2018 Biennial Meeting

Assessment of the effect of a pro-growth extracellular matrix protein, matrilin-2, on axonal sprouting in the post-stroke brain

S.P. Bridges, M. Machnicki, S.T. Carmichael

Society for Neuroscience 2018

Assessment of the effect of a pro-growth extracellular matrix protein, matrilin-2, on axonal sprouting in the post-stroke brain

S.P. Bridges, M. Machnicki, S.T. Carmichael

Leadership

Neuroscience Interdepartmental Program Admissions Committee

Graduate Student Representative 2017-2018 term

Chapter 1

Introduction

1.1 Stoke in the clinical context

Stroke remains a clinical challenge, affecting some 795,000 people annually at a cost of $52.8 billion (Tsao et al., 2022). On the whole, stroke is declining as a cause of mortality in the United States; despite this, there remains a substantial number of individuals who suffer the long-term effects of stroke, including motor deficits, cognitive decline, and psychological issues including depression. Of those who survive stroke, only 56% return to work after one year (Duong et al., 2019), 40% have chronic motor disability (Cramer et al., 1997), one in three suffer depression (Towfighi et al., 2017), and 30% develop vascular dementia, the second most common type of dementia (Savva & Stephan, 2010). Further, a substantial proportion of individuals who suffer stroke fail to recognize the symptoms and seek urgent intervention, leading them dependent on delayed treatments such as motor rehabilitation and preventative methods against subsequent strokes. These delayed treatments are limited in efficacy, leading to a continued need for improved therapies to return individuals to baseline ability. In fact, if measuring motor recovery after stroke, which is a particular focus of these studies, three of four patients have an impairment in performing ADLs at hospital admission, and only about one third of patients who have completed rehabilitation have achieved normal neurological function (Jorgensen et al., 1999)Every second patient does not regain function of the affected arm six months after stroke(Kwakkel et al., 2003)).

Currently, the most efficient treatments are limited to the acute phase of stroke. Recombinant tissue plasminogen activator (tPA) is the only approved pharmacological intervention for acute stroke. tPA, when administered intravenously, enzymatically breaks down clots and restores blood flow to areas affected by the stroke, resulting in improvement of symptoms by up to 50% (Saver et al., 2013). However, tPA must be administered within 4.5 hours of symptom onset, resulting in fewer than 10% of otherwise eligible patients receiving this treatment (Bambauer et al., 2006; Saver et al., 2013). An alternative treatment for stroke is

endovascular mechanical thrombectomy, in which blood clots are directly removed. Thrombectomy has the benefit of being available for use up to 24 hours after symptom onset, but is limited to strokes affecting large vessels only, resulting in being a therapy option for 3-22% of ischemic stroke patients (Mokin et al., 2019). Further, mechanical thrombectomy requires specialized training and equipment, resulting in the therapy being disproportionately unavailable to patients in rural areas and underserved populations (Kamel et al., 2021).

The degree to which an individual spontaneously recovers from stroke is highly variable, depending on a variety of factors including genetics, comorbidities and therapies offered during the recovery period. Post-stroke rehabilitation is beneficial, but limited in the degree of recovery, leading to lasting motor and cognitive deficits in many patients requiring long term specialized care (Wattanapan et al., 2020). As such, the need for therapies to further enhance functional recovery in the chronic stroke phase remains an area of high interest in the stroke field.

1.2 Effects of ischemic stroke on the brain

Of the two main types of stroke, ischemic and hemorrhagic, ischemic is the most common type, representing 87% of all strokes (Tsao et al., 2022). In ischemic stroke, blood supply is interrupted to an area of the brain by a blood clot or embolus. This interruption leads to a cascade of cellular and environmental changes, affecting all cells in the affected area. Initial events following interruption include excitotoxicity of energy deprived neurons due to depolarization and inability to maintain membrane potential. The process is rapidly irreversible and given the near absolute inability of the central nervous system to regenerate neurons, leads to persistent deficits.

As time progresses, surviving tissue adjacent to the stroke, also known as the peri-infarct, undergoes significant changes that form the basis for limited endogenous recovery, although some studies in human patients indicate long term remodeling of corticospinal projections may be a greater predictor of functional recovery in stroke patients (Branscheidt et al., 2022). In the weeks following stroke, surviving tissue undergoes dramatic changes that partially recapitulate lost connections that initially arose from neurons in the infarct core (Carmichael et al., 2016; Frost et al., 2003; Jones & Adkins, 2015). A primary mechanism through which this reorganization occurs is axonal sprouting, in which surviving neurons extend collaterals as explained in the subsequent section. This region is thus a prime target for intervention to further enhance functional recovery.

1.3 Axonal Sprouting as a form of endogenous repair

Axonal sprouting refers to the process through which surviving neurons in the peri-infarct region extend new collaterals in response to injury. At a baseline, axonal sprouting can first be detected within one week after stroke (Li et al., 2010), and depending on the location and size of stroke, can be seen projecting towards premotor, motor and somatosensory areas. Functional imaging and cortical stimulation and inactivation studies in human stroke patients show evidence of cortical reorganization through axonal sprouting, with reorganization of ipsilesional peri-infarct cortex being closely associated with post-stroke functional recovery (Benowitz & Carmichael, 2010; Chunyong et al., 2022; Dancause, 2006). Neurons that sprout have a unique transcriptome that was characterized in the foundational study for the work described herein (Li

et al., 2010). This baseline level of axonal sprouting occurs in response to injury and represents the limit of endogenous repair, remapping cortical connections to partially restore lost function.

Increases in ipsilesional axonal sprouting has been causally associated with improved motor recovery (Li et al., 2015; Overman et al., 2012), while blockade of factors known to induce axonal sprouting is associated with worse functional outcomes (Li et al., 2015). This increased axonal sprouting, also termed reparative axonal sprouting (Carmichael et al., 2017), generally follows a similar growth pattern as that seen in untreated stroke but with a greater magnitude. This increase can be due to reactivation of growth programs, such as that seen with growth factor administration (Li et al., 2015) or blocking the effect of known inhibitors of axonal outgrowth (Overman et al., 2012; Tsai et al., 2011) during the subacute stroke phase, after the inflammatory processes have largely resolved but neurons are sensitive to manipulation. These studies show that increasing axonal sprouting is causally associated with better functional recovery, although the mechanism is unknown.

Axonal sprouting is associated with functional recovery, but is limited in the post-stroke brain. Extracellular factors limit the ability of axons to sprout to a greater degree, including secretion of ephrin-A5 by reactive astrocytes (Overman et al., 2012), and secretion of chondroitin sulfate proteoglycans (CSPGs) (Galtrey & Fawcett, 2007b), both of which restrict axonal sprouting. Secretion of these factors does have some benefit, however, acting as neuroprotective elements to prevent injury to surviving cells from the highly inflammatory stroke core (Duan et al., 2015). Further, ablation of CSPGs, long thought to be the primary component of the ECM that restricts the ability of axons to regenerate following injury, has resulted in only partial return of function (Soleman et al., 2012), indicating that limited axonal sprouting and

functional recovery is not solely due to inhibitory factors, but may also include intrinsic factors that restrict repair

The studies described here have focused on ipsilesional axonal sprouting arising from the peri-infarct motor cortex. While characterized as beneficial, axonal sprouting can be detrimental to functional recovery, in what is termed "unbounded" axonal sprouting. Unbounded axonal sprouting can occur when glial growth inhibitors, such as a Nogo-A function blocking antibody, are administered simultaneously with increasing the activity of reforming circuits, such as motor rehabilitation (Wahl et al., 2014), and results in a worse overall recovery. This process of unbounded axonal sprouting after stroke is also seen with blockade of an astrocyte growth inhibitory molecule, in conjunction with forced overuse of the stroke-affected forelimb, in which axonal sprouting occurs throughout almost the entire cortex (Overman et al., 2012). This phenomenon may occur due to the uncontrolled directionality of new axon collaterals, or an instability in the sprouting connections such that they are exquisitely driven by high patterns of behavioral activity. Such an exuberant process of axonal sprouting results in aberrant connections to areas that are otherwise not implicated in functional recovery.

1.4 Transcriptomic signature of sprouting neurons

A sprouting transcriptome was initially characterized by the Carmichael lab, identifying differentially regulated genes in neurons that spontaneously extend collaterals compared to those that do not (Li et al., 2010) and confirmed in additional studies with the identification of a specific growth factor in this process (Li et al., 2015). Neurons in the peri-infarct were initially labeled with a retrograde fluorescent tracer, and seven days later a different tracer was injected

into the same location. Neurons that expressed only the second tracer were identified as having extended new axons into the injection site and were characterized compared to those neurons that did not express a new projection pattern. Sprouting neurons were characterized by, among others, increased expression of axonal guidance, growth factor and cytoskeleton remodeling genes. Surprisingly, several ECM genes are differentially regulated in sprouting neurons after stroke

1.5 The extracellular matrix of uninjured and injured brain

The extracellular matrix (ECM) consists of the three-dimensional scaffold that supports all cells. Within the brain, the ECM is composed of proteoglycans, glycoproteins, linker and adapter proteins such as Matrilin-2 (Deák et al., 1999), and more common ECM proteins such as collagens limited to specific domains such as the basement membrane. During development, the ECM plays a role in formation of neural circuits through facilitation of axonal pathfinding (Myers et al., 2011). Various components of the developing ECM, such as laminin and its interaction with integrin receptors on extending growth cones, facilitate neurite outgrowth (Baeten & Akassoglou, 2011; McKerracher et al., 1996; Pires-Neto et al., 1999). Additionally, components of the ECM ensure extending axons terminate in the correct location through growth-attractive and repulsive guidance cues. Such cues include netrin-1, which was initially believed to be a diffusible guidance cue (Kennedy et al., 1994) but has recently been characterized as more of a haptotactic guidance cue that guides developing axons along a set path (Dominici et al., 2017; Varadarajan et al., 2017). Haptotaxis refers to the tendency of cells, and in the case of neurons axons, to migrate directionally along a gradient of specific cues bound

to a substrate such as the ECM (Alvarez et al., 2021; Ricoult et al., 2015), An additional growth-attractive guidance cue characterized *in vitro* is nerve growth factor (Vahlsing et al., 1991), in which axons extend preferentially towards an increasing gradient of a growth factor to a certain point. Growth repulsive cues are also critically important, ensuring that axonal tracts are directed to the correct destination and preventing innervation of incorrect targets (Maeda, 2015; Mukhopadhyay et al., 2004). Such repulsive cues, including ephrin-a5 (Canty & Murphy, 2008) and semaphorin 3a (Y. Luo et al., 1993), are well characterized in development. However, ephrin-A5 has also been targeted after ischemic stroke. An earlier study found that astrocytes upregulate ephrin-A5 after stroke in peri-infarct cortex, and administration of a neutralizing antibody against ephrin-A5 post-stroke enhances axonal sprouting and accelerates motor recovery (Overman et al., 2012). Injection of the ECM protein glypican or an enzyme that degrades CPSGs chondroitinase ABC (ChABC) directly into the infarct core seven days after stroke partially recovered behavior, reduced GFAP intensity as a measure of astrocyte reactivity, and increased MAP2 expression, indicating the possibility of increased axonal sprouting (Hill et al., 2012).This result indicates that sprouting neurons are sensitive to components of the ECM and factors secreted into the extracellular space, whether through sensitivity remaining after development or reversion of these neurons to a more development-like state (Poplawski et al., 2020).

The ECM of the adult brain was long thought to be static, serving only as a scaffold and support system for cells once development has completed. In recent years, however, the ECM has been better characterized as a dynamic component of the mature ECM (Benarroch, 2015). A core function of the ECM of the brain is support of neural circuits, but these circuits are sensitive to stimulation and change in response to stimulation (Cirillo, 2021; von Bastian et al., 2022).

Peri-neuronal nets (PNNs) are specialized ECM subdomains that surround the cell body of numerous types of neurons and are composed of chondroitin sulfate proteoglycans (CSPGs), hyaluronic acid and various other specialized ECM proteins (Carulli et al., 2006). Most of these components are synthesized and deposited by the neurons themselves in addition to neighboring cells (Bosiacki et al., 2019; Lander et al., 1998) PNNs function in part through stabilization of synapses and anchoring of specific receptor types, manipulating the excitability of components of a circuit. PNNs thus must change in response to stimuli, in order to allow strengthening of new synapses and elimination of synapses no longer needed.

As the ECM is a dynamic component of the adult brain that can respond to change, so does it change in response to stroke (Baeten & Akassoglou, 2011; Ellison et al., 1999). Numerous ECM proteins are upregulated following the initial insult, including osteopontin (Ellison et al., 1998; Liu et al., 2017; Stanic et al., 2016), collagens (Williamson et al., 2021), and CSPGs (Asher et al., 2002). Some changes, such as the upregulation in osteopontin, can be beneficial, as osteopontin has a stimulating effect on axonal outgrowth and is beneficial for functional recovery (Liu et al., 2017). Others have a mixed effect. Upregulation of CSPGs has an initial neuroprotective effect, segregating the infarct area and protecting healthy tissue from reactive immune cells (Duan et al., 2015), while also exerting a growth suppressive effect on axons that would otherwise sprout (Galtrey & Fawcett, 2007b). These effects demonstrate two key points for the studies described throughout this work, that surviving neurons are impacted by changes in the ECM post-stroke, and that the post-stroke ECM is a target for enhancing functional recovery.

1.6 Targeting the ECM for stroke recovery

As described before, the ECM undergoes changes in composition and function in the post-stroke brain. A large volume of previous work has focused on manipulating putative growth-inhibitory components of the post-stroke brain, with particular focus on CSPG upregulation. Such studies often used a bacterial enzyme, chondroitinase ABC, which degrades CSPGs under the belief that removal of CSPGs in the peri-infarct area will result in a growth permissive environment more conducive to repair (García-Alías et al., 2009; Hill et al., 2012; Soleman et al., 2012). These studies show some degree of recovery following digestion of CSPGs, but it is incomplete. As CSPGs interact with various receptors, including protein tyrosine phosphatase receptors (PTPσ), inhibition of this receptor can enhance functional recovery (F. Luo et al., 2021). However, in other models of CNS injury, such as partial spinal cord transection, treatment degrading CSPGs only allows partial recovery and regeneration (Bradbury et al., 2002), indicating that it is not enough to remove a growth inhibitory environment.

Studies evaluating pro-recovery ECM proteins following stroke are lacking, despite the known ability of surviving neurons to respond to their extracellular environment and their production of ECM components (Li et al., 2015; Liu et al., 2017). It has been shown, following spinal cord injury, that axons are capable of growing through a growth inhibitory environment when given sufficient stimulus, such as exposure to growth factors (Anderson et al., 2016). Treatment with components of the ECM can enhance functional recovery following stroke (Lee et al., 2011; Trout et al., 2021), and hydrogels incorporating ECM proteins have been shown to be beneficial for repair processes in stroke and spinal cord injury (Anderson et al., 2016; Nih et al., 2018; Tukmachev et al., 2016). Given these results, combined with knowledge that neurons

are capable of reverting to a more embryonic-like, and thus development-like phenotype (Poplawski et al., 2020), modulation of the ECM through addition of pro-growth factors is an area that merits further study.

1.7 ECM proteins differentially regulated in sprouting neurons as potential therapeutics

Following stroke, sprouting neurons have a unique transcriptomic signature (Li et al., 2010). These changes in gene expression offer insight to the biology underlying endogenous recovery, and potential reasons for why this recovery is limited. Of note, no components of CSPGs, such as aggrecan, brevican or phosphacan, showed changes in gene expression in either young or aged sprouting neurons (Li et al., 2015). This is significant, as previous work has shown the negative impact these ECM proteins have on neurite extension (Inatani et al., 2001; Snow et al., 1990; Snow & Letourneau, 1992; Yamada et al., 1997). The lack of differential expression of these growth inhibitory proteins, despite their being generated by neurons at baseline (Bosiacki et al., 2019; Lander et al., 1998), limits the impact these proteins have on endogenous axonal sprouting. These studies focus on ECM proteins that maintain the pro-growth state of sprouting neurons.

Many ECM genes are differentially regulated in sprouting neurons post-stroke, with some showing a paradoxical down-regulation, and others showing up-regulation in young animals and down-regulation in aged. In this study, I focused on one ECM protein, matrilin-2 (Matn2), that enhances axon regeneration in peripheral nerve injury (Malin et al., 2009) and promotes functional recovery in white matter stroke (Sozmen et al., 2019) but is significantly down-regulated by 52% in young animals (Li et al., 2010). This down-regulation was seen as

paradoxical given Matn2's importance in peripheral nerve regeneration (Malin et al., 2009), and was seen as one component that explains differences seen in peripheral versus central nervous system regeneration (Chandran et al., 2016). As such, overexpression of Matn2 in the post-stroke brain was hypothesized to overcome this limitation and enhance functional recovery.

Another gene of interest is unique cartilage matrix associated protein (Ucma). In young animals, sprouting neurons had upregulated Ucma expression by 69% seven days post-stroke, making it one of the most upregulated genes in sprouting neurons (Li et al., 2010). In aged animals, Ucma expression was almost completely abolished, with a 98% reduction observed (Li et al., 2010). This remarkable divergence was of interest, as age is a factor in determining degree of recovery after suffering stroke (Kugler et al., 2003; Saposnik et al., 2008). Ucma is a newly characterized ECM protein, but does show aggrecanase inhibitor activity outside the brain (Seuffert et al., 2018). Aggrecanase, also known as ADAMTS4, is an enzyme that degrades aggrecan and is involved in ECM remodeling (Gottschall & Howell, 2015). As stated previously, aggrecan is a major type of CSPG (Dauth et al., 2016), is inhibitory to neurite outgrowth (Snow et al., 1990; Snow & Letourneau, 1992) and is one of several ECM proteins that has been blamed for the lack of regeneration in the post-stroke brain (Galtrey & Fawcett, 2007a; Kwok et al., 2014). As such, overexpression of Ucma could have a detrimental effect, inhibiting the ability of the brain to remodel the ECM and remove a growth inhibitory environment. Alternative, Ucma overexpression may aid in strengthening the new circuits formed during the recovery process through stabilization of perineuronal nets formed during recovery. Given the minimal information about Ucma at present, evaluation of its ability enhance neurite outgrowth is merited.

1.8 Conclusions and motivation for the study

Ischemic stroke results in dramatic changes to the surrounding surviving tissue, priming it for cortical reorganization and recovery of function in part through axonal sprouting. Axonal sprouting is one of the few identified intrinsic means of functional recovery after stroke, and yet it is limited. Sprouting neurons have a unique transcriptional profile, but changes in gene expression is both limited, returning to baseline prior to full functional recovery, and in some instances paradoxical, as neurons downregulate expression of certain genes that are beneficial in other types of nerve injury, as in the case of Matn2 (Li et al., 2010; Sozmen et al., 2019).

Identification of ECM proteins that promote axonal sprouting is an area ripe for review. Rather than remove a growth inhibitory environment, this study shall identify ECM proteins that promote axonal sprouting and functional recovery in spite of a growth inhibitory environment through direct stimulation of neurons. It is hoped that these studies will result in a better understanding of the role of the pro-growth ECM in functional recovery after stroke, and the identification of novel therapeutics for future studies.

1.9 References

Alvarez, S., Varadarajan, S. G., & Butler, S. J. (2021). Dorsal commissural axon guidance in the developing spinal cord. *Current Topics in Developmental Biology*, *142*, 197–231. https://doi.org/10.1016/BS.CTDB.2020.10.009

Anderson, M. A., Burda, J. E., Ren, Y., Ao, Y., O'Shea, T. M., Kawaguchi, R., Coppola, G., Khakh, B. S., Deming, T. J., & Sofroniew, M. V. (2016). Astrocyte scar formation aids CNS axon regeneration. *Nature*, *532*(7598), 195. https://doi.org/10.1038/NATURE17623

Asher, R. A., Morgenstern, D. A., Shearer, M. C., Adcock, K. H., Pesheva, P., & Fawcett, J. W. (2002). Versican Is Upregulated in CNS Injury and Is a Product of Oligodendrocyte Lineage Cells. *The Journal of Neuroscience*, *22*(6), 2225. https://doi.org/10.1523/JNEUROSCI.22-06-02225.2002

Baeten, K. M., & Akassoglou, K. (2011). Extracellular matrix and matrix receptors in blood-brain barrier formation and stroke. *Developmental Neurobiology*, *71*(11), 1018–1039. https://doi.org/10.1002/dneu.20954

Bambauer, K. Z., Johnston, S. C., Bambauer, D. E., & Zivin, J. A. (2006). Reasons Why Few Patients With Acute Stroke Receive Tissue Plasminogen Activator. *Archives of Neurology*, *63*(5), 661–664. https://doi.org/10.1001/ARCHNEUR.63.5.661

Benarroch, E. E. (2015). Extracellular matrix in the CNS. *Neurology*, *85*(16), 1417–1427. https://doi.org/10.1212/WNL.0000000000002044

Benowitz, L. I., & Carmichael, S. T. (2010). Promoting axonal rewiring to improve outcome after stroke. *Neurobiology of Disease*, *37*(2), 259–266.

https://doi.org/10.1016/J.NBD.2009.11.009

Bosiacki, M., Gąssowska-Dobrowolska, M., Kojder, K., Fabiańska, M., Jeżewski, D., Gutowska, I., & Lubkowska, A. (2019). Perineuronal Nets and Their Role in Synaptic Homeostasis. *International Journal of Molecular Sciences*, *20*(17). https://doi.org/10.3390/IJMS20174108

Bradbury, E. J., Moon, L. D. F., Popat, R. J., King, V. R., Bennett, G. S., Patel, P. N., Fawcett, J. W., & McMahon, S. B. (2002). Chondroitinase ABC promotes functional recovery after spinal cord injury. *Nature 2002 416:6881*, *416*(6881), 636–640. https://doi.org/10.1038/416636a

Branscheidt, M., Ejaz, N., Xu, J., Widmer, M., Harran, M. D., Cortés, J. C., Kitago, T., Celnik, P., Hernandez-Castillo, C., Diedrichsen, J., Luft, A., & Krakauer, J. W. (2022). No evidence for motor-recovery-related cortical connectivity changes after stroke using resting-state fMRI. *Journal of Neurophysiology*, *127*(3), 637–650. https://doi.org/10.1152/JN.00148.2021/ASSET/IMAGES/LARGE/JN.00148.2021_F006.JPEG

Canty, A. J., & Murphy, M. (2008). Molecular mechanisms of axon guidance in the developing corticospinal tract. *Progress in Neurobiology*, *85*(2), 214–235. https://doi.org/10.1016/J.PNEUROBIO.2008.02.001

Carmichael, S. T., Kathirvelu, B., Schweppe, C. A., & Nie, E. H. (2016). Molecular, cellular and functional events in axonal sprouting after stroke. *Experimental Neurology*. https://doi.org/10.1016/j.expneurol.2016.02.007

Carmichael, S. T., Kathirvelu, B., Schweppe, C. A., & Nie, E. H. (2017). Molecular, Cellular and

Functional Events in Axonal Sprouting after Stroke. *Experimental Neurology*, *287*(Pt 3), 384. https://doi.org/10.1016/J.EXPNEUROL.2016.02.007

Carulli, D., Rhodes, K. E., Brown, D. J., Bonnert, T. P., Pollack, S. J., Oliver, K., Strata, P., & Fawcett, J. W. (2006). Composition of perineuronal nets in the adult rat cerebellum and the cellular origin of their components. *Journal of Comparative Neurology*, *494*(4), 559–577. https://doi.org/10.1002/CNE.20822

Chandran, V., Coppola, G., Nawabi, H., Omura, T., Versano, R., Huebner, E. A., Zhang, A., Costigan, M., Yekkirala, A., Barrett, L., Blesch, A., Michaelevski, I., Davis-Turak, J., Gao, F., Langfelder, P., Horvath, S., He, Z., Benowitz, L., Fainzilber, M., … Geschwind, D. H. (2016). A Systems-Level Analysis of the Peripheral Nerve Intrinsic Axonal Growth Program. *Neuron*, *89*(5), 956. https://doi.org/10.1016/J.NEURON.2016.01.034

Chunyong, L., Yingkai, L., Fuda, L., Jiang, C., & Liu, Y. (2022). Longitudinal changes of motor cortex function during motor recovery after stroke. *Https://Doi.Org/10.1080/10749357.2022.2051829*. https://doi.org/10.1080/10749357.2022.2051829

Cirillo, J. (2021). Physical activity, motor performance and skill learning: a focus on primary motor cortex in healthy aging. *Experimental Brain Research 2021 239:12*, *239*(12), 3431–3438. https://doi.org/10.1007/S00221-021-06218-1

Cramer, S. C., Nelles, G., Benson, R. R., Kaplan, J. D., Parker, R. A., Kwong, K. K., Kennedy, D. N., Finklestein, S. P., & Rosen, B. R. (1997). A Functional MRI Study of Subjects Recovered From Hemiparetic Stroke. *Stroke*, *28*(12), 2518–2527. https://doi.org/10.1161/01.STR.28.12.2518

Dancause, N. (2006). Vicarious function of remote cortex following stroke: Recent evidence from human and animal studies. *Neuroscientist*, *12*(6), 489–499. https://doi.org/10.1177/1073858406292782

Dauth, S., Grevesse, T., Pantazopoulos, H., Campbell, P. H., Maoz, B. M., Berretta, S., & Parker, K. K. (2016). Extracellular matrix protein expression is brain region dependent. *Journal of Comparative Neurology*, *524*(7), 1309–1336. https://doi.org/10.1002/cne.23965

Deák, F., Wagener, R., Kiss, I., & Paulsson, M. (1999). The matrilins: a novel family of oligomeric extracellular matrix proteins. *Matrix Biology*, *18*(1), 55–64. https://doi.org/10.1016/S0945-053X(98)00006-7

Dominici, C., Moreno-Bravo, J. A., Puiggros, S. R., Rappeneau, Q., Rama, N., Vieugue, P., Bernet, A., Mehlen, P., & Chédotal, A. (2017). Floor-plate-derived netrin-1 is dispensable for commissural axon guidance. *Nature 2017 545:7654*, *545*(7654), 350–354. https://doi.org/10.1038/nature22331

Duan, C. L., Liu, C. W., Shen, S. W., Yu, Z., Mo, J. L., Chen, X. H., & Sun, F. Y. (2015). Striatal astrocytes transdifferentiate into functional mature neurons following ischemic brain injury. *Glia*, *63*(9), 1660. https://doi.org/10.1002/GLIA.22837

Duong, P., Sauvé-Schenk, K., Egan, M. Y., Meyer, M. J., & Morrison, T. (2019). Operational Definitions and Estimates of Return to Work Poststroke: A Systematic Review and Meta-Analysis. *Archives of Physical Medicine and Rehabilitation*, *100*(6), 1140–1152. https://doi.org/10.1016/J.APMR.2018.09.121

Ellison, J. A., Barone, F. C., & Feuerstein, G. Z. (1999). Matrix Remodeling after Stroke: De Novo Expression of Matrix Proteins and Integrin Receptors. *Annals of the New York*

Academy of Sciences, *890*(1), 204–222. https://doi.org/10.1111/J.1749-6632.1999.TB07996.X

Ellison, J. A., Velier, J. J., Spera, P., Jonak, Z. L., Wang, X., Barone, F. C., & Feuerstein, G. Z. (1998). Osteopontin and its Integrin Receptor αvβ3 Are Upregulated During Formation of the Glial Scar After Focal Stroke. *Stroke*, *29*(8), 1698–1707. https://doi.org/10.1161/01.STR.29.8.1698

Frost, S. B., Barbay, S., Friel, K. M., Plautz, E. J., & Nudo, R. J. (2003). Reorganization of remote cortical regions after ischemic brain injury: A potential substrate for stroke recovery. *Journal of Neurophysiology*, *89*(6), 3205–3214. https://doi.org/10.1152/JN.01143.2002/ASSET/IMAGES/LARGE/9K0633155007.JPEG

Galtrey, C. M., & Fawcett, J. W. (2007a). The role of chondroitin sulfate proteoglycans in regeneration and plasticity in the central nervous system. *Brain Research Reviews*, *54*(1), 1–18. https://doi.org/10.1016/J.BRAINRESREV.2006.09.006

Galtrey, C. M., & Fawcett, J. W. (2007b). The role of chondroitin sulfate proteoglycans in regeneration and plasticity in the central nervous system. *Brain Research Reviews*, *54*(1), 1–18. https://doi.org/10.1016/J.BRAINRESREV.2006.09.006

García-Alías, G., Barkhuysen, S., Buckle, M., & Fawcett, J. W. (2009). Chondroitinase ABC treatment opens a window of opportunity for task-specific rehabilitation. *Nature Neuroscience*, *12*(9), 1145–1151. https://doi.org/10.1038/nn.2377

Gottschall, P. E., & Howell, M. D. (2015). ADAMTS expression and function in central nervous system injury and disorders. *Matrix Biology : Journal of the International Society for Matrix Biology*, *44–46*, 70. https://doi.org/10.1016/J.MATBIO.2015.01.014

Hill, J. J., Jin, K., Mao, X. O., Xie, L., & Greenberg, D. A. (2012). Intracerebral chondroitinase ABC and heparan sulfate proteoglycan glypican improve outcome from chronic stroke in rats. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(23), 9155–9160. https://doi.org/10.1073/PNAS.1205697109/SUPPL_FILE/PNAS.201205697SI.PDF

Inatani, M., Honjo, M., Otori, Y., Oohira, A., Kido, N., Tano, Y., Honda, Y., & Tanihara, H. (2001). Inhibitory effects of neurocan and phosphacan on neurite outgrowth from retinal ganglion cells in culture. *Investigative Ophthalmology & Visual Science*, *42*(8), 1930–1938. http://intl.iovs.org/cgi/content/full/42/8/1930

Jones, T. A., & Adkins, D. L. (2015). Motor System Reorganization After Stroke: Stimulating and Training Toward Perfection. *Physiology*, *30*(5), 358. https://doi.org/10.1152/PHYSIOL.00014.2015

Jorgensen, H. S., Nakayama, H., Raaschou, H. O., & Olsen, T. S. (1999). Stroke: Neurologic and Functional Recovery The Copenhagen Stroke Study. *Physical Medicine and Rehabilitation Clinics of North America*, *10*(4), 887–906. https://doi.org/10.1016/S1047-9651(18)30169-4

Kamel, H., Parikh, N. S., Chatterjee, A., Kim, L. K., Saver, J. L., Schwamm, L. H., Zachrison, K. S., Nogueira, R. G., Adeoye, O., Diáz, I., Ryan, A. M., Pandya, A., & Navi, B. B. (2021). Access to Mechanical Thrombectomy for Ischemic Stroke in the United States. *Stroke*, *52*, 2554–2561. https://doi.org/10.1161/STROKEAHA.120.033485

Kennedy, T. E., Serafini, T., de la Torre, J. R., & Tessier-Lavigne, M. (1994). Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. *Cell*, *78*(3), 425–435. https://doi.org/10.1016/0092-8674(94)90421-9

Kugler, C., Altenhöner, T., Lochner, P., & Ferbert, A. (2003). Does age influence early recovery from ischemic stroke? A study from the Hessian Stroke Data Bank. *Journal of Neurology*, *250*(6), 676–681. https://doi.org/10.1007/S00415-003-1054-8

Kwakkel, G., Kollen, B. J., Van der Grond, J. V., & Prevo, A. J. H. (2003). Probability of regaining dexterity in the flaccid upper limb: Impact of severity of paresis and time since onset in acute stroke. *Stroke*, *34*(9), 2181–2186. https://doi.org/10.1161/01.STR.0000087172.16305.CD

Kwok, J. C. F., Yang, S., & Fawcett, J. W. (2014). Neural ECM in regeneration and rehabilitation. In *Progress in Brain Research* (Vol. 214, pp. 179–192). Elsevier B.V. https://doi.org/10.1016/B978-0-444-63486-3.00008-6

Lander, C., Zhang, H., & Hockfield, S. (1998). Neurons Produce a Neuronal Cell Surface-Associated Chondroitin Sulfate Proteoglycan. *Journal of Neuroscience*, *18*(1), 174–183. https://doi.org/10.1523/JNEUROSCI.18-01-00174.1998

Lee, B., Clarke, D., Al Ahmad, A., Kahle, M., Parham, C., Auckland, L., Shaw, C., Fidanboylu, M., Orr, A. W., Ogunshola, O., Fertala, A., Thomas, S. A., & Bix, G. J. (2011). Perlecan domain V is neuroprotective and proangiogenic following ischemic stroke in rodents. *The Journal of Clinical Investigation*, *121*(8), 3005–3023. https://doi.org/10.1172/JCI46358

Li, S., Nie, E. H., Yin, Y., Benowitz, L. I., Tung, S., Vinters, H. V, Bahjat, F. R., Stenzel-Poore, M. P., Kawaguchi, R., Coppola, G., & Carmichael, S. T. (2015). GDF10 is a signal for axonal sprouting and functional recovery after stroke. *Nature Neuroscience*, *18*(12), 1737–1745. https://doi.org/10.1038/nn.4146

Li, S., Overman, J. J., Katsman, D., Kozlov, S. V., Donnelly, C. J., Twiss, J. L., Giger, R. J.,

Coppola, G., Geschwind, D. H., & Carmichael, S. T. (2010). An age-related sprouting transcriptome provides molecular control of axonal sprouting after stroke. *Nature Neuroscience*, *13*(12), 1496–1504. https://doi.org/10.1038/nn.2674

Liu, Y., Wang, X., Li, W., Zhang, Q., Li, Y., Zhang, Z., Zhu, J., Chen, B., Williams, P. R., Zhang, Y., Yu, B., Gu, X., & He, Z. (2017). A Sensitized IGF1 Treatment Restores Corticospinal Axon-Dependent Functions. *Neuron*, *95*(4), 817-833.e4. https://doi.org/10.1016/j.neuron.2017.07.037

Luo, F., Wang, J., Zhang, Z., You, Z., Bedolla, A., Okwubido-Williams, F., Huang, L. F., Silver, J., & Luo, Y. (2021). Inhibition of Chondroitin Sulfate Proteoglycan Interactions With Receptor PTPσ Promotes Neurogenesis and Recovery From Stroke. *SSRN Electronic Journal*. https://doi.org/10.2139/SSRN.3858066

Luo, Y., Raible, D., & Raper, J. A. (1993). Collapsin: A protein in brain that induces the collapse and paralysis of neuronal growth cones. *Cell*, *75*(2), 217–227. https://doi.org/10.1016/0092-8674(93)80064-L

Maeda, N. (2015). Proteoglycans and neuronal migration in the cerebral cortex during development and disease. *Frontiers in Neuroscience*, *9*, 98. https://doi.org/10.3389/fnins.2015.00098

Malin, D., Sonnenberg-Riethmacher, E., Guseva, D., Wagener, R., Aszódi, A., Irintchev, A. A., Riethmacher, D., Irintchev, A. A., & Riethmacher, D. (2009). The extracellular-matrix protein matrilin 2 participates in peripheral nerve regeneration. *Journal of Cell Science*, *122*(Pt 7), 995–1004. https://doi.org/10.1242/jcs.040378

McKerracher, L., Chamoux, M., & Arregui, C. O. (1996). Role of laminin and integrin

interactions in growth cone guidance. *Molecular Neurobiology*, *12*(2), 95–116. https://doi.org/10.1007/BF02740648

Mokin, M., Ansari, S. A., McTaggart, R. A., Bulsara, K. R., Goyal, M., Chen, M., & Fraser, J. F. (2019). Indications for thrombectomy in acute ischemic stroke from emergent large vessel occlusion (ELVO): report of the SNIS Standards and Guidelines Committee. *Journal of NeuroInterventional Surgery*, *11*(3), 215–220. https://doi.org/10.1136/NEURINTSURG-2018-014640

Mukhopadhyay, R., Kumar, S., & Hoh, J. H. (2004). Molecular mechanisms for organizing the neuronal cytoskeleton. In *BioEssays* (Vol. 26, Issue 9, pp. 1017–1025). https://doi.org/10.1002/bies.20088

Myers, J. P., Santiago-Medina, M., & Gomez, T. M. (2011). Regulation of axonal outgrowth and pathfinding by integrin–ecm interactions. *Developmental Neurobiology*, *71*(11), 901–923. https://doi.org/10.1002/DNEU.20931

Nih, L. R., Gojgini, S., Carmichael, S. T., & Segura, T. (2018). Dual-function injectable angiogenic biomaterial for the repair of brain tissue following stroke. *Nature Materials 2018 17:7*, *17*(7), 642–651. https://doi.org/10.1038/s41563-018-0083-8

Overman, J. J., Clarkson, A. N., Wanner, I. B., Overman, W. T., Eckstein, I., Maguire, J. L., Dinov, I. D., Toga, A. W., & Carmichael, S. T. (2012). A role for ephrin-A5 in axonal sprouting, recovery, and activity-dependent plasticity after stroke. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(33). https://doi.org/10.1073/PNAS.1204386109/SUPPL_FILE/PNAS.201204386SI.PDF

Pires-Neto, M. A., Braga-De-Souza, S., & Lent, R. (1999). Extracellular matrix molecules play

diverse roles in the growth and guidance of central nervous system axons. *Brazilian Journal of Medical and Biological Research*, *32*(5), 633–638. https://doi.org/10.1590/S0100-879X1999000500017

Poplawski, G. H. D., Kawaguchi, R., Van Niekerk, E., Lu, P., Mehta, N., Canete, P., Lie, R., Dragatsis, I., Meves, J. M., Zheng, B., Coppola, G., & Tuszynski, M. H. (2020). Injured adult neurons regress to an embryonic transcriptional growth state. *Nature*, 1–6. https://doi.org/10.1038/s41586-020-2200-5

Ricoult, S. G., Kennedy, T. E., & Juncker, D. (2015). Substrate-Bound Protein Gradients to Study Haptotaxis. *Frontiers in Bioengineering and Biotechnology*, *3*(MAR). https://doi.org/10.3389/FBIOE.2015.00040

Saposnik, G., Cote, R., Phillips, S., Gubitz, G., Bayer, N., Minuk, J., & Black, S. (2008). Stroke outcome in those over 80: A multicenter cohort study across Canada. *Stroke*, *39*(8), 2310–2317. https://doi.org/10.1161/STROKEAHA.107.511402

Saver, J. L., Fonarow, G. C., Smith, E. E., Reeves, M. J., Grau-Sepulveda, M. V., Pan, W., Olson, D. W. M., Hernandez, A. F., Peterson, E. D., & Schwamm, L. H. (2013). Time to Treatment With Intravenous Tissue Plasminogen Activator and Outcome From Acute Ischemic Stroke. *JAMA*, *309*(23), 2480–2488. https://doi.org/10.1001/JAMA.2013.6959

Savva, G. M., & Stephan, B. C. M. (2010). Epidemiological studies of the effect of stroke on incident dementia: A systematic review. *Stroke*, *41*(1). https://doi.org/10.1161/STROKEAHA.109.559880

Seuffert, F., Weidner, D., Baum, W., Schett, G., & Stock, M. (2018). Upper zone of growth plate and cartilage matrix associated protein protects cartilage during inflammatory arthritis.

Arthritis Research & Therapy, *20*(1), 88. https://arthritis-research.biomedcentral.com/articles/10.1186/s13075-018-1583-2

Snow, D. M., Lemmon, V., Carrino, D. A., Caplan, A. I., & Silver, J. (1990). Sulfated proteoglycans in astroglial barriers inhibit neurite outgrowth in vitro. *Experimental Neurology*, *109*(1), 111–130. https://doi.org/10.1016/S0014-4886(05)80013-5

Snow, D. M., & Letourneau, P. C. (1992). Neurite outgrowth on a step gradient of chondroitin sulfate proteoglycan (CS-PG). *Journal of Neurobiology*, *23*(3), 322–336. https://doi.org/10.1002/NEU.480230311

Soleman, S., Yip, P. K., Duricki, D. A., & Moon, L. D. F. (2012). Delayed treatment with Chondroitinase ABC promotes sensorimotor recovery and plasticity after stroke in aged rats. *Brain : A Journal of Neurology*, *135*(Pt 4), 1210. https://doi.org/10.1093/BRAIN/AWS027

Sozmen, E. G., DiTullio, D. J., Rosenzweig, S., Hinman, J. D., Bridges, S. P., Marin, M. A., Kawaguchi, R., Coppola, G., & Carmichael, S. T. (2019). White Matter Stroke Induces a Unique Oligo-Astrocyte Niche That Inhibits Recovery. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *39*(47), 9343–9359. https://doi.org/10.1523/JNEUROSCI.0103-19.2019

Stanic, K., Saldivia, N., Förstera, B., Torrejón, M., Montecinos, H., & Caprile, T. (2016). Expression patterns of extracellular matrix proteins during posterior commissure development. *Frontiers in Neuroanatomy*, *10*(SEP), 89. https://doi.org/10.3389/FNANA.2016.00089/BIBTEX

Towfighi, A., Ovbiagele, B., El Husseini, N., Hackett, M. L., Jorge, R. E., Kissela, B. M.,

Mitchell, P. H., Skolarus, L. E., Whooley, M. A., & Williams, L. S. (2017). Poststroke Depression: A Scientific Statement for Healthcare Professionals From the American Heart Association/American Stroke Association. *Stroke*, *48*(2), e30–e43. https://doi.org/10.1161/STR.0000000000000113

Trout, A. L., Kahle, M. P., Roberts, J. M., Marcelo, A., de Hoog, L., Boychuk, J. A., Grupke, S. L., Berretta, A., Gowing, E. K., Boychuk, C. R., Gorman, A. A., Edwards, D. N., Rutkai, I., Biose, I. J., Ishibashi-Ueda, H., Ihara, M., Smith, B. N., Clarkson, A. N., & Bix, G. J. (2021). Perlecan Domain-V Enhances Neurogenic Brain Repair After Stroke in Mice. *Translational Stroke Research*, *12*(1), 72–86. https://doi.org/10.1007/S12975-020-00800-5/FIGURES/7

Tsai, S. Y., Papadopoulos, C. M., Schwab, M. E., & Kartje, G. L. (2011). Delayed anti-Nogo-A therapy improves function after chronic stroke in adult rats. *Stroke*, *42*(1), 186–190. https://doi.org/10.1161/STROKEAHA.110.590083

Tsao, C. W., Aday, A. W., Almarzooq, Z. I., Alonso, A., Beaton, A. Z., Bittencourt, M. S., Boehme, A. K., Buxton, A. E., Carson, A. P., Commodore-Mensah, Y., Elkind, M. S. V., Evenson, K. R., Eze-Nliam, C., Ferguson, J. F., Generoso, G., Ho, J. E., Kalani, R., Khan, S. S., Kissela, B. M., … Martin, S. S. (2022). Heart Disease and Stroke Statistics—2022 Update: A Report From the American Heart Association. *Circulation*, *145*(8), e153–e639. https://doi.org/10.1161/CIR.0000000000001052

Tukmachev, D., Forostyak, S., Koci, Z., Zaviskova, K., Vackova, I., Vyborny, K., Sandvig, I., Sandvig, A., Medberry, C. J., Badylak, S. F., Sykova, E., & Kubinova, S. (2016). Injectable Extracellular Matrix Hydrogels as Scaffolds for Spinal Cord Injury Repair. *Tissue*

Engineering - Part A, *22*(3–4), 306–317. https://doi.org/10.1089/TEN.TEA.2015.0422/ASSET/IMAGES/LARGE/FIGURE7.JPEG

Vahlsing, H. L., Hagg, T., Spencer, M., Conner, J. M., Manthorpe, M., & Varon, S. (1991). Dose-dependent responses to nerve growth factor by adult rat cholinergic medial septum and neostriatum neurons. *Brain Research*, *552*(2), 320–329. https://doi.org/10.1016/0006-8993(91)90098-G

Varadarajan, S. G., Kong, J. H., Phan, K. D., Kao, T. J., Panaitof, S. C., Cardin, J., Eltzschig, H., Kania, A., Novitch, B. G., & Butler, S. J. (2017). Netrin1 Produced by Neural Progenitors, Not Floor Plate Cells, Is Required for Axon Guidance in the Spinal Cord. *Neuron*, *94*(4), 790-799.e3. https://doi.org/10.1016/J.NEURON.2017.03.007

von Bastian, C. C., Belleville, S., Udale, R. C., Reinhartz, A., Essounni, M., & Strobach, T. (2022). Mechanisms underlying training-induced cognitive change. *Nature Reviews Psychology 2022 1:1*, *1*(1), 30–41. https://doi.org/10.1038/s44159-021-00001-3

Wahl, A. S., Omlor, W., Rubio, J. C., Chen, J. L., Zheng, H., Schröter, A., Gullo, M., Weinmann, O., Kobayashi, K., Helmchen, F., Ommer, B., & Schwab, M. E. (2014). Asynchronous therapy restores motor control by rewiring of the rat corticospinal tract after stroke. *Science*, *344*(6189), 1250–1255. https://doi.org/10.1126/SCIENCE.1253050/SUPPL_FILE/WAHL.SM.PDF

Wattanapan, P., Lukkanapichonchut, P., Massakulpan, P., Suethanapornkul, S., & Kuptniratsaikul, V. (2020). Effectiveness of Stroke Rehabilitation Compared between Intensive and Nonintensive Rehabilitation Protocol: A Multicenter Study. *Journal of Stroke and Cerebrovascular Diseases*, *29*(6), 104809.

https://doi.org/10.1016/J.JSTROKECEREBROVASDIS.2020.104809

Williamson, M. R., Fuertes, C. J. A., Dunn, A. K., Drew, M. R., & Jones, T. A. (2021). Reactive astrocytes facilitate vascular repair and remodeling after stroke. *Cell Reports*, *35*(4), 109048. https://doi.org/10.1016/J.CELREP.2021.109048

Yamada, H., Fredette, B., Shitara, K., Hagihara, K., Miura, R., Ranscht, B., Stallcup, W. B., & Yamaguchi, Y. (1997). The brain chondroitin sulfate proteoglycan brevican associates with astrocytes ensheathing cerebellar glomeruli and inhibits neurite outgrowth from granule neurons. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *17*(20), 7784–7795. https://doi.org/10.1523/JNEUROSCI.17-20-07784.1997

Chapter 2

In vitro analysis of ECM mediated neurite outgrowth

2.1 Introduction

2.1.1. Ischemic stroke induces changes in the surviving tissue

Ischemic stroke induces dramatic changes in the area immediately surrounding the infarct area, causing transcriptomic changes resulting in limited recovery (Carmichael et al., 2016; Li & Carmichael, 2006). Axonal sprouting is one such method through which surviving neurons collateralize and remap lost connections. Neurons that undergo post-stroke axonal sprouting have an unique transcriptomic profile with potential targets for enhancing post-stroke recovery (Li et al., 2010b). This "sprouting transcriptome" was identified through previous work in the Carmichael lab, in which peri-infarct neurons were labeled with two retrograde tracers at different time points after stroke, allowing for identification of neurons that had sprouted new collaterals as a result of CNS injury. These neurons were collected via laser capture microdissection and RNA sequenced via microarray. The resulting transcriptome identified 558 genes differentially regulated in sprouting vs. non-sprouting neurons, forming the initial basis for this study. Among the differentially regulated genes were various extracellular matrix (ECM) genes, indicating a role of sprouting neurons in shaping the post-stroke ECM.

The ECM of the post-stroke brain has drawn attention as a target for manipulation, due in part to the long-standing belief that a growth inhibitory extracellular environment is a main cause for stunted recovery (Bikbaev et al., 2015; Galtrey & Fawcett, 2007; S. Soleman et al., 2013). Previous work degrading growth inhibitory chondroitin sulfate proteoglycans (CSPGs) in the peri-infarct has promoted some degree of recovery (Sara Soleman et al., 2012), but this recovery is incomplete, indicating that simply removing a growth inhibitory environment is insufficient to enable complete recovery. Other work, focusing on spinal cord injury, has demonstrated that

axons are capable of growing through a growth inhibitory environment if given sufficient stimulus, such as growth factors or a hydrogel substrate (Anderson et al., 2016; Li et al., 2015).

At the onset of this work, there were few studies that identified ECM proteins that enhanced axonal sprouting or functional recovery post-stroke. The sprouting transcriptome, however, identified several ECM proteins that were either upregulated in spontaneously sprouting neurons, or downregulated despite previous literature indicating their importance in nerve injury recovery, as in the case of matrilin-2 (Malin et al., 2009). This chapter will describe a low throughput but high specificity screen to identify whether select ECM proteins identified from the sprouting transcriptome are targets for post-stroke manipulation. An *in vitro* model using murine primary cortical neurons is used to characterize dose-response and neurite extension in growth-permissive and inhibitory environments, using both early and late post-natal mice. The aim for the studies described in this chapter was identification of ECM proteins that enhance neurite outgrowth to translate to proteins that may increase axonal-sprouting and functional recovery post-stroke.

2.1.2 Primary cortical neurons as a screening system for axonal sprouting

Primary neurons are an ideal tool for the identification of ECM proteins that enhance axonal sprouting, as such a platform enables the experimenter to evaluate not only neurite length, as a proxy for axon growth, but also dose-response effects, an important factor for experiments performed in later chapters of this work. In combination with a semi-automated imaging and analysis platform, the use of primary cortical neurons allows for evaluation of numerous experimental conditions, such as amount of ECM protein exposed and/or growth inhibitory environments, in a relatively short period of time with consistency across analysis methods.

Several factors came into play when developing this platform. The use of cortical tissue for isolation of neurons was important, given the stroke model used later is a cortical stroke. Many screens for identification of drug targets use embryonic neurons, which differ from mature neurons in numerous ways, including susceptibility to growth inhibitory cues, axon development and differentiation. Culture from post-natal mice was necessary to more closely mimic the effect of the target ECM proteins on axonal sprouting. Post-natal neurons are ideal for this, as they have sufficiently matured to mimic the inability of adult neurons to regenerate axons, while still enabling easy collection and culture (Blackmore et al., 2010; Lerch et al., 2014). In this study early post-natal (post-natal day 3, P3) and late post-natal (post-natal day 12, P12) mice are used to isolate mixed cortical cultures for subsequent analysis of neurite outgrowth upon growth on a substrate of the experimental ECM proteins. The use of this experimental design allows for identification of pro-growth ECM proteins for subsequent *in vivo* axonal sprouting and functional recovery studies.

2.1.3 Use of recombinant ECM proteins

The experiments in this chapter use recombinant ECM proteins rather than viral overexpression of those proteins, as done in subsequent chapters. This was done for several reasons. First, use of recombinant proteins allows for more precise "dose" of the applied protein, to evaluate for potential dose responses and toxic effects. Various growth factors have been identified as beneficial in lower doses, but having toxic or opposite effects as neurons are exposed to increasing amounts (Mamounas et al., 2000; Vahlsing et al., 1991). Second, concerns over a ceiling effect arose, especially with P3 derived cortical neurons, as they still showed a remarkable capacity for rapid neurite extension in a short amount of time. Use of recombinant protein as a growth substrate bypassed the period of time needed for transduction via lentivirus

(which is faster in transduction than AAV), which would otherwise require 36-48 hours after infection for significant expression of the target gene (Philippe et al., 2006). Taken together, use of recombinant proteins allows for precise knowledge of the effect of the proteins themselves, including dose, on neurite outgrowth while bypassing the need for extending culture time beyond the target total time *in vitro*.

2.2 Methods

2.2.1 Primary Cortical Neuron Culture

Primary cortical neurons were cultured from post-natal day 3 and 12 C57Bl/6 mice using a modified protocol utilizing the Adult Brain Dissociation kit (Miltenyi Biotec). Briefly, mouse pups were anesthetized on ice and decapitated. Brains were removed following a midline skull incision, subcortical structures, hippocampus, underlying white matter and cerebellum were removed, and cortices were rinsed with ice-cold DPBS containing calcium, magnesium, sodium pyruvate and glucose. Cortices were minced using fine tip forceps and transferred to tubes containing proprietary dissociation solutions and enzymes (Miltenyi Biotec). Cortices were dissociated for 30 minutes with heat and agitation using the GENTLE MACS Octo Dissociator with Heaters (Miltenyi Biotec). Dissociated cortices were passed through a 70-um strainer, rinsed twice with DPBS containing 5% BSA (Miltenyi Biotec), and centrifuged at 300g for 10 minutes at 4C. The supernatant was carefully removed, and tissue pellet was resuspended in 6.2 ml DPBS. 1.2 ml Debris Removal Solution was added and mixed with the resuspended cell pellet, and 4 ml ice-cold DPBS was carefully overlaid on top. Tubes were centrifuged at 3,000g for 10 min at 4C, forming three distinct layers, and the top two layers were aspirated completely. Ice-cold DPBS was added to a final volume of 15 ml, the tube was inverted several times to mix completely, and cells were centrifuged at 1,000g for 10 minutes at 4C. The supernatant was

carefully removed, and the resulting cell pellet was resuspended in proprietary Red Blood Cell Removal Solution (Miltenyi Biotec). Cells were incubated at 4C for 10 minutes in the dark, after which 10 ml ice-cold DPBS with 5% BSA was added. Cells were centrifuged for 10 minutes at 300g at 4C, after which they were resuspended in NbActive4 culture medium (Brain Bits LLC) supplemented with 1% penicillin/streptomycin (Life Tech) and counted. Cells were plated at a density of 20K cells per well for post-natal day 3 neurons, and 45K cells per well for post-natal day 12 neurons, for a total volume of 200 µl/well. At 1 day *in vitro*, 100 µl media was removed from each well, and 100 µl replacement media was carefully applied to avoid dislodging cells.

2.2.2 ECM treatments

96-well glass bottom plates were used for analysis of neurite outgrowth (Greiner). Wells were initially coated with poly-L-lysine solution and rinsed with DPBS. ECM proteins of interest were reconstituted according to supplier instructions, and include mouse matrilin-2, f-spondin and netrin-1 (R&D Systems), human UCMA (Abcam), and fibronectin (Life Tech). Specified concentrations of ECM proteins were made through dilution of stock solutions using DPBS and applied to wells at a volume of 30 µl. Plates were incubated overnight in the cell culture incubator at 37C. The day of cell plating, ECM solutions were aspirated, wells were briefly rinsed with DPBS and set aside to dry at room temperature. Each treatment was replicated at least 6 times per plate, and each plate was performed in triplicate. Exogenous CSPG treatment was performed either through coincubation of CSPGs with targeted ECM proteins at 25 ug/ml, or by application in media at 1 day *in vitro* at 2.5 ug/ml (EMD Millipore).

2.2.3 Immunocytochemistry

Cells were fixed at 3 days *in vitro* with 4% paraformaldehyde and washed in PBS. Cells were immunolabeled with primary antibodies overnight against neurites (mouse-anti Tau for P3 neurons (EMD Millipore) or rabbit anti-Tuj1 for P12 neurons (Abcam)) and neuronal cells bodies (rabbit anti-NeuN, Abcam). In instances where two rabbit antibodies were used, primary antibodies were first pre conjugated to secondary dyes using the Mix-N-Stain kit (Biotium). Donkey secondary antibodies (Jackson ImmunoResearch) against Mouse (Cy2) and Rabbit (Cy3 or Cy5) were used at 1:500 for indirect immunofluorescence, and incubated for 1 hour. Following the last wash, cells were left in PBS and stored at 4C until imaging.

2.2.4 Confocal imaging and quantification

Images of cells were acquired using a Nikon Eclipse confocal microscope with High Content Analysis software (Nikon). 20 images per well were acquired at 20x magnification. Following acquisition, neurite length and neuronal cell bodies were quantified using the High Content Analysis software (Nikon). Briefly, neurites were determined by setting a consistent threshold, skeletonized, and length determined. Cell bodies were detected via fluorescent threshold, size, and direct association with a neurite. A ratio of total neurite length/number of neurite-containing cell bodies was determined for each well (mean neurite length per cell) and used for statistical analysis.

2.2.5 Statistics

For each plate, the mean neurite length per cell of the control condition was determined and used as a baseline for comparison. The mean neurite length per cell of all treatment conditions was converted to a percentage of control neurite length specific to each plate. One-way ANOVA with Tukey's post-hoc test for multiple comparisons was used to compare treatment effects on neurite outgrowth.

2.3 Results

2.3.1 Identification of target ECM proteins

Target genes were identified from the sprouting transcriptome identified by this lab (Li et al., 2010a). Briefly, genes identified for ECM proteins were initially screened for up- or down-regulation in sprouting neurons, with either a 1.25-fold increase or 0.75-fold decrease as a threshold and a statistical cutoff of $P < 0.005$. Up-regulated genes were hypothesized to be beneficial to naturally occurring repair post-stroke, as part of the normal recovery process. Down-regulated genes were hypothesized, in the absence of known growth-inhibitory function, to be paradoxically down-regulated due to other biological processes seen in the post-stroke brain. A gene that has prior reports of enhancement of axonal sprouting, and yet is downregulated in cortical neurons that sprout a new connection after stroke, may be hypothesized as playing a role in the only-limited axonal sprouting in the CNS after injury. Genes were further screened for known function in the CNS, with particular emphasis on roles associated with regeneration and recovery or developmental function in axon pathfinding. Genes identified using these criteria were the ideal targets, as they were believed to either be beneficial in the context of stroke, and thus targeted for enhanced and prolonged overexpression to maintain the pro-recovery state these neurons entered, or paradoxically downregulated, in which

case exposure to elevated levels could rescue the neurite outgrowth phenotype and recapitulate axonal sprouting *in vitro*. Use of recombinant protein for this initial screen was also important, as it would allow for dose-response evaluation and preliminarily inform on how translatable these proteins are.

As noted in Chapter 1, with these screening principles, two ECM genes are differentially regulated in stroke, in sprouting neurons, that identify these genes as candidates for playing a role in post-stroke axonal sprouting. Matrilin-2 is one of the most down-regulated genes in sprouting neurons in the young adult after stroke, with expression decreasing by 52% despite its role in enhancing axonal sprouting in the PNS (Malin et al., 2009), indicating a potential role in the limited recovery seen in young animals. Unique cartilage matrix associated protein (Ucma) is one the highest and most significantly induced genes in young adult at day 7, transiently increasing by 69% and then returning to baseline levels of expression at later time points in the axonal sprouting response, a possible reason for the transient recovery phase in young animals. Ucma is one of highest and most significantly down regulated genes at day 7 in the aged axonal sprouting transcriptome, and also at day 21, decreasing by 98% and 60% respectively. As such, downregulation of Ucma may play a role in the decreased degree of recovery associated with age (Kugler et al., 2003).

2.3.2 P3 neuron outgrowth assay

Primary cortical neurons from post-natal day 3 (P3) mice were isolated and grown on varying doses of the identified ECM proteins for 3 days (Figure 2-1). P3 neurons were initially used for several reasons. First, use of P3 mice enables collection of a large number of cells, allowing for multiple conditions, in this case dose of protein exposed to. Second, P3 neurons are developmentally mature enough to show sensitivity to a growth inhibitory environment, allowing

for evaluation of neurite outgrowth rescue in a context similar to that seen in stroke. The results from this screen are shown in Figure 2-2. Growth on a Matn2 substrate resulted in a dose-dependent increase in neurite outgrowth, to a maximum increase of approximately 50% over neurons grown on a growth neutral substrate ($p < 0.0001$ vs control, ordinary 1-way ANOVA). When grown on Ucma, neurons showed a U-shaped dose response curve, with maximum outgrowth seen at 50ng/ml Ucma ($p = 0.0001$ vs control, ordinary 1-way ANOVA). F-spondin (Spon1), another identified ECM protein, was evaluated, and found to have no effect on neurite outgrowth at a range of concentrations. The lack of outgrowth observed with Spon1 exposure, despite it being an upregulated ECM protein in sprouting neurons (Li et al., 2010b), demonstrates that the presence of exogenous ECM proteins is not sufficient to induce neurite outgrowth by itself. Cytochrome C (CyC) and growth differentiation factor 10 (GDF10) were used as negative and positive controls, respectively, as CyC has previously been shown to have no impact on neurite outgrowth, whereas GDF10 is a known inducer of neurite outgrowth (Li et al., 2015).

As the growth inhibitory environment in the post-stroke brain is thought to contribute to the limited recovery seen, neurons were subjected to a growth inhibitory environment rich in CSPGs to evaluate the ability of targeted ECM proteins to rescue normal outgrowth. Both Matn2 and Ucma were able to rescue neurite outgrowth in this growth inhibitory environment, showing similar growth response curves as those seen in a growth permissive environment.

2.3.3 P12 neuron outgrowth assay

As both Matn2 and Ucma showed positive effects on neurite outgrowth in P3 neurons, these proteins were evaluated using more mature P12 neurons. P12 neurons were chosen as the most mature neurons that could reliably be isolated and grown utilizing the methods used with

P3 neurons. P12 neurons showed a marked slowing of basal neurite outgrowth and enhanced sensitivity to a growth inhibitory environment. The results using P12 neurons are shown in Figure 2-3. As such, they more readily represented the state of neurons *in situ* in the post-stroke brain. Poly-D-Lysine (PDL) and fibronectin were used as negative and positive controls respectively, as PDL is a growth-neutral protein substrate and fibronectin is known to induce neurite outgrowth (Akers et al., 1981).

In P12 neurons, growth on Matn2 resulted in an increase in neurite outgrowth by about 34%, showing a similar dose-response as seen with P3 neurons ($p=0.0032$ at 500ng/ml vs control, ordinary 1-way ANOVA). Further, growth on Matn2 resulted in a rescue of neurite outgrowth in a growth inhibitory environment. When Ucma was used as a growth substrate in a growth permissive environment, there was again a U-shaped dose-response (43% increase in neurite outgrowth, $p <0.0001$ at 1ng/ml vs control, ordinary 1-way ANOVA), with no rescue observed in a growth-inhibitory environment.

2.3.4 Other ECM proteins evaluated

F-spondin (Spon1) was another ECM protein differentially regulated in post-stroke sprouting neurons tested using this neurite-outgrowth paradigm. Netrin1 (Ntn1) expression was unchanged in sprouting neurons but was used as a control for growth on an ECM substrate. Spon1 failed to increase neurite outgrowth across a range of concentrations in P3 neurons, while Ntn1 was found to have no effect on neurite outgrowth in P12 neurons (Figure 2-4).

2.4 Discussion

ECM proteins differentially regulated in sprouting neurons, as identified in the sprouting transcriptome generated by our lab, were evaluated using an *in vitro* neurite outgrowth screen.

Matn2 was identified as significantly down-regulated in post-stroke sprouting neurons, despite previously being identified as important in peripheral nerve regeneration (Korpos et al., 2015; Malin et al., 2009). Exogenous Matn2 application has also been shown to improve motor recovery through OPC differentiation in a white matter stroke model (Sozmen et al., 2019). Ucma, despite being a protein of unknown function in the CNS, was identified as one of the most significantly upregulated genes post-stroke. Ucma has been identified as an aggrecanase inhibitor in cartilage networks (Lee et al., 2015; Seuffert et al., 2018), leading to the possibility that in the post-stroke brain it may play a role in early ECM remodeling. Spon1 was another significantly upregulated gene in sprouting neurons, and had been previously characterized as important for commissural axon pathfinding during development (Burstyn-Cohen et al., 1999). These characteristics, combined with availability of recombinant proteins and novelty in CNS injury, led to these proteins being evaluated in a low-throughput but high specificity neurite outgrowth assay.

The neurite outgrowth assay was initially performed using mixed cortical cultures from post-natal day 3 mice. Growth in exogenous Matn2 led to a dose-responsive increase in neurite outgrowth to a maximum of 50% above the control condition with no obvious upper limit in dosage, while growth on Ucma showed a much narrower therapeutic index, with a 50% increase in neurite outgrowth seen at 50ng/ml but sharp decreases in neurite outgrowth as the dose either increases or decreases. This effect, termed hormesis, is not unique to Ucma, and has been seen in various biological processes (Dattilo et al., 2015; Mattson, 2008). Spon1, initially identified as an axonal pathfinding protein upregulated in sprouting neurons post-stroke, failed to increase neurite outgrowth. This result served as an important control, indicating that simply growing on a

substrate of exogenous protein is not sufficient to induce neurite outgrowth and that the effects seen with Matn2 and Ucma are due to biological effects beyond that.

Initial observations from the neurite outgrowth assay used here raised concerns about ceiling effects, as both Ucma and Matn2 increased neurite outgrowth by approximately 50%. When grown on a neutral or permissive substrate, P3 neurons show robust neurite outgrowth such that, at 3 days *in vitro*, it can be difficult to observe increases beyond what is observed here. To accommodate this, a similar assay was performed using mixed cortical cultures from P12 animals. P12 neurons had a dramatically slower growth rate and increased sensitivity to a growth inhibitory environment. Further, utilizing mixed cortical cultures more closely matched the biological system that would be used for studies described in subsequent chapters of this dissertation. When grown on Matn2, P12 neurons, similar to the results seen with P3 neurons, showed an increase in neurite outgrowth in a dose-responsive manner, while P12 neurons grown on Ucma showed a hormetic response but similar maximum increase in neurite outgrowth. Netrin1 (Ntn1), a canonically important ECM protein in axon pathfinding during development (Varadarajan et al., 2017; Wu et al., 2019), was not differentially regulated in sprouting neurons, but was used as a control for exogenous ECM exposure; when P12 neurons were grown on a substrate of Ntn1, no change in neurite outgrowth was observed, further solidifying that mere growth on exogenous ECM is insufficient to induce neurite outgrowth.

One factor thought to limit post-stroke recovery is the presence of a growth inhibitory environment. To mimic this, P3 and P12 mixed cortical cultures were exposed to CSPGs, either as a growth substrate (P3) or soluble application (P12), and neurite outgrowth was measured in the same manner. In P3 cultures, both Matn2 and Ucma exposure rescued neurite outgrowth. In P12 cultures, growth on Matn2 rescued neurite outgrowth in a growth inhibitory environment,

but growth on Ucma failed to rescue outgrowth. In totality, Matn2 was shown to be an ECM protein that enhances neurite outgrowth in a dose-response like manner in both immature and mature post-natal neurons and rescued neurite outgrowth in a growth inhibitory environment, while Ucma enhances neurite outgrowth in a hormetic manner in immature and mature post-natal neurons, rescues neurite outgrowth in immature post-natal neurons but fails to rescue in mature neurons.

A limitation in this set of experiments is the inherent difference between *in vitro* and *in vivo* analysis of axon outgrowth. It would be impossible to mimic the intact brain using this neurite outgrowth assay, and given the focus on ECM proteins in this screen, there could be unintentional effects on non-neurons once experiments transition to an *in vivo* model. A further limitation is experiments performed in future chapters of this work use a viral overexpression system for endogenous production of these proteins, whereas work described here was performed with exogenous application of recombinant protein. Exogenous application was done to identify dose-response type effects, whereas viral overexpression, as described later, was used to ensure sustained production of target proteins throughout the stroke recovery process and clear identification of cells overexpressing said proteins. Despite these limitations, this neurite outgrowth screen provides sufficient evidence to proceed with axonal sprouting quantification in the post-stroke brain.

2.5 Figures

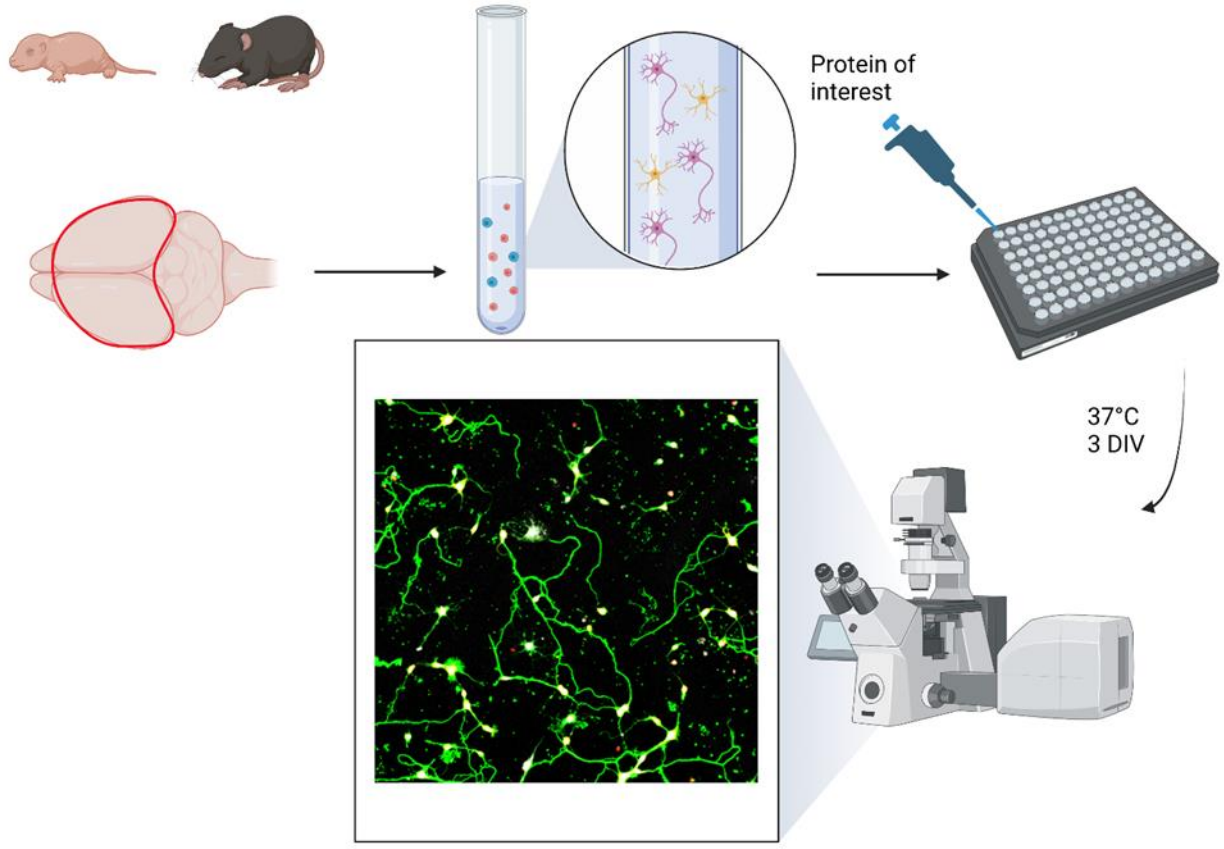


Figure 2-1. Primary neuron isolation and neurite outgrowth assay. Mixed cortical cultures were obtained from post-natal day 3 or 12 mice. Brains were extracted and cortices dissected from underlying white matter and subcortical tissue. Cortices were dissociated using the Adult Brain Dissociation Kit (Miltenyi), and debris and red blood cells removed. Cells were plated in 96-well glass bottom plates precoated with the desired ECM protein of interest in various concentrations. Cells were allowed to grow for 3 days, after which they were fixed, stained for neurites and neuronal cell bodies, and imaged on a confocal microscope. Neurite length is measured and compared to baseline to evaluate the effect of the ECM protein of interest on outgrowth.

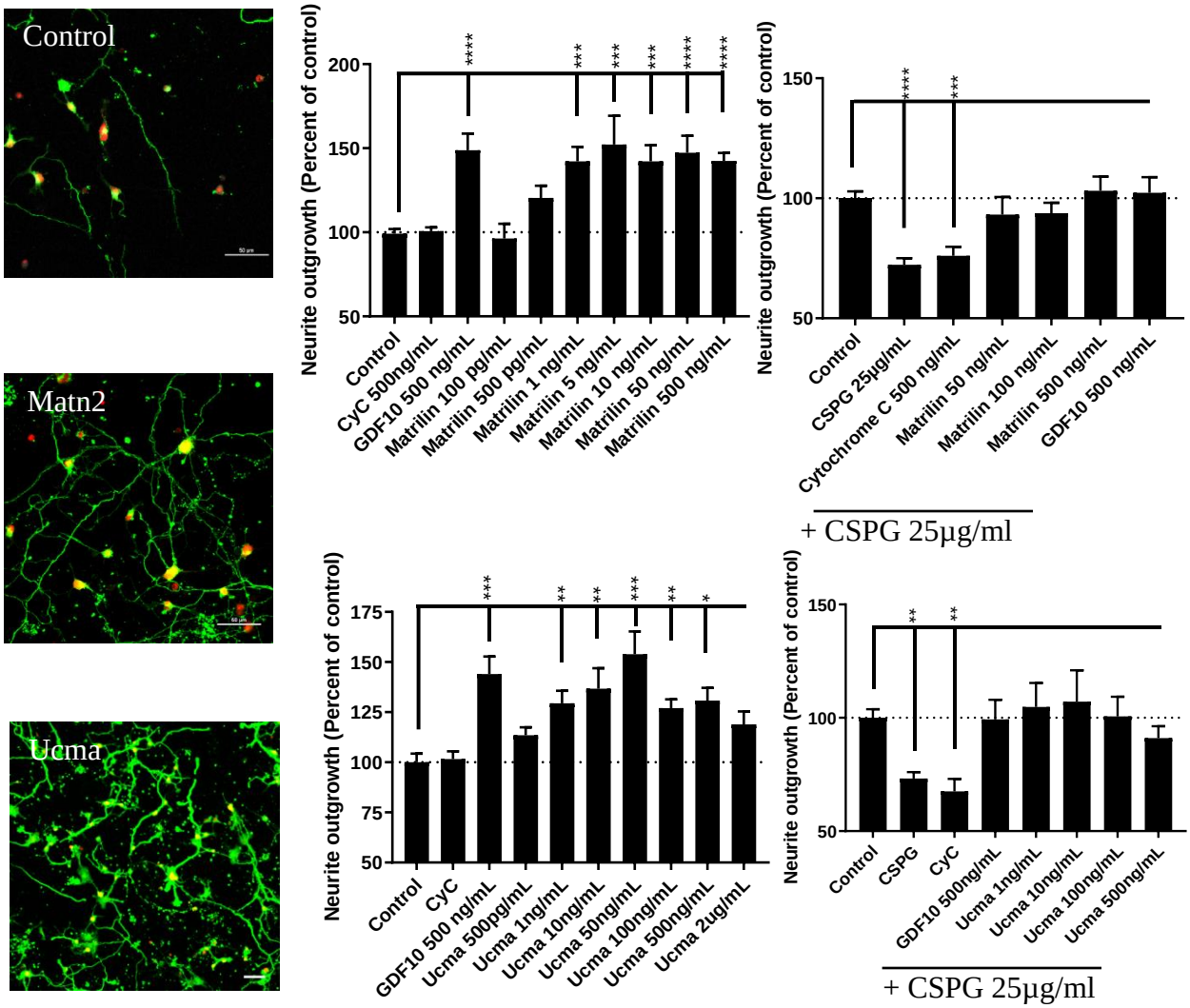


Figure 2-2. Effect of target ECM proteins on P3 neurite outgrowth. Mixed cortical cultures were grown on a substrate of the indicated ECM protein at stated concentrations, and neurite outgrowth was assessed at 3 DIV through Tuj-1 or Tau immunostaining. Growth on matrilin-2 resulted in an increase in neurite outgrowth in a dose-dependent manner (47% increase at 50 ng/ml, $p<0.0001$ via one-way ANOVA with post-hoc Tukey's test), and was sufficient to rescue neurite outgrowth in a growth inhibitory environment. Growth on Ucma resulted in an increase in neurite outgrowth in a hormesis-like response (53% increase at 50ng/ml, $p = 0.0001$ via one-way ANOVA with post-hoc Tukey's test) and was sufficient to rescue neurite outgrowth in a growth inhibitory environment. ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. CyC Cytochrome C, GDF10 growth differentiation 10, CSPG chondroitin sulfate proteoglycan.

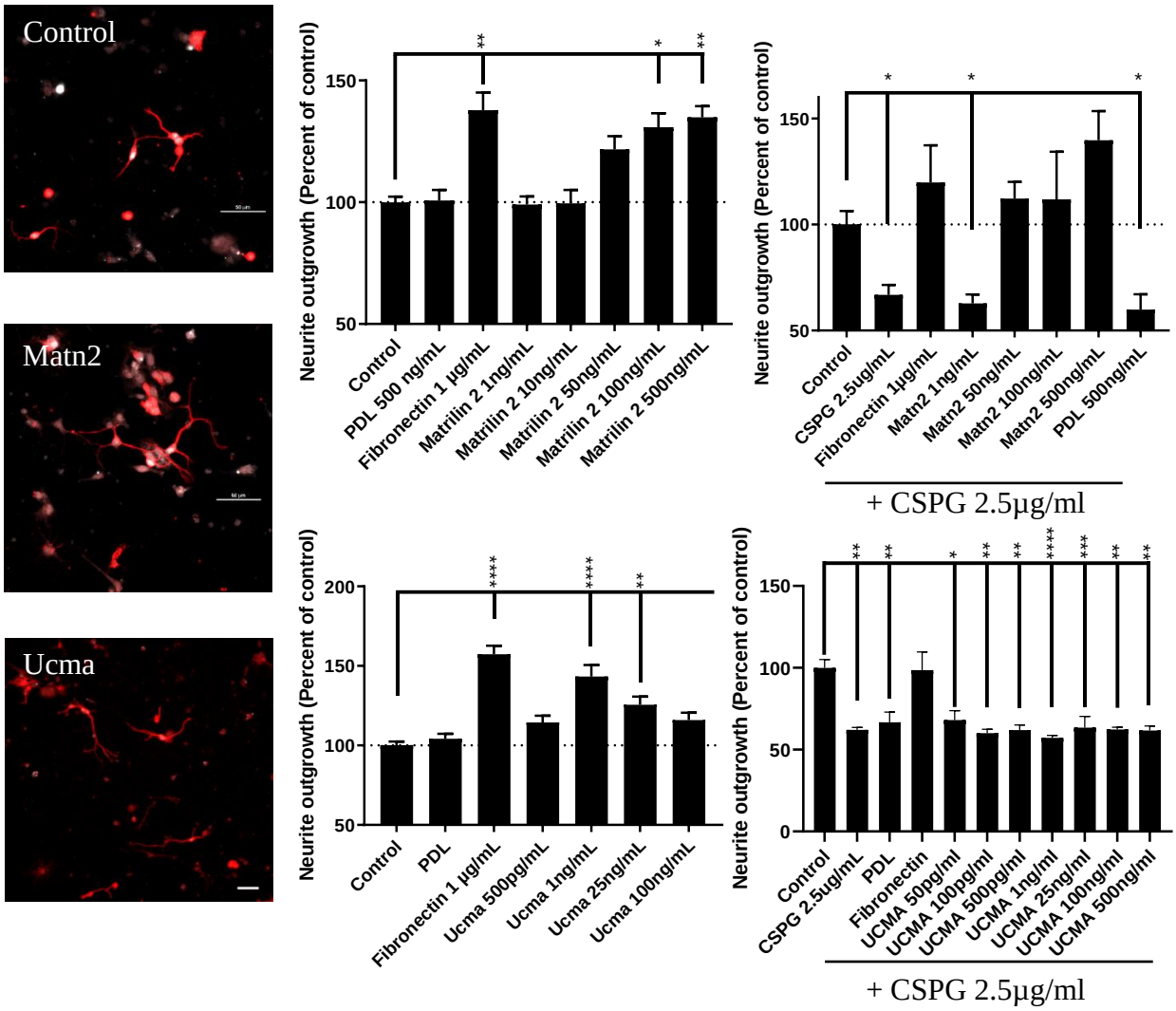


Figure 2-3. Effect of target ECM proteins on P12 neurite outgrowth. Mixed cortical cultures were grown on a substrate of the indicated ECM protein at stated concentrations, and neurite outgrowth was assessed at 3 DIV through Tuj-1 immunostaining. Growth on matrilin-2 resulted in an increase in neurite outgrowth in a dose-dependent manner (34% increase at 500 ng/ml, p=0.0032 via one-way ANOVA with post-hoc Tukey's test) and was sufficient to rescue neurite outgrowth in a growth inhibitory environment. Growth on Ucma resulted in an increase in neurite outgrowth in a hormesis-like response (43% increase at 1ng/ml, $p < 0.0001$ via one-way ANOVA with post-hoc Tukey's test) but did not rescue neurite outgrowth in a growth inhibitory environment. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. PDL Poly-D-Lysine.

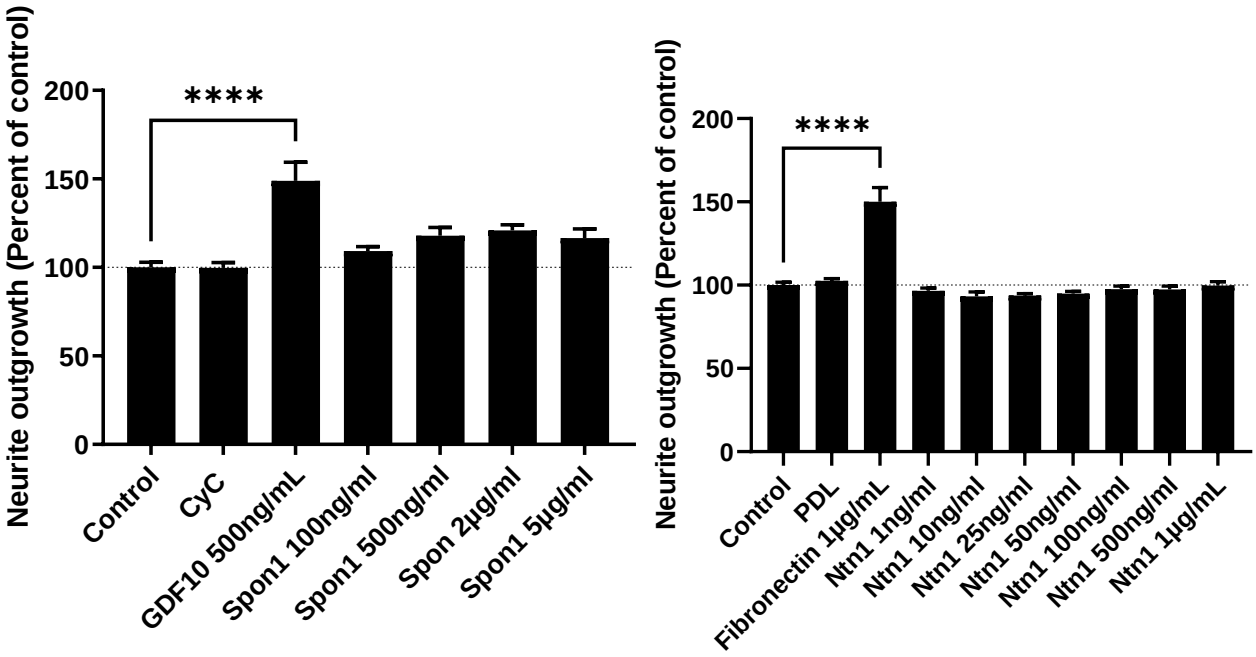


Figure 2-4. Enhanced neurite outgrowth is not a general feature of all ECM proteins. Mixed cortical cultures were grown on a substrate of the indicated ECM protein at stated concentrations, and neurite outgrowth was assessed at 3 DIV through Tuj-1 immunostaining. Growth on F-spondin (Spon1) with P3 neurons and netrin-1 (Ntn1) with P12 neurons failed to significantly increase neurite outgrowth. **** $p<0.0001$ via one-way ordinary ANOVA with post-hoc Tukey's test.

2.6 References

Akers, R. M., Mosher, D. F., & Lilien, J. E. (1981). Promotion of retinal neurite outgrowth by substratum-bound fibronectin. *Developmental Biology*, *86*(1), 179–188. https://doi.org/10.1016/0012-1606(81)90328-6

Anderson, M. A., Burda, J. E., Ren, Y., Ao, Y., O'Shea, T. M., Kawaguchi, R., Coppola, G., Khakh, B. S., Deming, T. J., & Sofroniew, M. V. (2016). Astrocyte scar formation aids CNS axon regeneration. *Nature*, *532*(7598), 195. https://doi.org/10.1038/NATURE17623

Bikbaev, A., Frischknecht, R., & Heine, M. (2015). Brain extracellular matrix retains connectivity in neuronal networks. *Scientific Reports*, *5*. https://doi.org/10.1038/srep14527

Blackmore, M. G., Moore, D. L., Smith, R. P., Goldberg, J. L., Bixby, J. L., & Lemmon, V. P. (2010). High content screening of cortical neurons identifies novel regulators of axon growth. *Molecular and Cellular Neuroscience*, *44*(1), 43–54. https://doi.org/10.1016/J.MCN.2010.02.002

Burstyn-Cohen, T., Tzarfaty, V., Frumkin, A., Feinstein, Y., Stoeckli, E., & Klar, A. (1999). F-Spondin Is Required for Accurate Pathfinding of Commissural Axons at the Floor Plate. *Neuron*, *23*(2), 233–246. https://doi.org/10.1016/S0896-6273(00)80776-X

Carmichael, S. T., Kathirvelu, B., Schweppe, C. A., & Nie, E. H. (2016). Molecular, cellular and functional events in axonal sprouting after stroke. *Experimental Neurology*. https://doi.org/10.1016/j.expneurol.2016.02.007

Dattilo, S., Mancuso, C., Koverech, G., Di Mauro, P., Ontario, M. L., Petralia, C. C., Petralia, A., Maiolino, L., Serra, A., Calabrese, E. J., & Calabrese, V. (2015). Heat shock proteins and hormesis in the diagnosis and treatment of neurodegenerative diseases. *Immunity & Ageing*

2015 12:1, *12*(1), 1–19. https://doi.org/10.1186/S12979-015-0046-8

Galtrey, C. M., & Fawcett, J. W. (2007). The role of chondroitin sulfate proteoglycans in regeneration and plasticity in the central nervous system. In *Brain Research Reviews* (Vol. 54, Issue 1, pp. 1–18). https://doi.org/10.1016/j.brainresrev.2006.09.006

Korpos, É., Deák, F., & Kiss, I. (2015). Matrilin-2, an extracellular adaptor protein, is needed for the regeneration of muscle, nerve and other tissues. *Neural Regeneration Research*, *10*(6), 866–869. https://doi.org/10.4103/1673-5374.158332

Kugler, C., Altenhöner, T., Lochner, P., & Ferbert, A. (2003). Does age influence early recovery from ischemic stroke? A study from the Hessian Stroke Data Bank. *Journal of Neurology*, *250*(6), 676–681. https://doi.org/10.1007/S00415-003-1054-8

Lee, Y.-J., Park, S.-Y., Lee, S.-J., Boo, Y. C., Choi, J.-Y., & Kim, J.-E. (2015). Ucma, a direct transcriptional target of Runx2 and Osterix, promotes osteoblast differentiation and nodule formation. *Osteoarthritis and Cartilage*, *23*(8), 1421–1431. https://doi.org/10.1016/j.joca.2015.03.035

Lerch, J. K., Martínez-Ondaro, Y. R., Bixby, J. L., & Lemmon, V. P. (2014). cJun promotes CNS axon growth. *Molecular and Cellular Neurosciences*, *59*, 97. https://doi.org/10.1016/J.MCN.2014.02.002

Li, S., & Carmichael, S. T. (2006). Growth-associated gene and protein expression in the region of axonal sprouting in the aged brain after stroke. *Neurobiology of Disease*, *23*(2), 362–373. https://doi.org/10.1016/j.nbd.2006.03.011

Li, S., Nie, E. H., Yin, Y., Benowitz, L. I., Tung, S., Vinters, H. V, Bahjat, F. R., Stenzel-Poore,

M. P., Kawaguchi, R., Coppola, G., & Carmichael, S. T. (2015). GDF10 is a signal for axonal sprouting and functional recovery after stroke. *Nature Neuroscience*, *18*(12), 1737–1745. https://doi.org/10.1038/nn.4146

Li, S., Overman, J. J., Katsman, D., Kozlov, S. V., Donnelly, C. J., Twiss, J. L., Giger, R. J., Coppola, G., Geschwind, D. H., & Carmichael, S. T. (2010a). An age-related sprouting transcriptome provides molecular control of axonal sprouting after stroke. *Nature Neuroscience*, *13*(12), 1496–1506. https://doi.org/10.1038/nn.2674

Li, S., Overman, J. J., Katsman, D., Kozlov, S. V., Donnelly, C. J., Twiss, J. L., Giger, R. J., Coppola, G., Geschwind, D. H., & Carmichael, S. T. (2010b). An age-related sprouting transcriptome provides molecular control of axonal sprouting after stroke. *Nature Neuroscience*, *13*(12), 1496–1504. https://doi.org/10.1038/nn.2674

Malin, D., Sonnenberg-Riethmacher, E., Guseva, D., Wagener, R., Aszódi, A., Irintchev, A. A., Riethmacher, D., Irintchev, A. A., & Riethmacher, D. (2009). The extracellular-matrix protein matrilin 2 participates in peripheral nerve regeneration. *Journal of Cell Science*, *122*(Pt 7), 995–1004. https://doi.org/10.1242/jcs.040378

Mamounas, L. A., Altar, C. A., Blue, M. E., Kaplan, D. R., Tessarollo, L., & Lyons, W. E. (2000). BDNF Promotes the Regenerative Sprouting, But Not Survival, of Injured Serotonergic Axons in the Adult Rat Brain. *Journal of Neuroscience*, *20*(2), 771–782. https://doi.org/10.1523/JNEUROSCI.20-02-00771.2000

Mattson, M. P. (2008). Awareness of Hormesis Will Enhance Future Research in Basic and Applied Neuroscience. *Http://Dx.Doi.Org/10.1080/10408440802026406*, *38*(7), 633–639. https://doi.org/10.1080/10408440802026406

Philippe, S., Sarkis, C., Barkats, M., Mammeri, H., Ladroue, C., Petit, C., Mallet, J., & Serguera, C. (2006). Lentiviral vectors with a defective integrase allow efficient and sustained transgene expression in vitro and vivo. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(47), 17684–17689. https://doi.org/10.1073/PNAS.0606197103/ASSET/703405C3-46BD-47DC-B69E-F642959834AC/ASSETS/GRAPHIC/ZPQ0370634690006.JPEG

Seuffert, F., Weidner, D., Baum, W., Schett, G., & Stock, M. (2018). Upper zone of growth plate and cartilage matrix associated protein protects cartilage during inflammatory arthritis. *Arthritis Research & Therapy*, *20*(1), 88. https://arthritis-research.biomedcentral.com/articles/10.1186/s13075-018-1583-2

Soleman, S., Filippov, M. A. A., Dityatev, A., & Fawcett, J. W. W. (2013). Targeting the neural extracellular matrix in neurological disorders. *Neuroscience*, *253*, 194–213. https://doi.org/10.1016/j.neuroscience.2013.08.050

Soleman, Sara, Yip, P. K., Duricki, D. A., & Moon, L. D. F. (2012). Delayed treatment with Chondroitinase ABC promotes sensorimotor recovery and plasticity after stroke in aged rats. *Brain : A Journal of Neurology*, *135*(Pt 4), 1210. https://doi.org/10.1093/BRAIN/AWS027

Sozmen, E. G., DiTullio, D. J., Rosenzweig, S., Hinman, J. D., Bridges, S. P., Marin, M. A., Kawaguchi, R., Coppola, G., & Carmichael, S. T. (2019). White Matter Stroke Induces a Unique Oligo-Astrocyte Niche That Inhibits Recovery. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *39*(47), 9343–9359. https://doi.org/10.1523/JNEUROSCI.0103-19.2019

Vahlsing, H. L., Hagg, T., Spencer, M., Conner, J. M., Manthorpe, M., & Varon, S. (1991). Dose-dependent responses to nerve growth factor by adult rat cholinergic medial septum and neostriatum neurons. *Brain Research*, *552*(2), 320–329. https://doi.org/10.1016/0006-8993(91)90098-G

Varadarajan, S. G., Kong, J. H., Phan, K. D., Kao, T.-J., Panaitof, S. C., Cardin, J., Eltzschig, H., Kania, A., Novitch, B. G., Butler, S. J., & al., et. (2017). Netrin1 Produced by Neural Progenitors, Not Floor Plate Cells, Is Required for Axon Guidance in the Spinal Cord. *Neuron*, *9*(0), 996–998. https://doi.org/10.1016/j.neuron.2017.03.007

Wu, Z., Makihara, S., Yam, P. T., Teo, S., Renier, N., Balekoglu, N., Moreno-Bravo, J. A., Olsen, O., Chédotal, A., Charron, F., & Tessier-Lavigne, M. (2019). Long-Range Guidance of Spinal Commissural Axons by Netrin1 and Sonic Hedgehog from Midline Floor Plate Cells. *Neuron*, *0*(0). https://doi.org/10.1016/j.neuron.2018.12.025

Chapter 3

In vivo analysis of ECM-enhanced axonal sprouting

3.1 Introduction

In Chapter 2, Matn2 and Ucma were identified as ECM proteins, differentially expressed by sprouting neurons following stroke, that enhance neurite outgrowth *in vitro* and, with Matn2, rescue neurite outgrowth in growth inhibitory environment in neurons derived from early and late post-natal mice. To further characterize the role of ECM in axonal growth, Chapter 2 utilized a common ECM associated with axonal growth in spinal cord development, Ntn1, as a comparator to the effects of Matn2 and Ucma. Ntn1 did not promote axonal growth in vitro in cortical neurons. Those results, serve as the basis for these *in vivo* studies quantifying the effect of viral overexpression on peri-infarct axonal sprouting.

3.1.1 Reintroduction of identified ECM proteins.

Matn2 and Ucma were selected from a sprouting transcriptome previously characterized by the Carmichael lab (Li et al., 2010). In this previous study, spontaneously sprouting neurons post-stroke were isolated and mRNA levels evaluated to determine differential gene expression dependent on stroke. Additionally, this study identified transcriptional differences between young and aged animals, in part to identify differences that can explain age-related decline in spontaneous recovery, as age is a key factor in predicting magnitude of recovery.

Matn2 is an adapter protein, operating as a component of filamentous ECM networks throughout the body and interacting with itself in addition to other ECM proteins such as fibronectin and various collagens (Korpos et al., 2015). As a component of ECM complexes, Matn2 has also been identified as necessary for the regeneration of peripheral nerves following injury (Malin et al., 2009). This is an interesting finding as it relates to brain responses to stroke: peripheral neurons successfully regenerate after injury, whereas central neurons do not (Chandran et al., 2016; Renthal et al., 2020). Specific differences in the transcriptomes between

peripheral and central injury have been identified, with a focus on networks of transcription factors that appear to coordinately induce axonal sprouting in the peripheral nervous system, but are either not induced in CNS injury or no as tightly regulated (Chandran et al., 2016; Lee & Cho, 2021). The role of Matn2 following CNS injury, however, is complicated. Following white matter stroke, Matn2 expression is increased during the acute stroke phase, and recombinant administration of Matn2 to the stroke area led to enhanced functional recovery through enhanced differentiation of oligodendrocyte progenitor cells and remyelination in the peri-infarct area (Sozmen et al., 2019). However, axonally derived Matn2 has been identified as proinflammatory during exacerbation of multiple sclerosis, with loss of Matn2 being correlated to improved recovery (Jonas et al., 2014). Further, Matn2 is among the most down-regulated genes in sprouting neurons following ischemic stroke in young animals, decreasing by 52%, while being unchanged in aged animals (Li et al., 2010). The significant downregulation of Matn2 in stroke, but its important role in the successful regeneration of connections in the PNS, suggests that Matn2 might be a candidate gene for the reduced axonal sprouting response in the brain after stroke.

Ucma is a relatively unknown ECM protein, having first been identified in 2008 (Tagariello et al., 2008). Within cartilage networks, Ucma acts as a stabilizing factor while also inhibiting aggrecanase activity during inflammatory pathologies such as osteoarthritis (Seuffert et al., 2018). Little is known of the function of Ucma within the brain, other than it is a significantly enriched gene within the insular cortex (Ibrahim et al., 2019). In sprouting neurons post-stroke, Ucma expression was significantly upregulated in young animals, and was the most down-regulated gene in aged animals at both acute and chronic time points, decreasing by 98% and 60% respectively (Li et al., 2010). Because plasticity and recovery after stroke decline with

age, this pattern of induction in sprouting neurons after stroke in young adults, and then profound inhibition in aged adults, associates Ucma with the reduced response to stroke with age.

Ntn1 is a canonical axon guidance ECM protein associated with formation of commissural axon tracts in the developing spinal cord (Varadarajan et al., 2017; Varadarajan & Butler, 2017). In the context of stroke, Ntn1 overexpression has been associated with angiogenesis resulting in some degree of functional recovery (Ding et al., 2014). Despite its fundamental role in axon pathfinding, Ntn1 has not been identified as a promoter of axonal sprouting in the post-stroke brain. This, in combination with the negative results seen in Chapter 2, made Ntn1 a negative control for axonal sprouting, as increased deposition of protein into the extracellular space may affect axonal sprouting due solely to changes in extracellular stiffness (Koser et al., 2016). Specifically, Ntn1 allows testing of the effects of inducing more of an ECM protein into the post-stroke brain and the region of recovery, to specifically test where Matn2 and Ucma distinctly influence axonal sprouting, or if such an effect is just a general one to altering levels of ECM proteins.

3.1.2 Enhancing axonal sprouting in the post-stroke brain

Axonal sprouting is one of limited ways in which the brain repairs following stroke (Carmichael et al., 2017). Axonal sprouting occurs when surviving neurons in the peri-infarct cortex extend collaterals to reform circuits lost as a result of the injury. Following stroke in motor cortex, axonal sprouting is seen arising from the surviving motor cortex and directed towards the premotor and somatosensory cortices as measured by direct quantification of axonal projections (Li et al., 2015; Overman et al., 2012). In the absence of injury, axonal sprouting does not occur, likely due to the mature state of neurons that results in a homeostatic state and the presence of growth inhibitory molecules such as CSPGs that restrict unbounded sprouting.

Axonal sprouting has also been demonstrated through reorganization of somatosensory maps, in which new areas of the brain are recruited and replace lost areas due to injury due to the formation of new circuits arising from sprouting axons (Brown et al., 2009; Caracciolo et al., 2018). Finally, there is evidence that axonal sprouting occurs in human patients post-stroke. EEG analysis of stroke patients who had significant recovery shows increased activation of new areas in the ipsilesional premotor cortex (Gerloff et al., 2006), At the molecular level, post-stroke human brain samples show increased levels of GAP-43 (Ng et al., 1988), a protein expressed in the growth cone of extending axons. Finally, patients showed a greater degree of functional recovery that correlated with an increase in structural remapping into the corticospinal tract with a correlated increase in functional connectivity (Guggisberg et al., 2017). Taken together, these previous studies show evidence that axonal sprouting is a conserved mechanism of post-stroke recovery, and studies to enhance axonal sprouting merit further attention.

The experiments detailed in this chapter evaluate the effect of ECM protein overexpression in the premotor cortex post-stroke, specifically evaluating those neurons that are overexpressing the protein in question. Multiple previous studies have identified ipsilesional cortico-cortical sprouting arising from the premotor cortex as predictive of functional recovery post-stroke (Li et al., 2010, 2015; Overman et al., 2012). As the ECM proteins tested within this chapter arose from a transcriptomic data set derived from peri-infarct sprouting neurons sprouting within the same hemisphere as the ischemic injury, ipsilesional cortico-cortical sprouting was assessed here.

3.2 Methods

3.2.1 Development of viral vectors

Lentiviruses were selected to allow for sustained overexpression of selected ECM proteins. The pCDH-EF1 third generation lentivirus backbone was a gift from Kazuhiro Oka (Addgene Plasmid #72266). EF1 was chosen as a promoter due to its strength at driving overexpression and to promote an increase in extracellular abundance of target proteins. Commercially available open reading frames of target proteins (Origene MC220646, MC210739, MC219613), with the addition of a Spot Tag (Chromotek) or FLAG-tag at the C-terminus for protein localization, and a P2A-cre recombinase were amplified using PCR and inserted downstream of the promoter. Clones were sequenced and DNA isolated for lentivirus preparation. An example vector is shown in Figure 3-1.

3.2.2 Virus Generation

Lentiviruses were prepared using a modified packaging protocol (Dull et al., 1998). Briefly, low passage HEK-293T/17 cells were cultured in DMEM containing high glucose, Glutamax supplement, sodium pyruvate, and 10% heat-inactivated FBS at 37C and 3% CO2. Packaging plasmids pMDLg/pRRE (Addgene #12251), pRSV-Rev (Addgene #12253) and pMD2.G (Addgene #12259) in addition to transfer plasmids were transfected into cells using calcium phosphate precipitation with BES. 16 hours after transfection, media was removed, cells were washed with DPBS and fresh media with 10mM sodium butyrate was applied. After 24 hours, media was removed and stored at 4C, and fresh media applied. At 72 hours post-transfection, media was collected and pooled, centrifuged at 2000g for 10 minutes to pellet debris, and filtered with a 0.45um vacuum filter. Viruses were purified from media with

sequential ultracentrifugation. Following a 2.5-hour, 20,000 rpm spin (Beckman), the viral pellet was resuspended in DPBS and overlaid over 20% sucrose in DPBS. Following a 2.5-hour 32,000 rpm spin (Beckman), the resulting pellet was resuspended in DPBS and purified with a final 5 minute 7,000 g spin. Viral particles in the supernatant were aliquoted and stored at -80C until use. AAV pCAG-FLEX-EGFP-WPRE was a gift from Hongkui Zeng (Addgene viral prep # 51502-AAV9)

3.2.3 Animals

All animals used for this study were C57BL/6 strain male mice aged 10-12 weeks at the time of surgery (n = 6-8) per group. Mice were obtained from the Jackson Laboratory (JAX # 000664). Mice were randomly assigned to treatment groups and housed 4 per cage with *ad lib* access to food and water. All experiments were performed in accordance with National Institutes of Health animal use guidelines and were approved by the University of California, Los Angeles Animal Research Committee (ARC protocol #00-159).

3.2.4 Photothrombotic Stroke and Virus Delivery

Focal ischemic stroke was induced in 10–12-week-old male mice via photothrombosis. Anesthesia was induced with 4% isoflurane with 100% oxygen and maintained at 1.5-2% isoflurane for the duration of surgery. The surgical area was clipped clean of hair and sterilized with alternating swabs of Betadine and 70% alcohol. Temperature was controlled via feedback-mediated heating pads, and maintained at 37.0C +/- 0.5C. After securing the mouse to the stereotaxic frame, skin over the skull was opened via a midline incision and the skull was cleared

of any connective tissue. When mice were at the appropriate temperature, a solution of the photosensitive dye Rose Bengal (100mg/kg in sterile PBS) was injected intraperitoneally and allowed to circulate for 5 minutes. Photothrombotic stroke was then performed using a 520nm wavelength laser (light source CLD1010L, ThorLabs; laser diode LP520-MF100, ThorLabs) set at 12-12.2 mW output targeted over the left forelimb motor cortex (A/P 0, M/L 1.5) for 10 minutes. When exposed to light in the green spectrum, Rose Bengal causes the formation of free radicals, which in turn induces platelet activation and formation of thrombi, leading to a focal loss of circulation.

Immediately following photothrombotic stroke, mice received virus injections in the anterior peri-infarct pre-motor cortex (from Bregma: A/P 1.5, M/L 1.75, D/V 0.75). A dental drill was used to thin the skull at the above referenced location, through which a pulled glass pipette was lowered to the desired depth and allowed to sit for 3 minutes. 300 nL of virus (composed of 3uL lentivirus mixed with 1uL FLEX-AAV) was injected at a rate of 1nL/sec. Following injection, the pipette was left in place for 5 minutes to prevent backflow. The pipette was then removed from the brain, the scalp closed using Vetbond tissue adhesive (3M), and mice were returned to their home cage for recovery. An experimental overview showing surgery process and regions targeted is shown in Figure 3-3.

3.2.5 Tissue collection and preparation

Four weeks post-stroke and virus injection, mice were sacrificed via transcardial perfusion of ice-cold PBS followed by 4% PFA. Brains were removed and cortices flattened as described previously (Li et al., 2010, 2015). Briefly, the cerebellum and olfactory bulbs were

removed. The hemispheres were split, and subcortical tissue including the hippocampus and white matter tracts were removed. The cortices were flattened between two glass slides separated by 1.1mm spacers. Tissue was then post-fixed in 4% PFA overnight at 4C, and cryoprotected for 4-5 days in a 30% sucrose solution. 40um tangential sections were cut on a cryostat (Leica CM 0530) and stored sequentially in a 50% glycerol antifreeze solution at -20C until mounting.

3.2.6 Imaging and analysis of axonal sprouting

For analysis of ipsilesional axonal sprouting, flattened sections representing every 160um dorsal-to-ventral were acquired at 20x using a Nikon Eclipse Ni epifluorescent microscope and Stereo Investigator software (MBF Bioscience). Images were then analyzed using Neurolucida 360 (MBF Bioscience). Using a semi-automated approach, markers were placed on areas of fluorescence, and Cartesian coordinates were generated relative to a reference point placed at the center of the virus injection. Coordinates were then formatted and analyzed using a custom program and script as described previously (Li et al., 2010, 2015). A sample flattened section with representative automated marker placement is shown in Figure 3-4.

3.2.7 Statistics

Sample sizes were consistent with previous similar studies within the lab. For quantification of axonal sprouting, Hotelling's T2 test for spatial correlation was performed as described previously (Li et al., 2010, 2015).

3.3 Results

3.3.1 Virus design and approach

In chapter 2 of this dissertation, primary neurons were exposed to exogenous recombinant proteins as a substrate for growth. While this approach could be recapitulated *in vivo,* whether through direct injection of recombinant protein (Sozmen et al., 2019) or administration through a hydrogel (Li et al., 2015), there would be a lack of specificity in any labeling approach of affected neurons, potentially diluting any effect seen. Specifically, Matn2 and Ucma were originally identified based on their differential expression in in peri-infarct cortex neurons that sprout a new connection after stroke. The goal of the present studies is to determine what effect these two ECM proteins have in peri-infarct neurons and if this effect is associated with an enhanced functional recovery. Techniques that just generally deposit more Matn2 or Ucma into the peri-infarct region do not test the selective role of neuronal induction. As such, lentivirus-mediated overexpression was used. All constructs were based on the pCDH backbone with the EF1α promoter, a strong ubiquitous promoter. This promoter was chosen due to the belief that these proteins would function primarily in the extracellular space, and therefore it would not be important to restrict generation of the proteins to a single cell type such as only neurons. Additionally, as the goal was to specifically label those cells overexpressing the target proteins and ensure that these cells were sufficiently labeled to allow tracing of axons to their terminals, a p2a-cre recombinase was inserted downstream of the gene of interested. The addition of cre-recombinase would allow the use of a FLEX AAV, which would simultaneously provide robust cell labeling while also restricting labeling to the area of lentivirus transduction and to those cells overexpressing the protein of interest. Representative images showing viral overexpression are shown in figure 3-2.

3.3.2 Matrilin 2 and Ucma overexpression enhance post-stroke axonal sprouting

To determine whether Matn2 or Ucma enhance axonal sprouting post-stroke, lentiviruses inducing overexpression of these genes, in addition to a cre-dependent AAV-GFP for cell labeling, were injected into the anterior peri-infarct motor cortex at the time of stroke. To control for virus injection, an empty vector was injected expressing only cre-recombinase. To control for ECM deposition alone inducing axonal sprouting through manipulation of tissue stiffness, a vector causing overexpression of Ntn1 was used. 28 days after stroke and virus injection, cortices were flattened as described. The results are shown in Figure 3-5. Matn2 overexpression in the peri-infarct motor cortex induced widespread radial axonal sprouting, with a significant increase of naturally occurring axonal sprouting ($p = 0.0305$, Hotelling's T2 test). Ucma overexpression also induced an increase in axonal sprouting, although the observed increase was more directional towards the somatosensory cortex ($p = 0.006$, Hotelling's T2 test).

To control for the biological effect of causing protein to be overexpressed into the extracellular space, the same experimental design was used while overexpressing Ntn1, an ECM protein that failed to induce neurite outgrowth as shown in Chapter 2. Ntn1 overexpression failed to increase axonal sprouting ($p = 0.1862$, Hotelling's T2 test).

3.4 Discussion

In this study, two ECM proteins, Matn2 and Ucma, were chosen for axonal sprouting studies after being identified as differentially regulated in sprouting neurons post-stroke (Li et al., 2010) and showing positive effects on neurite outgrowth in Chapter 2. Matn2 additionally had previously been shown as necessary for peripheral nerve regeneration (Malin et al., 2009). Ntn1 was also chosen as a negative control, as it did not increase neurite outgrowth in Chapter 2 but would control for increased deposition of ECM protein in the extracellular space. The target proteins were overexpressed via lentiviral transduction of cells in the premotor cortex following

photothrombotic stroke (Figure 3-2). Cells overexpressing these proteins were labeled through coadministration of a cre-dependent AAV expressing EGFP. 28 days following stroke, mice were sacrificed and cortices prepared for quantitative axonal sprouting measurement as previously performed (Li et al., 2010, 2015; Overman et al., 2012).

Both Matn2 and Ucma overexpression showed an increase in axonal sprouting from the premotor cortex, although the pattern of axonal sprouting differed. Matn2 overexpression showed a robust radial axonal sprouting pattern, with increased axonal density towards the premotor cortex, prefrontal cortex and somatosensory cortex. Ucma overexpression showed increased axonal density towards the somatosensory cortex. The differences seen in sprouting patterns is striking, given both proteins were overexpressed using the same system. One possible explanation is, at default, increased sprouting towards the somatosensory cortex appears to be the default post-stroke (Li et al., 2015). Therefore, it is possible that Ucma overexpression enhances this baseline sprouting, whereas Matn2 overexpression, through an unknown mechanism, is able to enhance sprouting not only towards the somatosensory cortex but also more anterior towards the premotor cortex. This is consistent with *in vitro* results seem in Chapter 2, where growth on Matn2 showed no negative effect at higher doses, whereas use of Ucma as a growth substrate had a U-shaped growth curve. However, Ucma overexpression did not appear to have a negative effect on axonal sprouting, despite results in Chapter 2 indicating a narrow dose-response profile. This could be due to several reasons, including viral overexpression of Ucma did not result in expression levels similar to that seen in the *in* vitro screen, or overexpression in the more complete biological context of an intact brain had more of a buffer against too much Ucma being present for enhanced axon outgrowth. Further studies on the biological profile of Ucma will be beneficial to determine how long it lasts in the brain. Post-stroke sprouting towards the prefrontal

and somatosensory cortices has been associated with increased functional recovery post-stroke (Li et al., 2015); therefore, Matn2 and Ucma are both targets for further functional studies as enhancers of post-stroke return of function.

Overexpression of Ntn1 failed to increase post-stroke axonal sprouting. Inclusion of Ntn1 in this study, despite it failing to increase neurite outgrowth in Chapter 2, addressed an important consideration in the effect of artificially increased deposited ECM protein into the extracellular space. Rather, this indicates that the increased axonal sprouting seen with Matn2 and Ucma overexpression is due to a mechanism specific to the protein itself, rather than through alteration of the extracellular environment alone. This is further demonstrated through the different patterns of axonal sprouting seen between Matn2 and Ucma.

Several limitations exist that limit the conclusions that can be drawn from this set of studies. First, only a limited number of surviving neurons are affected by the viral overexpression of target proteins, and, due to the combinatorial viral approach used here for labeling purposes, only these neurons are evaluated for axonal sprouting. This method of analysis omits those cells adjacent to area of cells transduced by the virus, which could be affected by the secreted proteins. This concern is addressed in later work. Second, an increase in ipsilesional axonal sprouting has been correlated with improved functional recovery, but it is unknown the degree to which axonal sprouting contributes to functional recovery. Previous work in the Carmichael lab on CCR5 showed that ipsilesional axonal sprouting is not necessary for functional recovery, while other studies have linked a decrease in axonal sprouting to worsened functional outcomes (Joy et al., 2019; Li et al., 2015). These outcomes could indicate that an increase in ipsilesional axonal sprouting is sufficient to improve functional recovery after stroke

but may not be necessary. Finally, it is unknown whether these axons are functional, are persistent post-stroke or subject to pruning, and whether they are beneficial for recovery.

Taken together, the study described in this chapter indicates Matn2 and Ucma induce an increase in axonal sprouting in the post-stroke brain and merit further study. In the next chapter the same stroke and viral approach are used to evaluate whether Matn2 and Ucma are able to improve functional recovery following stroke affecting the forelimb motor cortex.

3.5 Figures

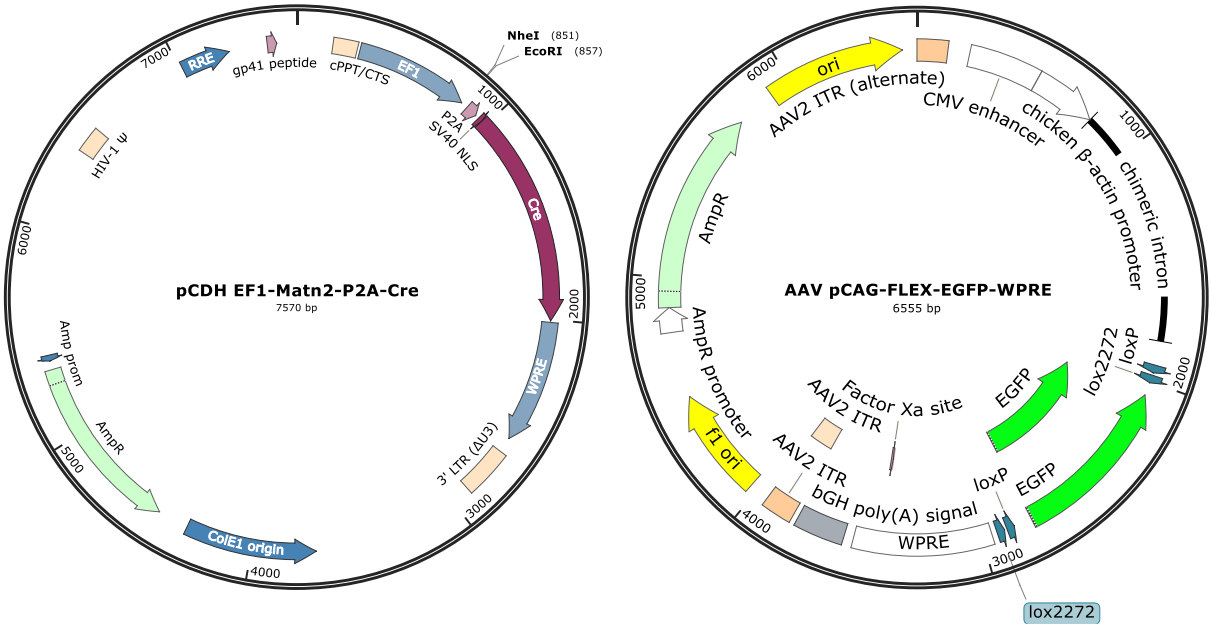


Figure 3-1. Representative viral vectors for overexpression of target ECM proteins and FLEX AAV construct. EF1, a strong ubiquitous promoter, was used to drive overexpression of target proteins (Matn2, Ucma or Ntn1) upstream of a P2A internal cleavage site, and cre recombinase. When used in combination with a FLEX, or cre-dependent, AAV, strong EGFP labeling was seen by those cells expressing cre recombinase, allowing for robust cell fill and visualization of axons to the terminal.

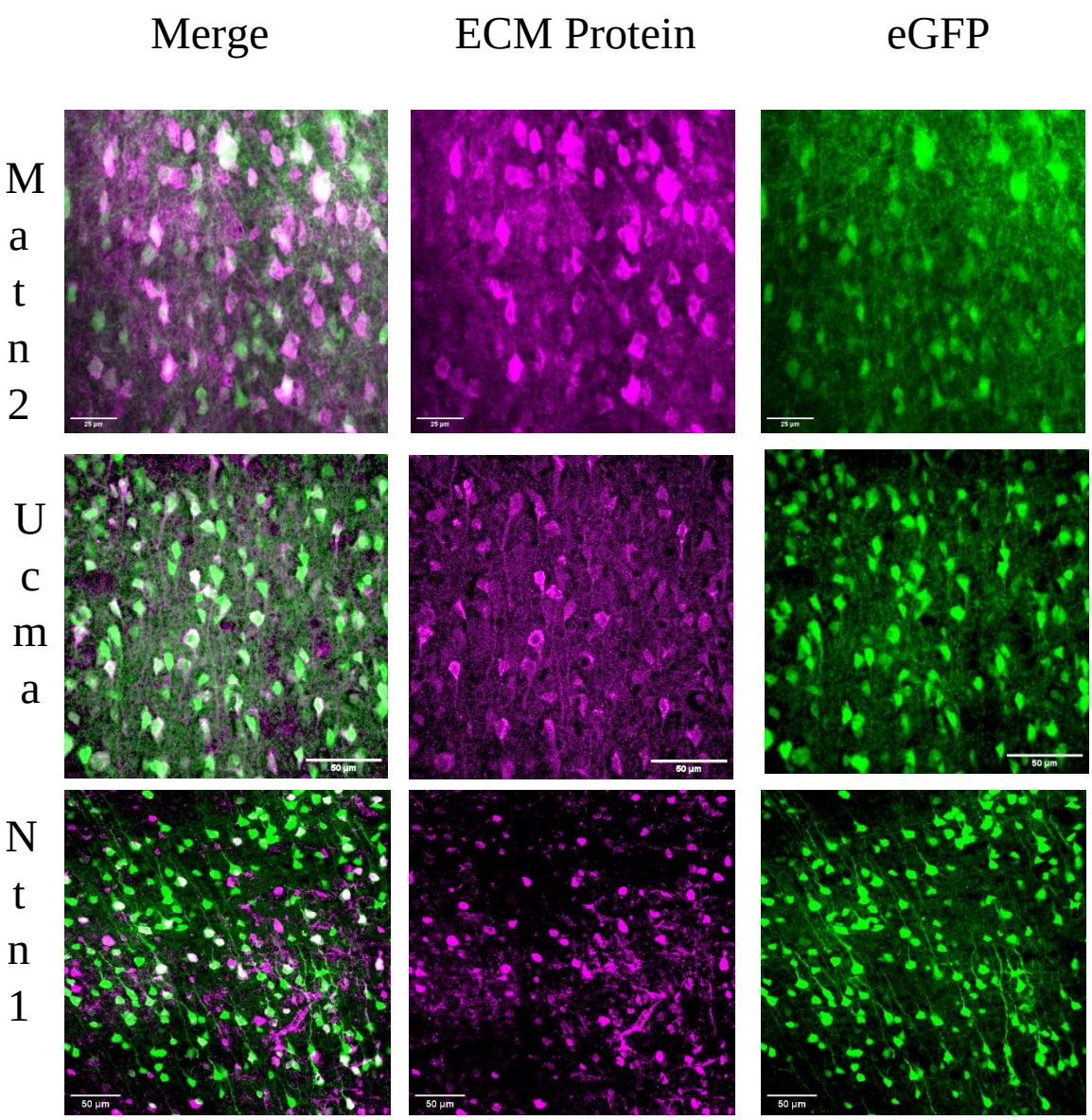
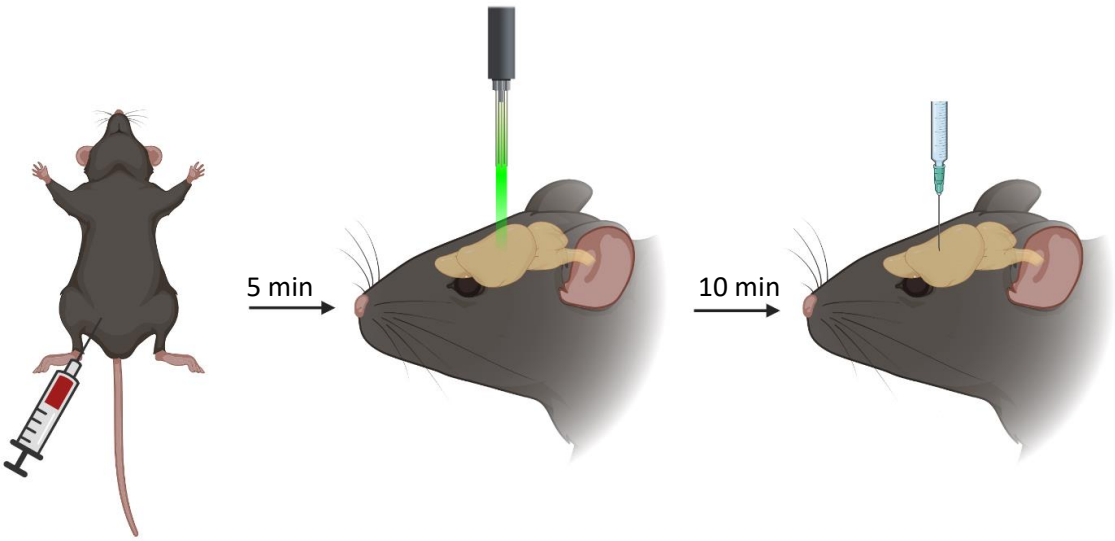


Figure 3-2. Lentiviruses drive overexpression of target proteins. Representative images of mice injected with lentivirus driving overexpression of Matn2 (top), Ucma (middle) or Ucma (bottom) in magenta, overlapping with eGFP expressed by cre-dependent AAV co-injected with lentivirus.

A



B

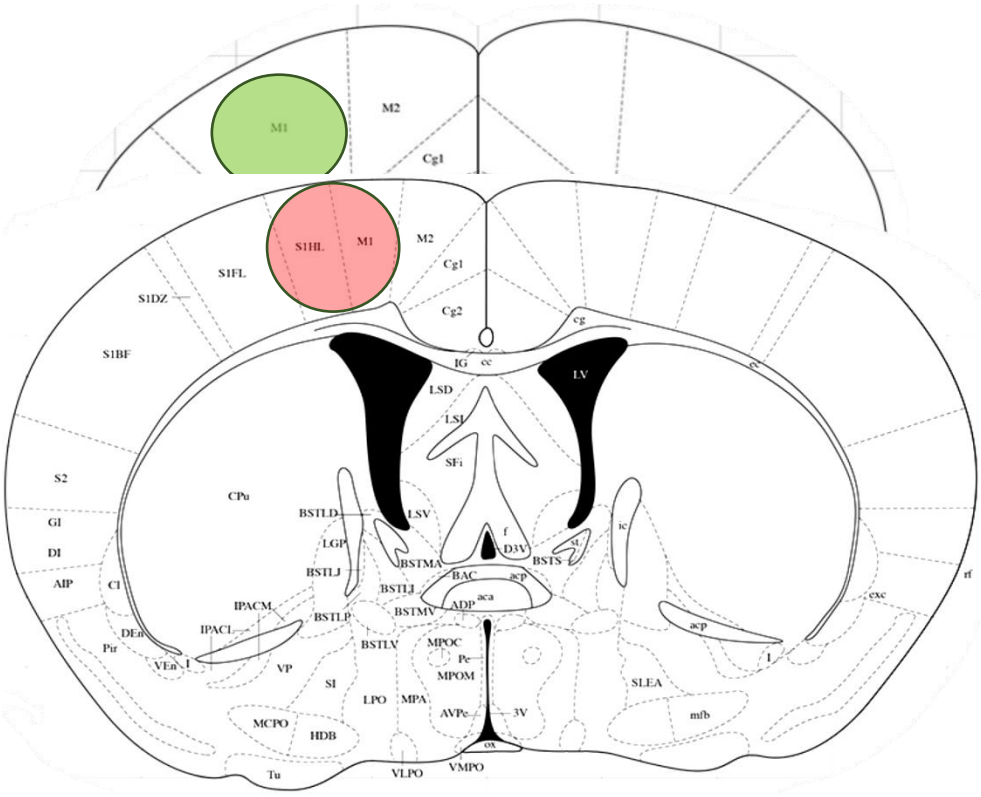
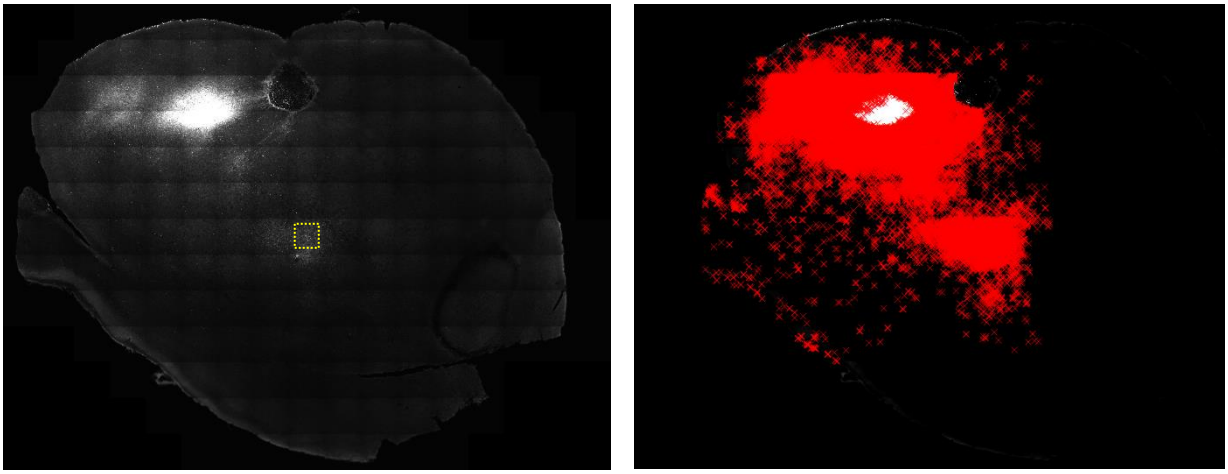


Figure 3-3. Experimental overview of surgery. A) Mouse were subjected to cortical stroke via photothrombosis, and a dual virus injection consisting of lentivirus driving overexpression of Matn2, Ucma, Ntn1 or empty vector, and cre-dependent AAV expression EGFP. B) Targets for viral overexpression and stroke. The circle in green indicated premotor cortex, the target for viral overexpression. Red indicated forelimb motor cortex, the target for photothrombotic stroke. Created with BioRender.com.

A



B

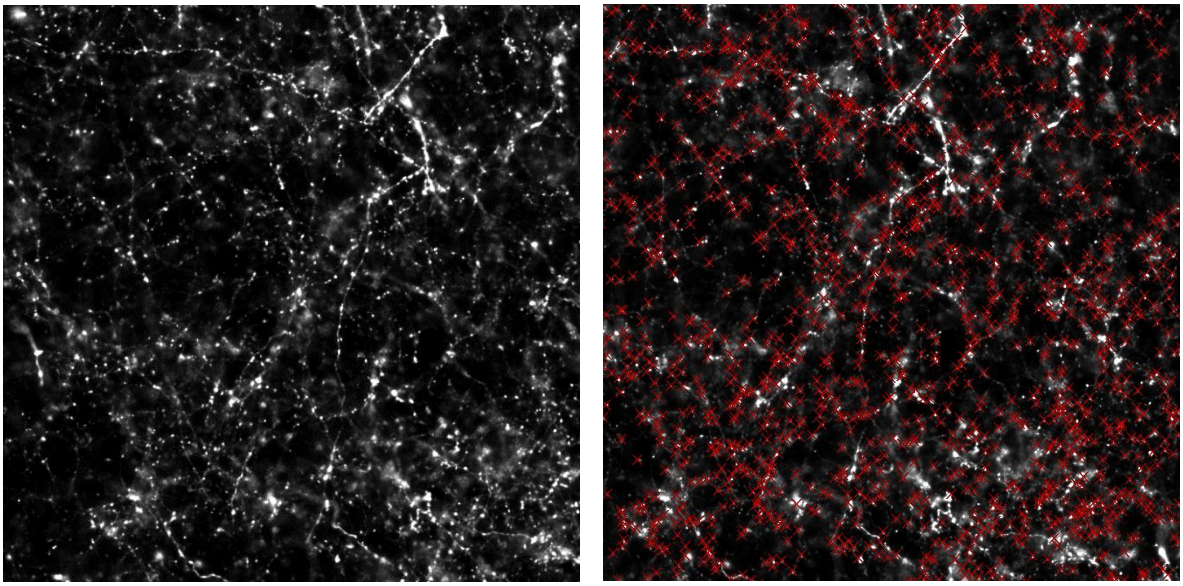
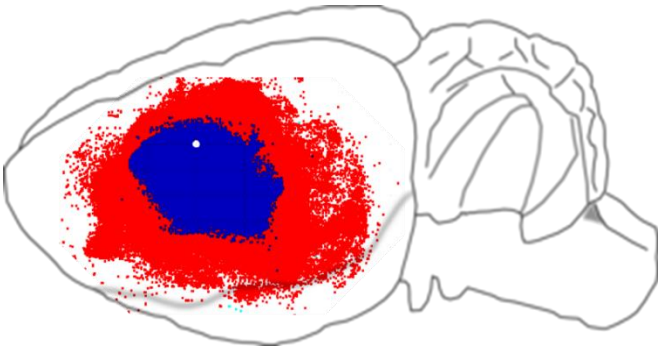
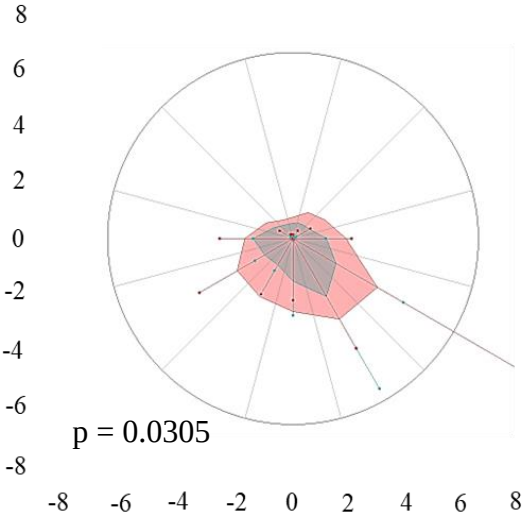


Figure 3-4. Representative flattened cortex with automated trace. 28 days post-stroke, mice were sacrificed, and brains prepared as flattened cortices. Cortices were sectioned tangentially and mounted. A representative flattened section is shown in A, as well as the same section after being analyzed using Neurolucida 360 as shown with the markers in red. In B, the region in the yellow box is shown at 20x magnification, with and without automated marker placement.

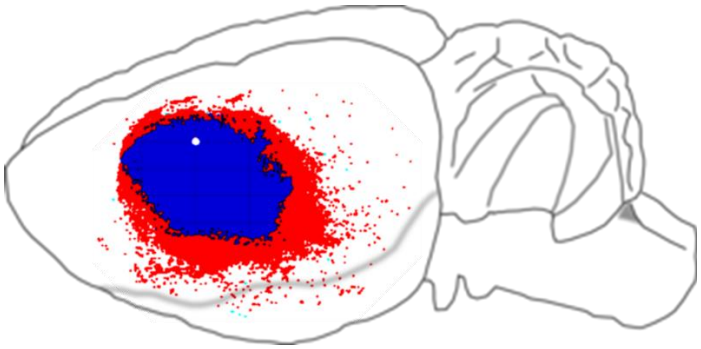
A Matn2 plus stroke vs control plus stroke



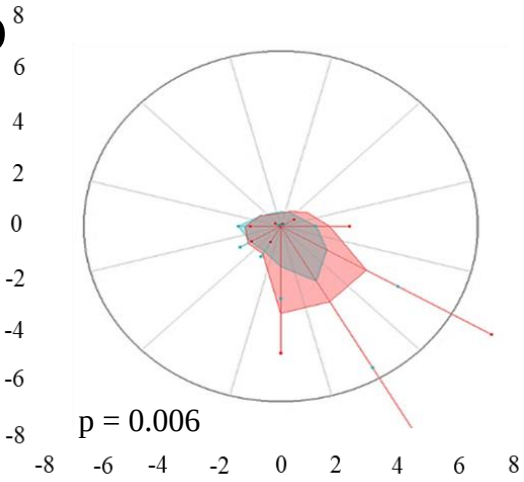
B



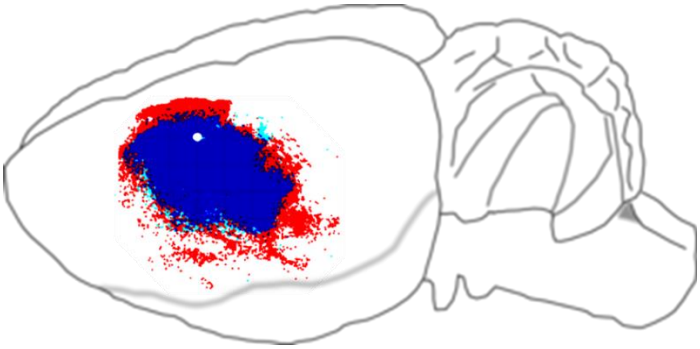
C Ucma plus stroke vs control plus stroke



D



E Ntn1 plus stroke vs control plus stroke



F

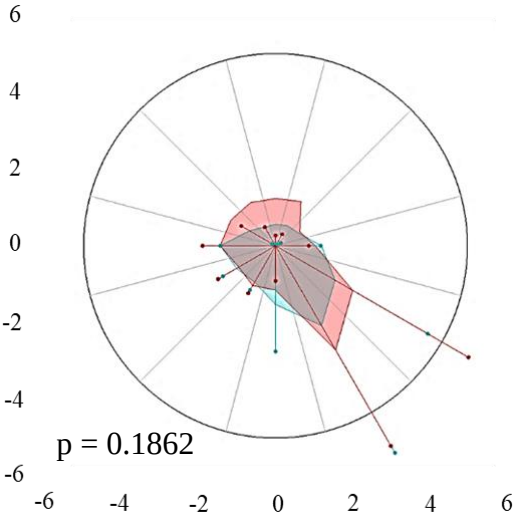


Figure 3-5. Matn2 and Ucma overexpression promote axonal sprouting in peri-infarct cortex after stroke. (A) Quantitative cortical mapping of axonal projections in ipsilateral peri-infarct cortex with empty vector lentivirus control (light blue, n = 6) and lentivirus overexpressing Matn2 (red, n = 8), with areas of overlap in dark blue. The empty circle indicates the injection site and reference point for generation of Cartesian coordinates. (B) Polar plot of axonal projections from the premotor cortex with the injection site as the origin. Shaded polygons represent the 70^{th} percentile of the distances of labeled axons from the origin in each segment of the graph. Weighted polar vectors represent the median number of connections present in a segment. Axes represent distance from origin site in millimeters. Matn2 overexpression promotes axonal sprouting radially, with increased connections identified in premotor and somatosensory cortex ($p = 0.0305$, Hotelling's T2 distribution test. (C) Quantitative cortical mapping of Ucma overexpression (red, n = 8), empty vector control (light blue, n = 6), and areas of overlap (dark blue) are presented as in (A). (D) Polar plot of Ucma versus empty vector control. Data are presented as in (B). Ucma promotes post-stroke axonal sprouting directionally towards the somatosensory cortex ($p = 0.006$, Hotelling's T2 distribution test. (E) Quantitative cortical mapping of Ntn1 overexpression (red, n = 6) and empty vector control (light blue, n = 6) with areas of overlap (dark blue) are presented as in (A). (F) Polar plot of Ntn1 overexpression vs. empty vector control. Data are presented as in (B). Ntn1 overexpression does not increase post-stroke axonal sprouting ($p = 0.1862$, Hotelling's T2 distribution test).

3.6 References

Brown, C. E., Aminoltejari, K., Erb, H., Winship, I. R., & Murphy, T. H. (2009). In Vivo Voltage-Sensitive Dye Imaging in Adult Mice Reveals That Somatosensory Maps Lost to Stroke Are Replaced over Weeks by New Structural and Functional Circuits with Prolonged Modes of Activation within Both the Peri-Infarct Zone and Distant Sites. *Journal of Neuroscience*, *29*(6), 1719–1734. https://doi.org/10.1523/JNEUROSCI.4249-08.2009

Caracciolo, L., Marosi, M., Mazzitelli, J., Latifi, S., Sano, Y., Galvan, L., Kawaguchi, R., Holley, S., Levine, M. S., Coppola, G., Portera-Cailliau, C., Silva, A. J., & Carmichael, S. T. (2018). CREB controls cortical circuit plasticity and functional recovery after stroke. *Nature Communications*, *9*(1), 2250. https://doi.org/10.1038/s41467-018-04445-9

Carmichael, S. T., Kathirvelu, B., Schweppe, C. A., & Nie, E. H. (2017). Molecular, Cellular and Functional Events in Axonal Sprouting after Stroke. *Experimental Neurology*, *287*(Pt 3), 384. https://doi.org/10.1016/J.EXPNEUROL.2016.02.007

Chandran, V., Coppola, G., Nawabi, H., Omura, T., Versano, R., Huebner, E. A., Zhang, A., Costigan, M., Yekkirala, A., Barrett, L., Blesch, A., Michaelevski, I., Davis-Turak, J., Gao, F., Langfelder, P., Horvath, S., He, Z., Benowitz, L., Fainzilber, M., … Geschwind, D. H. (2016). A Systems-Level Analysis of the Peripheral Nerve Intrinsic Axonal Growth Program. *Neuron*, *89*(5), 956. https://doi.org/10.1016/J.NEURON.2016.01.034

Ding, Q., Liao, S. J., & Yu, J. (2014). Axon guidance factor netrin-1 and its receptors regulate angiogenesis after cerebral ischemia. *Neuroscience Bulletin*, *30*(4), 683. https://doi.org/10.1007/S12264-013-1441-9

Dull, T., Zufferey, R., Kelly, M., Mandel, R. J., Nguyen, M., Trono, D., & Naldini, L. (1998). A

Third-Generation Lentivirus Vector with a Conditional Packaging System. *Journal of Virology*, *72*(11), 8463–8471. https://doi.org/10.1128/jvi.72.11.8463-8471.1998

Gerloff, C., Bushara, K., Sailer, A., Wassermann, E. M., Chen, R., Matsuoka, T., Waldvogel, D., Wittenberg, G. F., Ishii, K., Cohen, L. G., & Hallett, M. (2006). Multimodal imaging of brain reorganization in motor areas of the contralesional hemisphere of well recovered patients after capsular stroke. *Brain*, *129*(3), 791–808. https://doi.org/10.1093/BRAIN/AWH713

Guggisberg, A. G., Nicolo, P., Cohen, L. G., Schnider, A., & Buch, E. R. (2017). Longitudinal Structural and Functional Differences Between Proportional and Poor Motor Recovery After Stroke. *Neurorehabilitation and Neural Repair*, *31*(12), 1029–1041. https://doi.org/10.1177/1545968317740634

Ibrahim, C., Le Foll, B., & French, L. (2019). Transcriptomic Characterization of the Human Insular Cortex and Claustrum. *Frontiers in Neuroanatomy*, *13*. https://doi.org/10.3389/fnana.2019.00094

Jonas, A., Thiem, S., Kuhlmann, T., Wagener, R., Aszodi, A., Nowell, C., Hagemeier, K., Laverick, L., Perreau, V., Jokubaitis, V., Emery, B., Kilpatrick, T., Butzkueven, H., & Gresle, M. (2014). Axonally derived matrilin-2 induces proinflammatory responses that exacerbate autoimmune neuroinflammation. *The Journal of Clinical Investigation*, *124*(11), 5042–5056. https://doi.org/10.1172/JCI71385

Joy, M. T., Ben Assayag, E., Shabashov-Stone, D., Liraz-Zaltsman, S., Mazzitelli, J., Arenas, M., Abduljawad, N., Kliper, E., Korczyn, A. D., Thareja, N. S., Kesner, E. L., Zhou, M., Huang, S., Silva, T. K., Katz, N., Bornstein, N. M., Silva, A. J., Shohami, E., & Carmichael,

S. T. (2019). CCR5 Is a Therapeutic Target for Recovery after Stroke and Traumatic Brain Injury. *Cell*, *176*(5), 1143. https://doi.org/10.1016/J.CELL.2019.01.044

Korpos, É., Deák, F., & Kiss, I. (2015). Matrilin-2, an extracellular adaptor protein, is needed for the regeneration of muscle, nerve and other tissues. *Neural Regeneration Research*, *10*(6), 866–869. https://doi.org/10.4103/1673-5374.158332

Koser, D. E., Thompson, A. J., Foster, S. K., Dwivedy, A., Pillai, E. K., Sheridan, G. K., Svoboda, H., Viana, M., Costa, L. D. F., Guck, J., Holt, C. E., & Franze, K. (2016). Mechanosensing is critical for axon growth in the developing brain. *Nature Neuroscience 2016 19:12*, *19*(12), 1592–1598. https://doi.org/10.1038/nn.4394

Lee, J., & Cho, Y. (2021). Comparative gene expression profiling reveals the mechanisms of axon regeneration. *The FEBS Journal*, *288*(16), 4786–4797. https://doi.org/10.1111/FEBS.15646

Li, S., Nie, E. H., Yin, Y., Benowitz, L. I., Tung, S., Vinters, H. V, Bahjat, F. R., Stenzel-Poore, M. P., Kawaguchi, R., Coppola, G., & Carmichael, S. T. (2015). GDF10 is a signal for axonal sprouting and functional recovery after stroke. *Nature Neuroscience*, *18*(12), 1737–1745. https://doi.org/10.1038/nn.4146

Li, S., Overman, J. J., Katsman, D., Kozlov, S. V., Donnelly, C. J., Twiss, J. L., Giger, R. J., Coppola, G., Geschwind, D. H., & Carmichael, S. T. (2010). An age-related sprouting transcriptome provides molecular control of axonal sprouting after stroke. *Nature Neuroscience*, *13*(12), 1496–1504. https://doi.org/10.1038/nn.2674

Malin, D., Sonnenberg-Riethmacher, E., Guseva, D., Wagener, R., Aszódi, A., Irintchev, A. A., Riethmacher, D., Irintchev, A. A., & Riethmacher, D. (2009). The extracellular-matrix

protein matrilin 2 participates in peripheral nerve regeneration. *Journal of Cell Science*, *122*(Pt 7), 995–1004. https://doi.org/10.1242/jcs.040378

Ng, S. C., de la Monte, S. M., Conboy, G. L., Karns, L. R., & Fishman, M. C. (1988). Cloning of human GAP 43: Growth association and ischemic resurgence. *Neuron*, *1*(2), 133–139. https://doi.org/10.1016/0896-6273(88)90197-3

Overman, J. J., Clarkson, A. N., Wanner, I. B., Overman, W. T., Eckstein, I., Maguire, J. L., Dinov, I. D., Toga, A. W., & Carmichael, S. T. (2012). A role for ephrin-A5 in axonal sprouting, recovery, and activity-dependent plasticity after stroke. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(33). https://doi.org/10.1073/PNAS.1204386109/SUPPL_FILE/PNAS.201204386SI.PDF

Renthal, W., Tochitsky, I., Yang, L., Cheng, Y. C., Li, E., Kawaguchi, R., Geschwind, D. H., & Woolf, C. J. (2020). Transcriptional reprogramming of distinct peripheral sensory neuronsubtypes after axonal injury. *Neuron*, *108*(1), 128. https://doi.org/10.1016/J.NEURON.2020.07.026

Seuffert, F., Weidner, D., Baum, W., Schett, G., & Stock, M. (2018). Upper zone of growth plate and cartilage matrix associated protein protects cartilage during inflammatory arthritis. *Arthritis Research & Therapy*, *20*(1), 88. https://arthritis-research.biomedcentral.com/articles/10.1186/s13075-018-1583-2

Sozmen, E. G., DiTullio, D. J., Rosenzweig, S., Hinman, J. D., Bridges, S. P., Marin, M. A., Kawaguchi, R., Coppola, G., & Carmichael, S. T. (2019). White Matter Stroke Induces a Unique Oligo-Astrocyte Niche That Inhibits Recovery. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *39*(47), 9343–9359.

https://doi.org/10.1523/JNEUROSCI.0103-19.2019

Tagariello, A., Luther, J., Streiter, M., Didt-Koziel, L., Wuelling, M., Surmann-Schmitt, C., Stock, M., Adam, N., Vortkamp, A., & Winterpacht, A. (2008). Ucma - A novel secreted factor represents a highly specific marker for distal chondrocytes. *Matrix Biology*, *27*(1), 3–11. https://doi.org/10.1016/j.matbio.2007.07.004

Varadarajan, S. G., & Butler, S. J. (2017). Netrin1 establishes multiple boundaries for axon growth in the developing spinal cord. *Developmental Biology*, *430*(1), 177. https://doi.org/10.1016/J.YDBIO.2017.08.001

Varadarajan, S. G., Kong, J. H., Phan, K. D., Kao, T. J., Panaitof, S. C., Cardin, J., Eltzschig, H., Kania, A., Novitch, B. G., & Butler, S. J. (2017). Netrin1 Produced by Neural Progenitors, Not Floor Plate Cells, Is Required for Axon Guidance in the Spinal Cord. *Neuron*, *94*(4), 790-799.e3. https://doi.org/10.1016/J.NEURON.2017.03.007

Chapter 4

Effects of Matn2 and Ucma on functional recovery in the post-stroke brain

4.1 Introduction

Previous chapters have identified Matn2 and Ucma as ECM proteins that enhance axonal sprouting post-stroke. An increase in axonal sprouting has been shown to lead to enhanced functional recovery. In this chapter, Matn2 and Ucma are evaluated as enhancers of recovery of motor function through the use of two well characterized behavioral tests that require the use of forelimb motor cortex, the area affected by the stroke model used.

4.1.1 *In vivo* assessment of Matn2 and Ucma to promote post-stroke functional recovery

In Chapter 3, Matn2 and Ucma were able to enhance axonal sprouting post-stroke. As axonal sprouting has been causally linked to improved functional recovery (Li et al., 2015; Overman et al., 2012), a study to evaluate these proteins in promote motor recovery following cortical stroke was performed. Forelimb motor function was assessed through two well-characterized motor behavior tasks, the grid walk and pasta matrix. In the grid walk task, motor coordination is evaluated by observing mice spontaneously walking on a grid. Foot faults, defined as slippage of the limb through an opening, and total steps are recorded and counted. Following ischemic injury to the forelimb motor cortex, mice show an increase in percentage of foot faults relative to total number of steps taken (Chao et al., 2012; Joy et al., 2019; Li et al., 2015). In the pasta matrix task, mice are trained to break pieces of pasta by reaching through an opening in a plexiglass box, such that only the stroke-affected limb can be used, and are evaluated for ischemia related impact on grip strength and fine motor control (Tennant et al., 2010). These tasks have been well characterized in motor stroke, and evaluate gross and fine motor control. This is relevant, as among human patients with stroke affecting the motor cortex, approximately 70% have weakness impairing function in the arm on the affected side, and of those more than half have deficit 6 months post-stroke (Allison et al., 2016; Kwakkel et al.,

2003). As such, utilization of behavioral tasks in mice that recapitulate deficit seen in human patients, and evaluating whether the treatments put forward in this study can accelerate recovery allows for direct translation to whether these treatments are suitable for human stroke recovery.

Mice are trained on the pasta matrix for one month prior to stroke, and baseline behavior is assessed immediately prior to surgery. As in Chapter 3, mice are given a photothrombotic stroke to the forelimb motor cortex to induce ischemia. A combination of a lentivirus driving overexpression of the target protein and cre-recombinase and a cre-dependent AAV are injected into the peri-infarct premotor cortex. This dual virus approach was used to limit overexpression of the target proteins to the surviving motor cortex, as this allows for direct manipulation of an area known to contribute to stroke recovery through axonal sprouting (Li et al., 2015; Overman et al., 2012), while also selectively labeling those cells overexpressing the protein of interest to allow for tissue outcome measures such as evaluation of axonal sprouting in the chronic, potentially recovered brain. The area of viral transduction is limited to surviving motor cortex, extending anterior to the premotor cortex and posterior to just adjacent to the infarct core. This region has been identified as a key region from which axonal sprouting and where cortical remapping occur (Caracciolo et al., 2018; Li et al., 2015), and . Acute functional deficits are measured 7 days post-stroke, and behavior is evaluated periodically over the course of two months, with pasta matrix further revaluated 3-, 5-, 7-, and 9-weeks post-stroke, and grid walk further evaluated 4-, 6-, and 8-weeks post-stroke.

4.1.2 Linking an increase in axonal sprouting to functional recovery

An increase in axonal sprouting has previously been associated with improvement in functional recovery (Li et al., 2015; Overman et al., 2012), but increased axonal sprouting is not strictly necessary for recovery (Joy et al., 2019). Other mechanisms, including enhancing

plasticity of the peri-infarct in neuronal functional connectivity (Joy et al., 2019; Joy & Carmichael, 2020), increasing the activity of neural progenitors (Liang et al., 2019), and modulating the excitability of neurons involved in remapping of cortical circuits (Caracciolo et al., 2018; Latifi et al., 2020) all play a role in functional recovery, whether independently or in addition to axonal sprouting. Axonal sprouting may also be a transient response. While pruning of axons is typically seen in the developing brain (Luo & O'Leary, 2005), stroke or CNS injury reverts neurons to a more development-like state (Poplawski et al., 2020). Excess axonal sprouting, especially after treatment-induced enhancement, may in turn result in a need for pruning of excess connections. Such an observation is seen when an axonal growth inhibition is blocked in the setting of deliberate overuse of the affected brain circuits (Overman et al., 2012; Wahl et al., 2014). Here, evaluation of axonal sprouting two months post-stroke, after evaluation of functional outcomes, will lend insight to whether prolonged axonal sprouting is associated with return of function.

4.2 Methods

4.2.1 Mice

All animals used in this section were male C57Bl/6 mice obtained from Jackson Laboratories (JAX stock #000664). Mice were 8-10 weeks old at the start of the behavior training described below, and 12-14 weeks old at the time of surgery. All mice were randomly assigned to treatment groups, while ensuring adequate numbers for each behavior task. All experiments were performed in accordance with National Institutes of Health animal use guidelines and were approved by the University of California, Los Angeles Animal Research Committee (ARC protocol #00-159).

4.2.2 Photothrombotic stroke and virus injection

Stroke was induced in 12–14-week-old mice using the same approach described in Chapter 3. Immediately after stroke, mice received 250nl virus injections in the anterior peri-infarct premotor cortex as described in Chapter 3.

4.2.3 Gridwalk

The gridwalk task was administered as described previously (Chao et al., 2012). Baseline behavior was assessed 1 day prior to stroke. Ischemia-related deficits were analyzed at 1, 4, 6 and 8- weeks post-stroke. The gridwalking task was performed on an apparatus consisting of 12-mm square wire mesh over an area of 32 cm x 20 cm x 50 cm (length/width/height). A mirror was positioned underneath the grid, and a video camera was placed such that the entire grid field was visible to allow for visualizing total steps and stepping errors ("foot faults"). Each animal underwent a single trial at the previously mentioned time points in a dark location during their awake period. Mice were allowed to walk freely for a period of five minutes, after which they were returned to their home cage. The number of mice per condition was determined through comparison to previous studies using this model of stroke (Joy et al., 2019; Li et al., 2015; Overman et al., 2012).

Videos were analyzed by the investigator blinded to the condition. Each video was analyzed at 25% speed for either a period of 2 minutes after onset of movement, or 50 total steps taken by the mouse, whichever condition happened later. A total step was defined as intentional forward movement by the mouse using all four limbs. Foot faults were assessed for the stroke-

affected right limb, and defined as a loss of control of the limb resulting in the paw breaking the plane of the grid. The percentage of foot faults was calculated as [(number of foot faults / number of total steps) x 100]. A percentage of foot faults was used for statistical analysis to control for variability in total locomotion between animals and across trials.

4.2.4 Pasta Matrix

The pasta matrix task was adapted from (Tennant et al., 2010). In this task, mice are placed in a plexiglass box with a small opening that allows mice to reach for and retrieve small pieces of pasta (3.2 cm in length, 1 mm in diameter) arranged in a 5x5 grid in front of the opening. 4 weeks prior to stroke and virus injection, 8-10 week-old mice were food-deprived to 85% of initial body weight and subjected to daily training (6 days/week). Each mouse was trained once per day, during which they were placed in the plexiglass box for 15 minutes and allowed free movement. During early training, the grid of pasta was placed directly in front of the opening and lightly tapped against the box to encourage the mouse to reach for and break the pasta. As mice became more skilled at breaking the pasta, the grid was gradually moved over in front of the opening, thereby ultimately forcing the mouse to use only the right paw. At the end of each training session the number of broken pasta pieces was recorded. After 4 weeks of training, any mouse that failed to break at least 5 pieces of pasta was excluded from further analysis. The baseline number of pasta broken was assessed the 2 days immediately prior to stroke, with the average number of pasta pieces broken used as the baseline for statistical analysis. Mice were assessed for acute ischemia-related functional deficit 1 week after stroke, with repeated trials 3,5,7 and 9-weeks post-stroke. The percent of baseline was defined as [(number of pieces of pasta broken/baseline number of pieces of pasta broken) x 100].

4.2.5 Tissue collection and processing

1 day following the final pasta matrix trial, mice were sacrificed via transcardial perfusion with ice-cold PBS and 4% PFA. A subset of brains (n = 8 per experimental condition) was processed for flattened cortices as described in Chapter 3. Brains that were left whole were post-fixed in 4% PFA overnight at 4C, cryoprotected in 30% sucrose for 48 hours, and stored at -80C.

4.2.6 Axonal sprouting and infarct size analysis

Axonal sprouting was measured as described in Chapter 3. Infarct volume was determined by measuring the infarct core in flattened sections and extrapolating volume by distance between sections and the thickness of each section.

4.2.7 Statistics

Sample sizes were assessed by power analysis using a significance level of a = 0.05 with 80% power to detect differences by ANOVA. Behavioral data were analyzed using a two-way repeated measures ANOVA followed by Tukey's multiple comparisons test. Infarct size data were analyzed by ordinary one-way ANOVA followed by Tukey's multiple comparisons test. Two mice were excluded from analysis due to death in the first week post-stroke. Statistical analysis and graph generation was performed using GraphPad Prism software (version 9.1.0).

4.3 Results

4.3.1 Matrilin 2 overexpression accelerates post-stroke functional recovery in the grid walk, whereas Ucma only transiently enhances recovery

Baseline performance in the grid task was assessed 1 day prior to surgery (Figure 4-1). All mice showed a baseline foot fault percent of approximately 10% relative to total number of steps taken (Figure 4-2). One week post-surgery, all mice in stroke groups showed an approximate three-fold increase in foot faults (Baseline vs Week 1 Ctrl virus plus stroke $p < 0.0001$, Matn2 plus stroke $p < 0.0001$, Ucma plus stroke $p < 0.0001$), and had a significant motor deficit when compared to sham stroke mice (Ctrl virus vs Ctrl virus plus stroke $p < 0.0001$, Ctrl virus vs Matn2 plus stroke $p < 0.0001$, Ctrl virus vs Ucma plus stroke $p < 0.0001$). In contrast, mice receiving sham strokes showed no significant increase in foot faults. One-month post-stroke, Ctrl virus plus stroke mice continued showing a significant foot fault percentage (24.86%) compared to sham group (Ctrl vs Ctrl virus plus stroke $p < 0.0001$). Mice receiving Matn2 or Ucma overexpression showed accelerated recovery that is significant compared to Ctrl virus plus stroke mice (Ctrl virus plus stroke vs Matn2 plus stroke $p = 0.0102$, Ctrl plus stroke vs Ucma plus stroke $p = 0.0001$), while still showing deficit when compared to sham group (Ctrl virus vs Matn2 plus stroke $p = 0.0048$, Ctrl virus vs Ucma plus $p = 0.0082$). Six weeks post-stroke, Ctrl virus plus stroke mice continued showing a significant foot fault deficit (20.99%) compared to sham mice (Ctrl virus vs Ctrl virus plus stroke $p = 0.0007$). Mice overexpressing Matn2 or Ucma did not show a significant deficit compared to sham stroke mice, and both groups showed significant improvement compared to Ctrl virus plus stroke mice (Ctrl virus plus stroke vs Matn2 plus stroke $p = 0.0040$, Ctrl virus plus stroke vs Ucma plus stroke $p = 0.0408$). Two-months post-stroke, mice receiving control virus plus stroke show a significant foot fault

deficit (16.29%) when compared to sham mice (Ctrl virus vs Ctrl virus plus stroke p = 0.0019), and Ucma plus stroke mice show a return of deficit (15.83%, Ctrl virus vs Ucma plus stroke p = 0.0235). Matn2 plus stroke mice did not show a deficit, and were significantly improved compared to control plus stroke mice (Ctrl virus plus stroke vs Matn2 plus stroke p = 0.0334). Mice overexpressing Ucma alone show a trend of increased foot fault percentage when compared to sham mice with control virus (p = 0.057). All data were analyzed via two-way repeated measures ANOVA with post-hoc Tukey's multiple comparisons test.

4.3.2 Matrilin 2 overexpression post-stroke accelerated recovery in the pasta matrix task, whereas Ucma overexpression partially accelerated recovery.

Mice were trained for the pasta matrix task for four weeks prior to surgery, and baseline assessed as the average number of pasta pieces broken in the last two days of training (Figure 4-1). One week following stroke, all mice receiving stroke showed significant impairment when compared to baseline, averaging 15% performance (Ctrl virus vs Ctrl virus plus stroke p = 0.00156, Ctrl virus vs Matn2 plus stroke p = 0.00126, Ctrl virus vs Ucma plus stroke p = 0.00146). Three weeks following stroke, Ctrl virus plus stroke mice showed a deficit (8%) when compared to sham mice (Ctrl virus vs Ctrl virus plus stroke p = 0.00011). Mice overexpressing Matn2 or Ucma showed an improving performance, but still showed a significant deficit when compared to sham groups (Ctrl virus vs Matn2 plus stroke p = 0.02113, Ctrl virus vs Ucma plus stroke p = 0.03213), and mice overexpressing Matn2 showed significant improvement compared to mice injected with control virus (Ctrl virus plus stroke vs Matn2 plus stroke p = 0.01552). Five weeks post-stroke, stroke mice injected with control virus still showed a significant deficit (22%) when compared to sham (Ctrl virus vs Ctrl virus plus stroke p = 0.00172). Mice overexpressing Matn2 or Ucma no longer had a significant deficit compared to sham, and both

groups showed significant improvement compared to stroke mice injected with control virus (Ctrl virus plus stroke vs Matn2 plus stroke $p < 0.0001$, Ctrl virus plus stroke vs Ucma plus stroke $p = 0.01510$). Seven weeks post-stroke, mice with stroke and control virus continued showing a deficit compared to sham (Ctrl virus vs Ctrl virus plus stroke $p = 0.00762$) and mice with stroke overexpressing Matn2 (Ctrl virus plus stroke vs Matn2 plus stroke $p = 0.00581$). Stroke mice overexpressing Matn2 or Ucma no longer show a deficit. Nine weeks post-stroke, mice that received a stroke no longer show a deficit, although stroke mice overexpressing Matn2 show a significantly better performance when compared to stroke plus control virus mice (Ctrl virus plus stroke vs Matn2 plus stroke $p = 0.00274$). All data were analyzed via two-way repeated measures ANOVA with post-hoc Tukey's multiple comparisons test.

4.3.3. Matrilin2 and Ucma overexpression enhance axonal sprouting two months after stroke.

Following behavioral assessments, mice were sacrificed, and axonal sprouting assessed as described in Chapter 3. These results are shown in Figure 4-4. Matn2 overexpression ($p = 0.03255$, Hotelling's T2 test for spatial distribution) and Ucma overexpression ($p = 0.01265$, Hotelling's T2 test for spatial distribution) following stroke resulted in a significant increase in axonal sprouting compared to empty vector control. Neither Matn2 nor Ucma overexpression in the absence of stroke changed axonal sprouting patterns (Figure 4-5).

4.3.4 Post-stroke Ucma overexpression results in a larger infarct

Infarct size was estimated by measuring infarct area and extrapolating volume using section thickness and interval between sections evaluated in tissue from mice that received virus injections at time of stroke and were sacrificed two months post-stroke. These results are shown in Figure 4-6. Matn2 overexpression did not result in a larger infarct size compared to control virus plus stroke ($p = 0.9099$). Ucma overexpression post-stroke did result in significantly larger

infarct volume compared to both control virus plus stroke and Matn2 overexpression plus stroke (Ctrl virus plus stroke vs Ucma plus stroke p = 0.0009, Matn2 plus stroke vs Ucma plus stroke p = 0.0004, one-way ANOVA with post-hoc Tukey's multiple comparisons test).

4.4 Discussion

In Chapter 3, Matn2 and Ucma overexpression were correlated with significant post-stroke axonal sprouting. An increase in axonal sprouting has previously been shown to correlate with improved functional recovery (Carmichael et al., 2017; Li et al., 2015; Overman et al., 2012). Given this, Matn2 and Ucma overexpression were evaluated for their impact on improving functional recovery post-stroke as measured by motor coordination in the grid walk and fine motor control and strength with the pasta matrix tasks.

In the grid walk task, mice in stroke groups showed a significant deficit, as measured by the percentage of foot faults relative to total number of steps taken. One month following stroke, mice in the Matn2 and Ucma overexpression groups showed significant improvement in gait performance relative to mice in the control virus group, but still showed a deficit when compared to sham mice. Six weeks following stroke, mice injected with the control virus continued showing a deficit compared to sham controls in addition to mice with stroke and overexpressing Matn2 or Ucma. Stroke mice overexpressing Matn2 or Ucma no longer showed a deficit when compared to sham mice. Two months post stroke, stroke mice with control virus continued to show deficit, while stroke mice overexpressing Ucma showed a new deficit, indicating a worsening recovery. Stroke mice overexpressing Matn2 showed no deficit and a significant improvement compared to stroke mice receiving control virus. Of interest is mice in the Ucma overexpression plus sham stroke group appear to be trending worse 2 months post virus injection

when compared to sham plus control virus mice, indicating a possible biological effect of Ucma on baseline motor function. In the pasta matrix task, mice in the stroke groups showed significant impairment in fine motor control and strength one week post stroke, as measured by number of pieces of pasta broken relative to the baseline number of pieces broken. Over the course of biweekly testing, mice overexpressing Matn2 and Ucma show accelerated recovery compared to mice that received control virus, with a loss of deficit observed by five weeks post stroke. However, mice in these groups begin to diverge from that point, as Matn2 overexpression does show a significant performance compared to control virus plus stroke mice by week 9, whereas Ucma overexpression appears to have a plateau in terms of functional improvement. A limitation to this study is the variability in percent of baseline number of pieces of pasta broken that restricts the conclusions that can be drawn, likely due to the limited number of mice per group, although sample size was consistent with the power analysis performed and previous studies using this behavior test (Rosenzweig & Carmichael, 2013; Sozmen et al., 2016). The reduced number of mice is due to strict guidelines followed to ensure mice are adequately trained and capable of performing the task in question to ensure confidence in the performance of mice once training is concluded and mice are no longer exposed to the testing setup daily. These functional outcomes, especially with Matn2 overexpression, are on par with or surpass similar studies that evaluated enhancing axonal sprouting with delivery of a growth factor (Li et al., 2015), keeping the window of plasticity open for longer by inhibiting a receptor system that reduces CREB action (Joy et al., 2019), or blocking the growth inhibitory molecule ephrin-A5 or its receptor Nogo-A in the post-stroke brain (Lindau et al., 2014; Overman et al., 2012; Tsai et al., 2011). Of note, post-stroke Matn2 overexpression results in total recovery of functional outcome, a result not seen in studies that degraded CSPG deposits through injection of chondroitinase ABC in

other CNS injury models (Sara Soleman et al., 2012; Wiersma et al., 2017). This further demonstrates that stimulating neurons towards recovery may be a better route towards finding treatment for return of motor function.

An increase in axonal sprouting, as described in Chapter 3, was the impetus that led to evaluation of functional outcomes impacted by Matn2 and Ucma overexpression following stroke. Two months post-stroke, both Matn2 and Ucma overexpression showed significant increases in axonal sprouting, while overexpression of these proteins alone did not impact axonal distribution. The axonal sprouting pattern seen with Matn2 overexpression two months post-stroke was markedly different than that seen one-month post-stroke. One-month post-stroke, Matn2 overexpression resulted in a radial pattern, with increased projections towards the premotor and somatosensory cortices. Two months post-stroke, however, Matn2 overexpression appears to enhance axonal sprouting more so towards the somatosensory cortex, indicating that in the intervening month of recovery axons are pruned, indicating a change in both spatial magnitude and direction over the course of recovery. Further experiments could identify the mechanism through which projections are pruned, and whether different subpopulations, such as those initially towards the premotor cortex versus those towards the somatosensory cortex, contribute differentially towards functional recovery., as the main recovery effect is seen.

Ucma overexpression two months post-stroke also increased axonal sprouting, following a similar pattern to that seen one-month post-stroke. This result, in combination with the limited recovery seen in the grid walk and pasta matrix tasks, is surprising, as previous studies have causally associated an increase in ipsilateral axonal sprouting with improved functional recovery (Li et al., 2015; Overman et al., 2012). Unlike these previous studies, Ucma overexpression post-stroke is associated with a marked increase in infarct size, which has been associated with worse

clinical outcome (Ospel et al., 2021). Due to this, the beneficial effects of Ucma overexpression are partially outweighed by the negative tissue damage outcomes. This may be an effect of using viral overexpression as a therapeutic option; it is unknown whether a more limited delivery method, such as a hydrogel or use of a weaker viral promoter, could balance beneficial therapeutic effects with detrimental biological effects. The mechanism through which Ucma overexpression leads to a larger infarct volume during chronic stroke is unknown. One possibility is Ucma has previously been characterized as exhibiting aggrecanase activity (Seuffert et al., 2018). Aggrecan is a key type of chondroitin sulfate proteoglycan, which has previously been characterized as inhibitory to neurite outgrowth (Jin et al., 2018; Li et al., 2015), and is a core component of perineuronal nets (Carulli et al., 2006; Deepa et al., 2006). Interestingly, perineuronal net remodeling has been associated with improved recovery post-stroke (Dzyubenko et al., 2018b), perhaps through reopening a period of plasticity that allows for restructuring and remapping of cortical circuits. There may be more than one type of axonal or synaptic plasticity that is operative in recovering a motor function after stroke. Axonal plasticity in the formation of new and long distance intra-cortical connections may be occurring in parallel to plasticity associated with remodeling of the perineuronal net and local dendritic boutons and neuronal connections (Carulli et al., 2020; Carulli & Verhaagen, 2021). If Ucma overexpression impacts this remodeling, it could result in a decrease in this aspect of synaptic plasticity of the post-stroke brain, and thus impact the ability to fully recover and potentially disrupt even endogenous mechanisms of recovery.

Ultimately, Matn2 overexpression was identified as a potential treatment for stroke, while Ucma overexpression had mixed results that require additional study. The degree of functional recovery is quite striking, as Matn2 overexpression rapidly returned mice to baseline function,

on par with previous recovery studies (Joy et al., 2019; Li et al., 2015; Overman et al., 2012), while Ucma overexpression, at least in the short term, accelerated the recovery process. The mechanisms through which either of these proteins enhance recovery are not fully understood, although enhancing axonal sprouting can aid in remapping circuits lost due to injury. Questions also arise about how translational these proteins can be. Experiments in the next chapter begin to evaluate this, by determining whether the source of the protein impacts axonal sprouting, and other biological effects, such as chemoattractant capability, these proteins have after stroke.

More broadly, these results identify the benefits of remodeling the ECM to enhance post-stroke functional recovery. The ECM undergoes dramatic changes following ischemic injury, with increased expression of proteins that can both contribute to and inhibit functional recovery (Baeten & Akassoglou, 2011; Galtrey & Fawcett, 2007; S. Soleman et al., 2013). Some studies, however, have shown that remodeling of the ECM in the chronic phase of stroke is necessary for recovery. Inhibition of matrix metalloproteinase 9, an enzyme that degrades components of the ECM and is implicated in synaptic plasticity (Vafadari et al., 2016), seven days post-stroke impaired recovery through inhibition of plasticity and tissue remodeling (Zhao et al., 2006). Growth factors such as brain derived neurotrophic factor can be incorporated into ECM networks and released following remodeling (Dzyubenko et al., 2018a; Song & Dityatev, 2018), facilitating further axonal sprouting and formation of circuits. Taken together, these results demonstrate that the ECM is a dynamic component of post-stroke recovery, and manipulation of it during chronic stroke is a viable target for treatment of stroke.

4.5 Figures

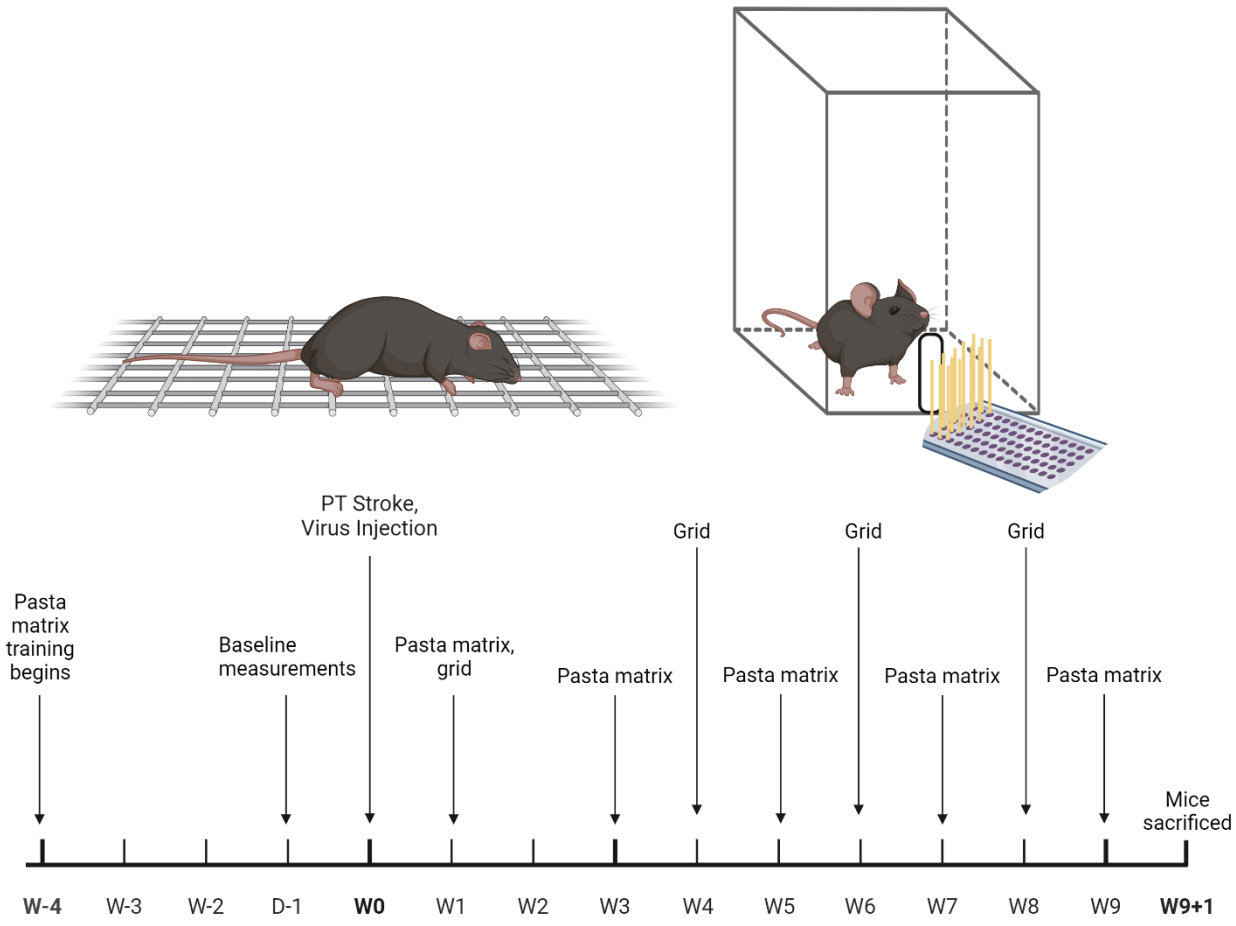


Figure 4-1: Schematic and timeline of *in vivo* behavior tests. Food deprived male C57Bl/6 mice (n=11-12 per group) were trained on the pasta matrix task for 4 weeks (6 days/week), with baseline measurements taken 1 day prior to surgeries. Behavioral changes due to ischemia were assessed 1 week after stroke with the pasta matrix and gridwalk tasks. Pasta matrix was further assessed 3, 5, 7 and 9 weeks post-stroke, and grid was assessed 4, 6 and 8 weeks post-stroke. Created with BioRender.com

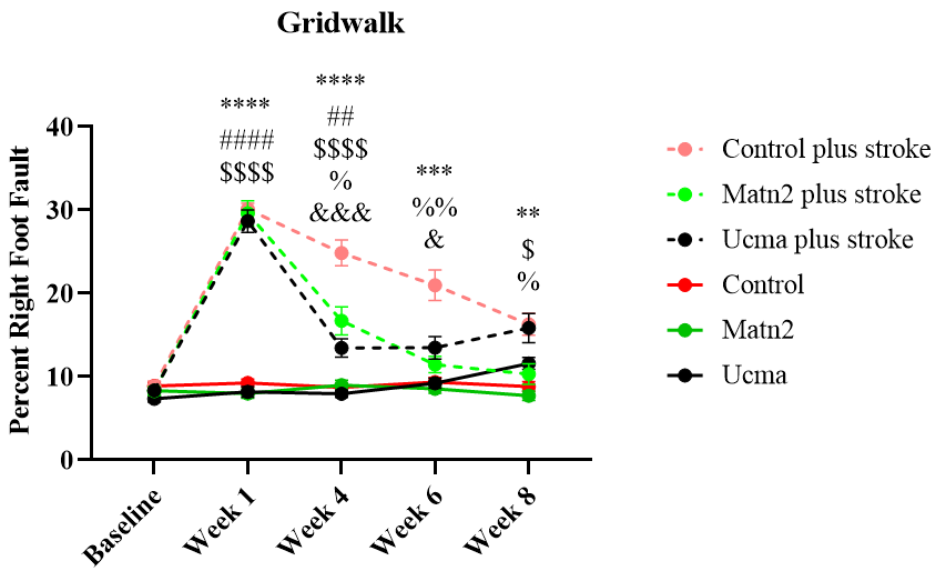


Figure 4-2. Matn2 overexpression enhances post-stroke functional recovery as measured by gridwalk, whereas Ucma only partially increases recovery. Percent foot faults was calculated as [(number of foot faults / number of total steps) x 100]. Baseline gridwalk was assessed at 1-2 days prior to stroke, with all groups having an approximate 10% foot fault rate. Acute ischemia related motor deficits were measured 1-, 4-, 6- and 8-weeks post-stroke. 1-week post stroke, mice in the stroke groups showed significant deficit compared to sham controls (Week 1 Ctrl vs ctrl plus stroke $p<0.001$, Ctrl vs Matn2 plus stroke $p<0.001$, Ucma vs Ucma plus stroke $p<0.001$). 4 weeks post-stroke, mice in stroke groups continued to show significant deficit when compared to no-stroke sham mice (Week 4 Ctrl vs ctrl plus stroke $p<0.001$, Ctrl vs Matn2 plus stroke $p = 0.0048$, Ctrl vs Ucma plus stroke $p<0.001$). Matn2 plus stroke and Ucma plus stroke showed significant recovery compared to Ctrl plus stroke (Matn2 plus stroke vs ctrl plus stroke $p = 0.0102$, Ucma plus stroke vs Ctrl plus stroke $p = 0.001$). 6 weeks post-stroke, Ctrl plus stroke mice continue to show deficit when compared to no stroke shams (Ctrl vs Ctrl plus stroke $p = 0.007$). Matn2 plus stroke mice no longer showed deficit when compared to no-stroke shams, and showed a significant improvement in recovery when compared to Ctrl plus stroke ($p = 0.0040$). Ucma plus stroke mice showed no deficit when compared to no-stroke shams and were significantly different compared to Ctrl plus stroke mice ($p = 0.0408$). 8 weeks post-stroke, Ctrl plus stroke mice still had a deficit compared to no-stroke shams ($p = 0.0019$). Matn2 plus stroke mice showed no deficit compared to no-stroke shams, and showed significantly fewer foot faults compared to Ctrl plus stroke mice ($p = 0.0334$). Ucma plus stroke mice were no longer significantly different compared to Ctrl plus stroke mice, and were showed significantly more foot faults compared to no-stroke shams (Ctrl vs Ucma plus stroke $p = 0.0235$). All data shown are mean ± SEM and analyzed via 2-way repeated measures ANOVA with post-hoc Tukey's multiple comparison test.

* Ctrl virus vs ctrl virus plus stroke # Ctrl virus vs Matn2 plus stroke % Matn2 plus stroke vs ctrl plus stroke $ Ctrl virus vs Ucma plus stroke & Ucma plus stroke vs Ctrl virus plus stroke

P = 0.057 @ 8 weeks Ctrl vs Ucma

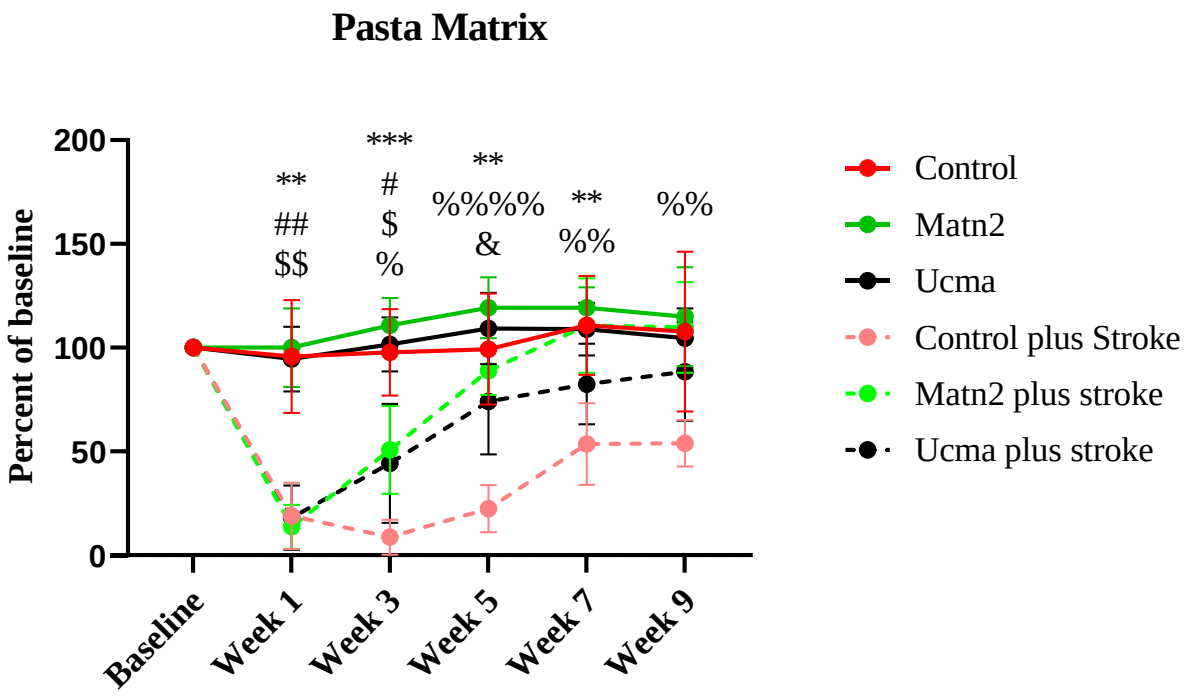


Figure 4-3. Matn2 and Ucma overexpression accelerate recovery in the pasta matrix assessment of grip strength. Percent of baseline was defined as (number of pasta pieces broken at baseline)/(number of pieces broken during trial)*100. 1 week following surgery, mice in the stroke groups showed a significant decline in performance compared to no-stroke shams (Ctrl vs Ctrl plus stroke p = 0.00156, Ctrl vs Matn2 plus stroke p = 0.00126, Ctrl vs Ucma plus stroke p = 0.00146). 3 weeks post-stroke, mice in the Matn2 group showed significant improvement compared to the Ctrl plus stroke group (p = 0.01552). When compared to sham no-stroke mice, all mice still had significant impairments in function (Ctrl vs Ctrl plus stroke p = 0.00011, Ctrl vs Matn2 plus stroke p = 0.02113, Ctrl vs Ucma plus p = 0.03213). % weeks post-stroke, mice in the Matn2 plus stroke and Ucma plus stroke groups no longer had a significant deficit compared to sham controls, and both showed significant improvement compared to Ctrl plus stroke mice (Ctrl vs Ctrl plus stroke p = 0.00172, Ctrl plus stroke vs Matn2 plus p<0.0001, Ctrl plus stroke vs Ucma plus stroke p = 0.01510). At 7 and 9 weeks post-stroke, Matn2 plus stroke mice remained deficit free and significantly improved compared to Ctrl plus stroke mice (Week 7 p = 0.00581, Week 9 p = 0.00274). Ucma plus stroke mice were no longer significantly improved compared to Ctrl plus stroke mice. All data are shown as mean ± SEM and analyzed via 2-way repeated measures ANOVA with post-hoc Tukey's multiple comparison test.

* Ctrl virus vs Ctrl virus plus stroke # Ctrl virus vs Matn2 plus stroke $ Ctrl virus vs Ucma plus stroke % Ctrl virus plus stroke vs Matn2 plus stroke & Ctrl virus plus stroke vs Ucma plus stroke

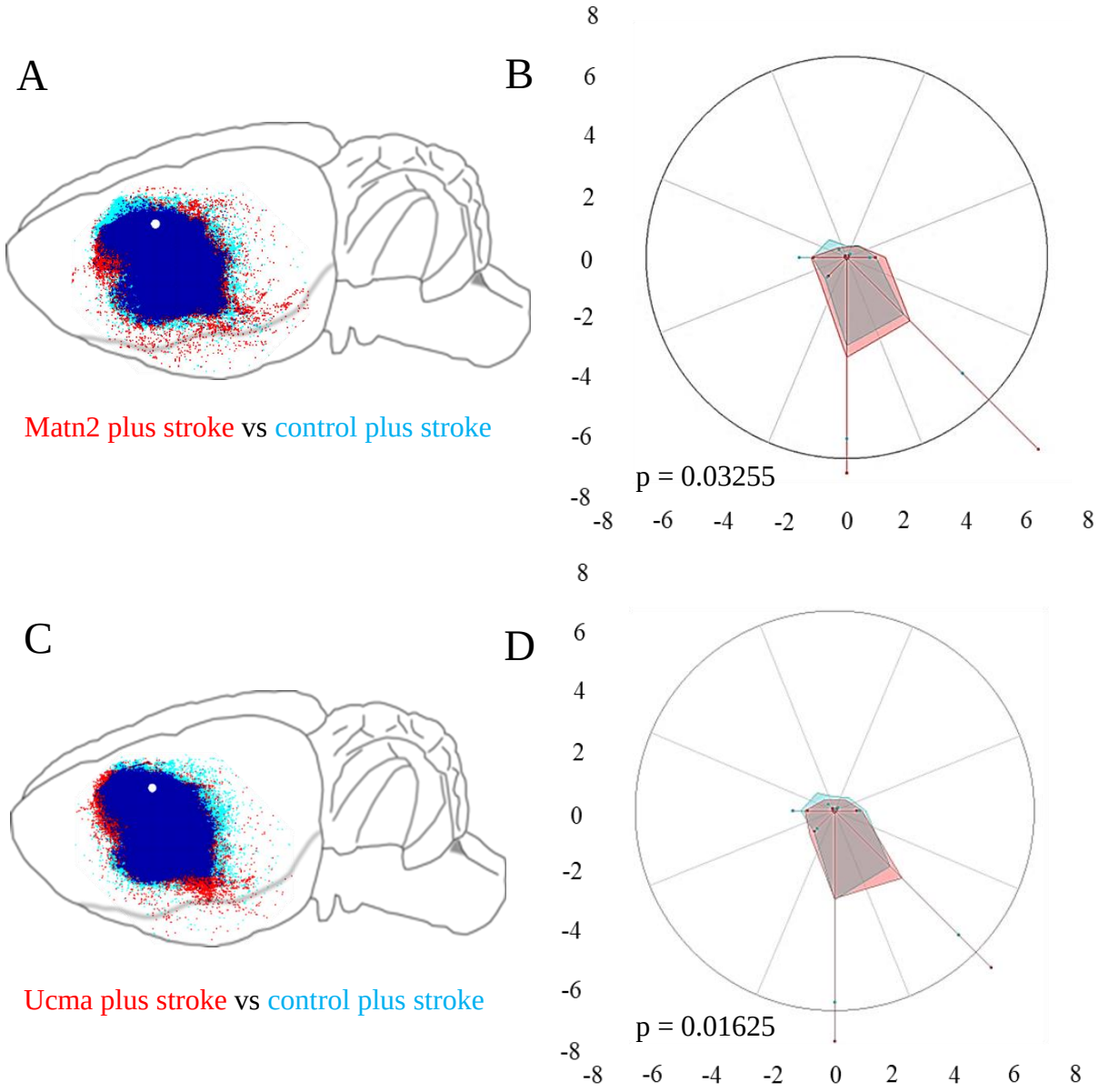


Figure 4-4. Matn2 and Ucma overexpression enhances axonal sprouting two months after stroke. Data are presented as in Chapter 3. A) Quantitative mapping of cortical projections comparing Matn2 plus stroke (red, n = 8) and empty vector control plus stroke (light blue, n = 8), with areas of dense overlap (dark blue). B) Polar plot of Matn2 plus stroke vs empty vector control virus plus stroke. Two months after stroke, Matn2 overexpression results in enhanced axonal projections towards the somatosensory cortex (p = 0.03255, Hotelling's T2 distribution test). C) Quantitative mapping of cortical projections comparing Ucma plus stroke (red, n = 8) and empty vector control virus plus stroke (light blue, n = 8) with areas of dense overlap (dark blue). D) Polar plot of Ucma plus stroke vs empty vector control plus stroke. Two months after stroke, Ucma overexpression results in enhanced axonal projections to somatosensory cortex (p = 0.01625, Hotelling's T2 distribution test).

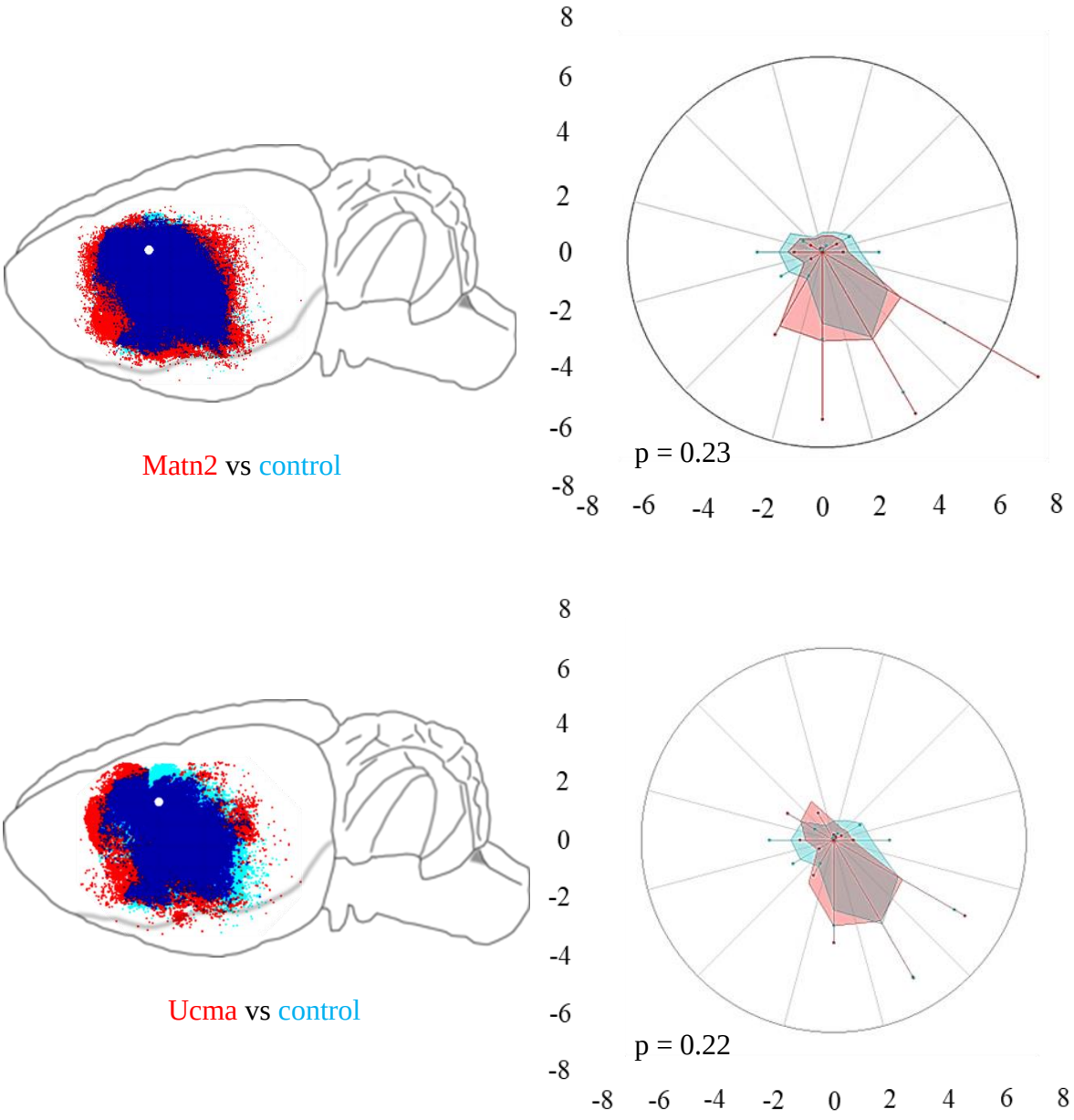


Figure 4-5. Matn2 and Ucma overexpression in the absence of stroke does not alter axonal projections.

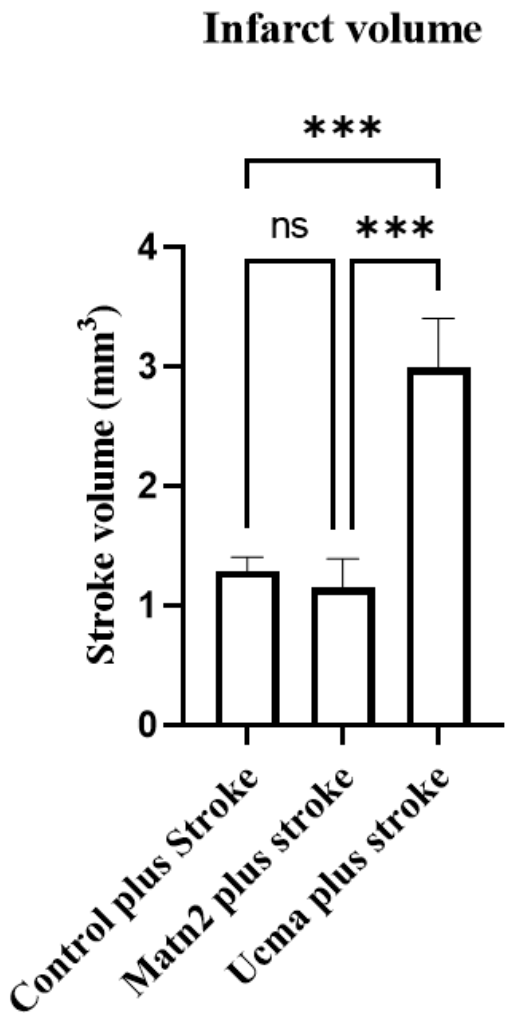


Figure 4-6. Prolonged Ucma overexpression in stroke increases the size of infarct. Stroke size was evaluated 2 months after surgery by measuring the infarct area and extrapolating total volume based on section thickness and distance between sections. Ucma plus stroke mice had significantly large stroke volumes compared to Ctrl plus stroke (p=0.0009) and Matn2 plus stroke (p = 0.0004). Data are mean ± SEM and were analyzed via one-way ANOVA with post-hoc Tukey's test for multiple comparisons.

4.6 References

Allison, R., Shenton, L., Bamforth, K., Kilbride, C., & Richards, D. (2016). Incidence, Time Course and Predictors of Impairments Relating to Caring for the Profoundly Affected arm After Stroke: A Systematic Review. *Physiotherapy Research International*, *21*(4), 210. https://doi.org/10.1002/PRI.1634

Baeten, K. M., & Akassoglou, K. (2011). Extracellular matrix and matrix receptors in blood-brain barrier formation and stroke. *Developmental Neurobiology*, *71*(11), 1018–1039. https://doi.org/10.1002/dneu.20954

Caracciolo, L., Marosi, M., Mazzitelli, J., Latifi, S., Sano, Y., Galvan, L., Kawaguchi, R., Holley, S., Levine, M. S., Coppola, G., Portera-Cailliau, C., Silva, A. J., & Carmichael, S. T. (2018). CREB controls cortical circuit plasticity and functional recovery after stroke. *Nature Communications*, *9*(1), 2250. https://doi.org/10.1038/s41467-018-04445-9

Carmichael, S. T., Kathirvelu, B., Schweppe, C. A., & Nie, E. H. (2017). Molecular, Cellular and Functional Events in Axonal Sprouting after Stroke. *Experimental Neurology*, *287*(Pt 3), 384. https://doi.org/10.1016/J.EXPNEUROL.2016.02.007

Carulli, D., Broersen, R., de Winter, F., Muir, E. M., Meškovic, M., de Waal, M., de Vries, S., Boele, H. J., Canto, C. B., de Zeeuw, C. I., & Verhaagen, J. (2020). Cerebellar plasticity and associative memories are controlled by perineuronal nets. *Proceedings of the National Academy of Sciences of the United States of America*, *117*(12), 6855–6865. https://doi.org/10.1073/PNAS.1916163117/-/DCSUPPLEMENTAL

Carulli, D., Rhodes, K. E., Brown, D. J., Bonnert, T. P., Pollack, S. J., Oliver, K., Strata, P., & Fawcett, J. W. (2006). Composition of perineuronal nets in the adult rat cerebellum and the

cellular origin of their components. *Journal of Comparative Neurology*, *494*(4), 559–577. https://doi.org/10.1002/CNE.20822

Carulli, D., & Verhaagen, J. (2021). An Extracellular Perspective on CNS Maturation: Perineuronal Nets and the Control of Plasticity. *International Journal of Molecular Sciences*, *22*(5), 1–26. https://doi.org/10.3390/IJMS22052434

Chao, O. Y., Pum, M. E., Li, J.-S., & Huston, J. P. (2012). The grid-walking test: assessment of sensorimotor deficits after moderate or severe dopamine depletion by 6-hydroxydopamine lesions in the dorsal striatum and medial forebrain bundle. *Neuroscience*, *202*, 318–325. https://doi.org/10.1016/J.NEUROSCIENCE.2011.11.016

Deepa, S. S., Carulli, D., Galtrey, C., Rhodes, K., Fukuda, J., Mikami, T., Sugahara, K., & Fawcett, J. W. (2006). Composition of perineuronal net extracellular matrix in rat brain: a different disaccharide composition for the net-associated proteoglycans. *The Journal of Biological Chemistry*, *281*(26), 17789–17800. https://doi.org/10.1074/JBC.M600544200

Dzyubenko, E., Manrique-Castano, D., Kleinschnitz, C., Faissner, A., & Hermann, D. M. (2018a). Role of immune responses for extracellular matrix remodeling in the ischemic brain. In *Therapeutic Advances in Neurological Disorders* (Vol. 11). SAGE Publications Ltd. https://doi.org/10.1177/1756286418818092

Dzyubenko, E., Manrique-Castano, D., Kleinschnitz, C., Faissner, A., & Hermann, D. M. (2018b). Topological remodeling of cortical perineuronal nets in focal cerebral ischemia and mild hypoperfusion. *Matrix Biology*, *74*, 121–132. https://doi.org/10.1016/J.MATBIO.2018.08.001

Galtrey, C. M., & Fawcett, J. W. (2007). The role of chondroitin sulfate proteoglycans in

regeneration and plasticity in the central nervous system. *Brain Research Reviews*, *54*(1), 1–18. https://doi.org/10.1016/J.BRAINRESREV.2006.09.006

Jin, J., Tilve, S., Huang, Z., Zhou, L., Geller, H. M., & Yu, P. (2018). Effect of chondroitin sulfate proteoglycans on neuronal cell adhesion, spreading and neurite growth in culture. *Neural Regeneration Research*, *13*(2), 289. https://doi.org/10.4103/1673-5374.226398

Joy, M. T., Ben Assayag, E., Shabashov-Stone, D., Liraz-Zaltsman, S., Mazzitelli, J., Arenas, M., Abduljawad, N., Kliper, E., Korczyn, A. D., Thareja, N. S., Kesner, E. L., Zhou, M., Huang, S., Silva, T. K., Katz, N., Bornstein, N. M., Silva, A. J., Shohami, E., & Carmichael, S. T. (2019). CCR5 Is a Therapeutic Target for Recovery after Stroke and Traumatic Brain Injury. *Cell*, *176*(5), 1143. https://doi.org/10.1016/J.CELL.2019.01.044

Joy, M. T., & Carmichael, S. T. (2020). Encouraging an excitable brain state: mechanisms of brain repair in stroke. *Nature Reviews Neuroscience 2020 22:1*, *22*(1), 38–53. https://doi.org/10.1038/s41583-020-00396-7

Kwakkel, G., Kollen, B. J., Van der Grond, J. V., & Prevo, A. J. H. (2003). Probability of regaining dexterity in the flaccid upper limb: Impact of severity of paresis and time since onset in acute stroke. *Stroke*, *34*(9), 2181–2186. https://doi.org/10.1161/01.STR.0000087172.16305.CD

Latifi, S., Mitchell, S., Habibey, R., Hosseini, F., Donzis, E., Estrada-Sanchez, A. M., Rezaei Nejad, H., Levine, M., Golshani, P., & Thomas Carmichael, S. (2020). Neuronal Network Topology Indicates Distinct Recovery Processes after Stroke. *Cerebral Cortex*, *30*(12), 6363–6375. https://doi.org/10.1093/CERCOR/BHAA191

Li, S., Nie, E. H., Yin, Y., Benowitz, L. I., Tung, S., Vinters, H. V, Bahjat, F. R., Stenzel-Poore,

M. P., Kawaguchi, R., Coppola, G., & Carmichael, S. T. (2015). GDF10 is a signal for axonal sprouting and functional recovery after stroke. *Nature Neuroscience*, *18*(12), 1737–1745. https://doi.org/10.1038/nn.4146

Liang, H., Zhao, H., Gleichman, A., Machnicki, M., Telang, S., Tang, S., Rshtouni, M., Ruddell, J., & Thomas Carmichael, S. (2019). Region-specific and activity-dependent regulation of SVZ neurogenesis and recovery after stroke. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(27), 13621–13630. https://doi.org/10.1073/pnas.1811825116

Lindau, N. T., Bänninger, B. J., Gullo, M., Good, N. A., Bachmann, L. C., Starkey, M. L., & Schwab, M. E. (2014). Rewiring of the corticospinal tract in the adult rat after unilateral stroke and anti-Nogo-A therapy. *Brain*, *137*(3), 739–756. https://doi.org/10.1093/BRAIN/AWT336

Luo, L., & O'Leary, D. D. M. (2005). AXON RETRACTION AND DEGENERATION IN DEVELOPMENT AND DISEASE. *Http://Dx.Doi.Org/10.1146/Annurev.Neuro.28.061604.135632*, *28*, 127–156. https://doi.org/10.1146/ANNUREV.NEURO.28.061604.135632

Ospel, J. M., Hill, M. D., Menon, B. K., Demchuk, A., McTaggart, R., Nogueira, R., Poppe, A., Haussen, D., Qiu, W., Mayank, A., Almekhlafi, M., Zerna, C., Joshi, M., Jayaraman, M., Roy, D., Rempel, J., Buck, B., Tymianski, M., & Goyal, M. (2021). Strength of Association between Infarct Volume and Clinical Outcome Depends on the Magnitude of Infarct Size: Results from the ESCAPE-NA1 Trial. *American Journal of Neuroradiology*, *42*(8), 1370–1374. https://doi.org/10.3174/AJNR.A7183

Overman, J. J., Clarkson, A. N., Wanner, I. B., Overman, W. T., Eckstein, I., Maguire, J. L., Dinov, I. D., Toga, A. W., & Carmichael, S. T. (2012). A role for ephrin-A5 in axonal sprouting, recovery, and activity-dependent plasticity after stroke. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(33). https://doi.org/10.1073/PNAS.1204386109/SUPPL_FILE/PNAS.201204386SI.PDF

Poplawski, G. H. D., Kawaguchi, R., Van Niekerk, E., Lu, P., Mehta, N., Canete, P., Lie, R., Dragatsis, I., Meves, J. M., Zheng, B., Coppola, G., & Tuszynski, M. H. (2020). Injured adult neurons regress to an embryonic transcriptional growth state. *Nature*, 1–6. https://doi.org/10.1038/s41586-020-2200-5

Rosenzweig, S., & Carmichael, S. T. (2013). Age-dependent exacerbation of white matter stroke outcomes: a role for oxidative damageand inflammatory mediators. *Stroke; a Journal of Cerebral Circulation*, *44*(9), 2579. https://doi.org/10.1161/STROKEAHA.113.001796

Seuffert, F., Weidner, D., Baum, W., Schett, G., & Stock, M. (2018). Upper zone of growth plate and cartilage matrix associated protein protects cartilage during inflammatory arthritis. *Arthritis Research & Therapy*, *20*(1), 88. https://arthritis-research.biomedcentral.com/articles/10.1186/s13075-018-1583-2

Soleman, S., Filippov, M. A. A., Dityatev, A., & Fawcett, J. W. W. (2013). Targeting the neural extracellular matrix in neurological disorders. *Neuroscience*, *253*, 194–213. https://doi.org/10.1016/j.neuroscience.2013.08.050

Soleman, Sara, Yip, P. K., Duricki, D. A., & Moon, L. D. F. (2012). Delayed treatment with Chondroitinase ABC promotes sensorimotor recovery and plasticity after stroke in aged rats. *Brain : A Journal of Neurology*, *135*(Pt 4), 1210.

https://doi.org/10.1093/BRAIN/AWS027

Song, I., & Dityatev, A. (2018). Crosstalk between glia, extracellular matrix and neurons. *Brain Research Bulletin*, *136*, 101–108. https://doi.org/10.1016/J.BRAINRESBULL.2017.03.003

Sozmen, E. G., Rosenzweig, S., Llorente, I. L., DiTullio, D. J., Machnicki, M., Vinters, H. V., Havton, L. A., Giger, R. J., Hinman, J. D., & Carmichael, S. T. (2016). Nogo receptor blockade overcomes remyelination failure after white matter stroke and stimulates functional recovery in aged mice. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(52), E8453–E8462. https://doi.org/10.1073/PNAS.1615322113/SUPPL_FILE/PNAS.201615322SI.PDF

Tennant, K. A., Asay, A. L., Allred, R. P., Ozburn, A. R., Kleim, J. A., & Jones, T. A. (2010). The vermicelli and capellini handling tests: simple quantitative measures of dexterous forepaw function in rats and mice. *Journal of Visualized Experiments : JoVE*, *41*. https://doi.org/10.3791/2076

Tsai, S. Y., Papadopoulos, C. M., Schwab, M. E., & Kartje, G. L. (2011). Delayed anti-Nogo-A therapy improves function after chronic stroke in adult rats. *Stroke*, *42*(1), 186–190. https://doi.org/10.1161/STROKEAHA.110.590083

Vafadari, B., Salamian, A., & Kaczmarek, L. (2016). MMP-9 in translation: from molecule to brain physiology, pathology, and therapy. *Journal of Neurochemistry*, *139*, 91–114. https://doi.org/10.1111/JNC.13415

Wahl, A. S., Omlor, W., Rubio, J. C., Chen, J. L., Zheng, H., Schröter, A., Gullo, M., Weinmann, O., Kobayashi, K., Helmchen, F., Ommer, B., & Schwab, M. E. (2014). Asynchronous therapy restores motor control by rewiring of the rat corticospinal tract after

stroke. *Science*, *344*(6189), 1250–1255. https://doi.org/10.1126/SCIENCE.1253050/SUPPL_FILE/WAHL.SM.PDF

Wiersma, A. M., Fouad, K., & Winship, I. R. (2017). Enhancing Spinal Plasticity Amplifies the Benefits of Rehabilitative Training and Improves Recovery from Stroke. *Journal of Neuroscience*, *37*(45), 10983–10997. https://doi.org/10.1523/JNEUROSCI.0770-17.2017

Zhao, B. Q., Wang, S., Kim, H. Y., Storrie, H., Rosen, B. R., Mooney, D. J., Wang, X., & Lo, E. H. (2006). Role of matrix metalloproteinases in delayed cortical responses after stroke. *Nature Medicine 2006 12:4*, *12*(4), 441–445. https://doi.org/10.1038/nm1387

Chapter 5

Mechanism of Matn2 and Ucma overexpression in post-stroke recovery

5.1 Introduction

In Chapter 4, Matn2 and Ucma overexpression in the peri-infarct cortex were evaluated for enhancement of functional recovery. Matn2 overexpression was found to accelerate motor recovery with a return to baseline normal levels of function, whereas Ucma overexpression initially accelerated recovery but failed to return behavior to baseline levels, and may be detrimental to long term recovery by potentiating the brain damage of the stroke. Protein overexpression post-stroke was also associated with increased axonal sprouting two months post-stroke. Interestingly, the pattern of axonal sprouting at two-months, when a stable motor control pattern of recovery is present, is less and more spatially restricted compared to the initial axonal sprouting at one-month, when the motor recovery is first detected—indicating a possible two-phase of axonal sprouting in outgrowth and then refinement. There are several questions remaining specific to the mechanism through which these proteins affect the endogenous recovery process in the brain, such as whether these proteins can act as guidance cues for sprouting axons, and whether the source of the protein is important for axonal sprouting.

5.1.1 Axonal sprouting and guidance cues

Axonal guidance cues have been well characterized in development as an important factor through which extending CNS axons navigate to the correct location through short- and long-range attractive and repulsive cues (Benarroch, 2015; Varadarajan & Butler, 2017; Xiao et al., 2022; Yu & Bargmann, 2001). Attractive cues include sonic hedgehog (X. Li et al., 2021; Wu et al., 2019), glial derived neurotrophic factor (Onesto et al., 2021), and netrin-1 (Varadarajan et al., 2017), which can act as long-range chemoattractants or contact dependent haptotactic cues, and repulsive cues including semaphorins (Giger et al., 2010; Vecino & Kwok, 2016), ephrins (Kaprielian et al., 2000; Yu & Bargmann, 2001), and CSPGs (Laabs et al., 2005),

which in concert with attractive cues ensure developing axons form functional circuits. Previous work has shown the sensitivity of sprouting neurons to repulsive cues such as Ephrin A5 (Overman et al., 2012) and semaphoring 3A (Hira et al., 2018), but little there are few studies evaluating attractive guidance cues. Recovering neurons have also been found to revert to a development-like state after injury, meaning they may be sensitive to guidance cues (Carmichael et al., 2017; S. Li et al., 2010a; Poplawski et al., 2020) that are normally not a factor in the more static architecture of the adult brain. The gene expression data for sprouting neurons after stroke suggests this biology. Matn-2 and Ucma are strongly and differentially expressed in neurons stimulated by stroke to enter an axonal growth state. Matn2 and Ucma have not been characterized as proteins with guidance cue effects, but it stands to reason they may play this role during post-stroke increased plasticity. If so, overexpressing Matn2 or Ucma in a location where sprouting neurons are sparse, but still present, would result in a greater magnitude or density of axons present in that area. If these two ECM proteins are functioning in way that alters the extracellular environment of the post-stroke cortex, and establishing resident cues that a growing axon detects, then placing Matn-2 and Ucma within the ECM, and not in the growing axon, as was done in Chapters 3 and 4, would test this type of interaction. Here, through use of a viral vector with limited spatial expression is used to generate a deposit of overexpressed Matn2 or Ucma outside of the neurons in the peri-infarct cortex that are measured for their sprouting response, to evaluate their ability to draw or guide axons towards an ectopic location.

5.1.2 Source specific effects

In previous chapters, Matn2 and Ucma were overexpressed using lentiviruses under the EF1a promoter, a strong ubiquitous promoter that would drive expression in the majority of cell types(Qin et al., 2010; Yaguchi et al., 2013). The sprouting of axons distant to the stroke site

ensured that a neuronal response was measured, as local effects within the injection site were not measured. In fact, the dense accumulation of axons at the site of the injection, from the profuse local projections of labeled neurons, prevents an analysis of very local axonal projections. However, proteins are synthesized and packaged into the secretory pathway, allowing for secretion well beyond the cell body (Kennedy & Hanus, 2019). As the EF1a promoter causes expression in neurons, among other cells, there is the possibility that Matn2 and Ucma could be transported and secreted to sites distant from the peri-infarct by the growing axons themselves.. This biology would result in Matn2 and Ucma not being ideal translational targets for post-stroke treatment, as directly inducing neurons in the human post-stroke brain is not a practical treatment strategy. One possible translational approach in this molecular system is for protein delivery in humans through a hydrogel in the infarct core or cavity (Cook et al., 2017; Ghuman et al., 2016).

To disassociate an effect of Matn-2 or Ucma from the growing axon vs the environment that the growing axon detects, overexpression must be limited to a cell population that is limited to a restricted location, such as astrocytes. While astrocytes are proliferative post-stroke (Barreto et al., 2011), their migration is limited. As such, limiting overexpression of Matn2 and Ucma by astrocytes in the peri-infarct motor cortex, an area important in functional recovery after stroke (S. Li et al., 2015), would test the cell-autonomous vs non-cell autonomous question, and would more closely mimic secretion of the proteins from a hydrogel.

5.2 Methods

5.2.1 Animals

All animals used for this study were C57BL/6 strain male mice aged 10-12 weeks at the time of surgery (n = 8) per group. Mice were obtained from the Jackson Laboratory (JAX # 000664). Mice were randomly assigned to treatment groups and housed 4 per cage with *ad lib* access to food and water. All experiments were performed in accordance with National Institutes of Health animal use guidelines and were approved by the University of California, Los Angeles Animal Research Committee (ARC protocol #00-159).

5.2.2 Virus design and generation

To ensure limited protein spread, open reading frames for proteins of interest were cloned into a backbone with the shortened GFAP promoter, with a FLAG epitope tag added to the C terminal. To further ensure expression by astrocytes only, a miR124T microRNA targeting motif was included following the stop codon (Taschenberger et al., 2017). This microRNA targeting motif results in mRNA generated from the overexpression vector is degraded without translation in neurons, further restricting protein deposition to the site of viral injection.

5.2.3 Photothrombotic stroke and virus injection

Photothrombotic stroke was performed using the same approach as described in Chapter 3.

Virus injections were performed immediately following photothrombotic stroke using the same approach as described in Chapter 3. For ectopic ECM overexpression studies, 300nl of lentivirus was injected at a rate of 1 nl/sec in the barrel cortex (A/P -2. M/L 2.8, D/V 0.75), a

region with minimal axonal sprouting post-stroke as shown in Figure 3-5 in control virus plus stroke mice. If Matn2 or Ucma expression results in distant guidance cue effects, there should be enhanced axonal sprouting to this location. For peri-infarct limited ECM overexpression studies, two 150nl injections of lentivirus were performed in anterior peri-infarct motor cortex (A/P 1.5, M/L 1.5 and 1.9, D/V 0.75). For both sets of experiments, 21 days post-stroke 250nl of 10% biotinylated dextran amine (BDA) in saline was injected in the anterior peri-infarct motor cortex (A/P 1.5, M/L 1.75, D/V 0.75).

5.2.4 Tissue collection and processing

28 days post-stroke, mice were sacrificed, and flattened cortices prepared as described in Chapter 3. This is the time point of initially robust axonal sprouting in both Matn-2 and Ucma neuronal over-expression.

5.2.5 Immunohistochemistry, imaging and analysis

Flattened sections were stained for axonal projections from the peri-infarct as described previously (S. Li et al., 2010b, 2015). Briefly, sections representing every 160 were removed from antifreeze solution, washed with PBS and blocked in a solution of 5% normal donkey serum and 0.3% Triton X. Following blocking, a staining solution of streptavidin conjugated to AlexaFluor 647 (ThermoFisher, 1:1000) was applied to sections and allowed to incubate overnight at 4C. Sections were rinsed and mounted on triple-subbed slides. Slides were then imaged and analyzed using the same approach described in Chapter 3.

5.2.6 Primary Neuron Culture and Immunocytochemistry

Primary neurons were isolated from post-natal day 3 mice as described in Chapter 2. Neurons were transduced with overexpression viruses at 1 day *in vitro*, and allowed to grow under the conditions described in Chapter 2 for 7 days. Neurons were fixed and stained for Matn2 (R&D Systems 1:50) and Tuj1 (Abcam). For cell surface labeling of Matn2, cells were incubated with Matn2 antibody for one hour, washed with PBS and fixed. Cultures were imaged on a Nikon Eclipse Ti or Zeiss LSM 880 with AiryScan for super-resolution imaging.

5.3 Results

5.3.1 Ectopic overexpression of Matrilin-2 and Ucma fails to exert a guidance cue effect

To evaluate potential guidance cue effects of Matn2 and Ucma, the approach used in Chapters 3 and 4 was modified to ensure protein overexpression was limited to a defined location. This was achieved through use of the astrocyte specific GFAP promoter in combination with a microRNA targeting sequence, miR124T, to further limit expression in non-astrocytes. This is shown in figure 5-1. Mice were subjected to photothrombotic stroke in the motor cortex, and virus injected in the medial barrel cortex, an area with sparse axonal projections following stroke. Three weeks later, biotinylated dextran amine (BDA), an anterograde axonal tracer, was injected into the peri-infarct. One week following BDA injection, mice were sacrificed and cortices prepared for axonal tracing as described in Chapter 3. These results are shown in Figure 5-2. Overexpression of Matn2 and Ucma in an ectopic location did not result in any significant changes in axonal projections (Ctrl plus stroke vs Matn2 plus stroke $p = 0.857$, Ctrl plus stroke vs Ucma plus $p = 0.7985$, Hotelling's T2 test for spatial distribution).

5.3.2 Limiting overexpression of Matn2 to the peri-infarct enhances axonal sprouting, whereas limiting Ucma overexpression to the peri-infarct is detrimental to axonal sprouting

To evaluate potential cell-trafficking and cell-source dependent mechanisms of the overexpressed ECM proteins, Matn2 and Ucma were overexpressed in the peri-infarct using astrocyte specific lentiviruses as described earlier following photothrombotic stroke in the motor cortex. As before, three weeks following stroke mice were injected with BDA in the peri-infarct, and brains were processed and analyzed for axonal sprouting. These results are shown in Figure 5-3. Limiting Matn2 overexpression to the peri-infarct motor cortex resulted in a significant increase in axonal sprouting towards the somatosensory cortex (Ctrl plus stroke vs Matn2 plus stroke $p = 0.045$, Hotelling's T2 test for spatial distribution). Limiting Ucma overexpression to the peri-infarct resulted in a near global decrease in axonal sprouting (Ctrl plus stroke vs Ucma plus stroke $p = 0.003$, Hotelling's T2 test for spatial distribution). The axonal sprouting distribution seen with Matn2 overexpression here is limited and more directional when compared to that seen in Figure 3-5, which was more radial and affected multiple anatomical locations including premotor and temporal cortices. Ucma overexpression limited to astrocytes resulted in more limited axonal sprouting, similar to that seen with blockade of growth factors that enhance axonal sprouting (S. Li et al., 2015).

5.4 Discussion

In Chapters 3 and 4 of this work, peri-infarct Matn2 and Ucma overexpression were found to enhance axonal sprouting in the post-stroke brain, in addition to enhancing functional recovery either totally (in the case of Matn2) or partially (in the case of Ucma). Overexpression

was achieved through use of a lentivirus under the EF1a promoter, a strong ubiquitous promoter. However, use of the EF1a promoter, and in particular overexpression in neurons themselves, results in the potential for trafficking of the overexpressed proteins beyond the cell body, resulting in secretion outside of the peri-infarct, and the potential for a combination of cell-autonomous effects—Matn-2 and Ucma secreted from the growing axon, vs local tissue effects from these two proteins deposited into the ECM. Further, neuronal production of Matn-2 or Ucma may have intracellular effects in the neurons, in addition to extracellular interactions post-secretion. Matn2 has been shown to be trafficked outside of the soma (Fleetwood et al., 2014), and ECM receptors such as integrins can be activated intracellularly by proteins (Ginsberg, 2014).Immunofluorescent staining of these proteins has proven to be especially difficult to visualize them in subcellular domains in intact tissue, so direct visualization has not been possible beyond cell bodies. To address the potential confound of proteins having an interaction with cell compartments other than the cell body, Matn2 and Ucma were overexpressed in lentiviruses operating under the shortened GFAP promoter, to drive expression in astrocytes, with the addition of a microRNA targeting sequence, miR124T, to further limit expression of proteins to astrocytes only as shown in Figure 5-1 (Taschenberger et al., 2017). Use of this viral approach allows for production of overexpressed protein in a single location, thus allowing for evaluation of both potential guidance cue effects and source-specific effects of the target proteins.

Components of the ECM have been well characterized as exerting guidance cue effects during CNS development, whether through attraction, such as sonic hedgehog and glial derived neurotrophic factor (X. Li et al., 2021; Onesto et al., 2021; Wu et al., 2019), or repulsion, as seen with semaphorins and ephrins (Canty & Murphy, 2008; Giger et al., 2010; Kaprielian et al.,

2000; Vecino & Kwok, 2016).To evaluate guidance cue effects of Matn2 and Ucma, astrocyte-specific lentiviruses were injected into the barrel cortex at the time of stroke, and biotinylated dextran amine (BDA) was injected in the peri-infarct one week prior to tissue collection. The barrel cortex was chosen as relatively few axons were identified as sprouting towards that location post-stroke, but some were still present. As such, we hypothesized that by generating a localized depot of either Matn2 or Ucma, there would be an increased density of sprouting axons towards that area either through interaction with the growth cone of extending axons, or by drawing additional axons to an ectopic location. Matn2 and Ucma overexpression, when limited to an ectopic location, failed to increase axonal sprouting towards that ectopic location. This would indicate that Matn2 and Ucma do not exert long range chemoattractant cues similar to that seen with sonic hedgehog in development (X. Li et al., 2021). There are several reasons why this may be the case, beyond these proteins not having any such effects. First, it is possible that either of proteins may promote outgrowth through interaction with an extending growth cone with exerting a contact dependent guidance cue effect, but do not cause axons to change the direction in which they are growing or cause axons to fasciculate as seen in development (Van Vactor, 1998). It is also possible that the limited area of overexpression was insufficient to attract axons due to lack of a large enough gradient. Both of these possibilities could be tested through overexpressing Matn2 or Ucma in the lateral somatosensory cortex at the boundary where axonal sprouting is typically limited in untreated post-stroke brain. This area was not chosen for this initial study as there was a desire to evaluate whether there is significant impact on the directionality of axonal sprouting and ability to of these proteins to influence formation of new circuits. The barrel cortex was chosen to test for this putative guidance cue effect as sparse axons are present there in untreated stroke brains. If Matn2 or Ucma did exert a long-range guidance

cue effect, overexpression in an area with sparse but present sprouting axons could both enhance the sprouting of axons already present, and cause more axons to change direction and trend towards an ectopic location. This would recapitulate the type of axon guidance seen in development through sonic hedgehog expression (X. Li et al., 2021), and would impact the translational impact of these proteins, as such an effect would impact axonal sprouting beyond the peri-infarct motor cortex.

As stated previously, the direct translational impact of these studies is limited by the use of viral vectors for treatment. As the most likely translational outcome of these experiments is a hydrogel, in which Matn2 or Ucma would be incorporated into a hydrogel in the stroke site that allows for secretion of the proteins in a set amount locally to the neurons that form new connections after stroke, we needed to evaluate whether the source of the protein impacts the observed axonal sprouting. To achieve this, Matn2 and Ucma were overexpressed in the peri-infarct motor cortex using astrocyte-specific lentiviruses, and BDA was again used to evaluate axonal sprouting one-month post-stroke. With this experimental design, Matn2 and Ucma expression would be limited to the peri-infarct motor cortex, mimicking the effect of a hydrogel continuously secreting the proteins for interaction with neurons that are then evaluated for axonal sprouting. Limiting Matn2 overexpression to the peri-infarct motor cortex did result in a significant increase in axonal sprouting compared to stroke mice injected with a control virus ($p = 0.045$), but the observed sprouting was much more restricted than that seen in Chapter 3. This could indicate that, while Matn2 does have interactions with receptors on the neuronal cell body, it may also have cell-compartment specific effects, such as being trafficked to the axon terminal and interaction with the growth cone. Matn2 has been localized to axons in the spinal cord following an inflammatory disease model (Fleetwood et al., 2014), and preliminary stains of

primary neurons overexpressing Matn2 show localization of Matn2 outside of the cell body, deposited alongside extending neurites, and present in neurite terminals (Figure 5-4). Experiments to determine whether preventing Matn2 trafficking into the axon would shed light on potential interactors in the growth cone, which would show a self-generated haptotactic effect (Sarkar et al., 2020). Limiting Ucma overexpression to the peri-infarct had a detrimental effect on axonal sprouting when compared to stroke mice injected with a control virus ($p = 0.003$). There are several reasons this may have occurred. First, as seen in Chapter 2, Ucma has a relatively narrow range where it increases neurite outgrowth *in vitro* (Figure 2-2 and 2-3). As the peri-infarct motor cortex is a highly reactive area, it is feasible that the level of Ucma in the extracellular space resulted in a net negative effect on axonal sprouting. Second, Ucma has previously been identified as having aggrecanase-inhibitor function (Seuffert et al., 2018). Aggrecan is a major of component of perineuronal nets (Deepa et al., 2006), which play an important role in post-stroke plasticity through remodeling (Dzyubenko et al., 2018). As the brain enters the chronic stroke phase and recovery processes proceed, perineuronal nets undergo remodeling in response to the formation of new circuits, with nets that are important for recovery being strengthened, while those that are no longer needed being degraded (Dzyubenko et al., 2018). This period of remodeling appears to correlate with the changes in axonal sprouting observed between 4 and 8 weeks post-stroke, and may indicate that there is an initial overabundance of axonal sprouting, and as circuits that are beneficial to recovery are formed, extraneous circuits and the axons that formed them are pruned. While the critical period for plasticity reopening occurs early in post-stroke recovery (Biernaskie et al., 2004), recovery continues, albeit at a slower pace, indicating that plasticity to some degree remains an important component of post-stroke recovery (Ballester et al., 2019). If chronic Ucma overexpression

blocks this extended period of plasticity through artificial stabilization of perineuronal nets, it could explain the plateau in functional recovery observed. This may seem paradoxical, as there is a direct relationship between increases in axonal sprouting and better functional recovery, as seen in this study and others (S. Li et al., 2015; Overman et al., 2012). In fact, it makes sense from a biological perspective, as unbounded axonal sprouting can be detrimental to functional recovery (Wahl et al., 2014). It is possible that there is an initial overabundance of axonal sprouting, that does play a role in initiating functional recovery, but then become unneeded and thus targets for pruning for refinement of the newly formed circuits.

5.4.1 Future directions

In this body of work, Matn2 was identified as an ECM protein that enhances axonal sprouting and functional recovery post-stroke, while Ucma was identified as an ECM protein that initially accelerates functional recovery, but has detrimental effects on motor outcomes associated with chronic exposure and potential inhibitory effects on axonal sprouting dependent on the cellular source or level. This work contributes to therapies for those suffering from chronic disability associated with stroke, but additional areas of research specific to these proteins remains. Matn2 expression was down-regulated in sprouting neurons, as shown in the foundational work for these studies (S. Li et al., 2010a). This was initially thought to be a paradoxical down-regulation, as Matn2 had been identified as important for peripheral nerve regeneration (Malin et al., 2009); however, more recent studies identified axonally-derived Matn2 as contributing to increased inflammation and injury in a multiple sclerosis model (Jonas et al., 2014). Therefore, it is possible that the down-regulation is a neuroprotective effect, and that delayed expression of Matn2, as done throughout these studies, would be beneficial. This could be tested by driving overexpression prior to induction of the stroke and evaluation of

axonal sprouting as done before. Additionally, dramatic differences are present in the distribution of sprouting axons seen 4 and 9 weeks post-stroke (Figures 3-5 and 4-4). In this time period, functional recovery still proceeds towards baseline levels, despite the apparent pruning of axons that is occurring. This raises the question of whether the axons projecting towards the premotor and temporal cortices seen 4 weeks post-stroke, but absent once function is fully restored, are necessary for functional recovery. One could evaluate the time course of this refinement of axonal sprouting through evaluation of axonal projections at time points prior to 4 weeks post-stroke and at 6 weeks post-stroke, where motor behavior is no longer distinguishable from sham stroke mice. Ideally, axons would be traced in the same mouse throughout the recovery period, such as through two-photon imaging of entire circuits, but that is not experimentally feasible at this time.

Ucma overexpression was found to enhance axonal sprouting and initially accelerate functional recovery, but this recovery was incomplete. Further, Ucma overexpression limited to peri-infarct astrocytes was found to be detrimental to axonal sprouting. These limitations could be due to an overabundance of Ucma in the peri-infarct. This could be tested through administration of Ucma through a hydrogel, as this would allow for more precise knowledge of the amount of Ucma present in the peri-infarct. Further evaluation of the impact Ucma overexpression has on perineuronal composition and remodeling could also be evaluated through immunofluorescent staining in the area with overexpression.

For both Matn2 and Ucma, further knowledge of their binding partners and signaling pathways is critical for less invasive treatments, such as small molecule administration. Recent advances in protein-protein interaction technologies, including proximity biotinylation techniques including TurboID (Branon et al., 2018; Takano et al., 2020), would be suitable

applications. Such techniques would not only identify receptors that these proteins interact with, but also other ECM proteins (Piecha et al., 1999), which in turn would aid in better understanding of the post-stroke ECM environment. Ultimately, these studies represent one of the first bodies of work that identify a pro-growth ECM protein, Matn2, whose overexpression alone is sufficient to accelerate functional recovery and return motor function to normal levels. Ucma overexpression is also unique in accelerating functional recovery initially, but fails to return mice to baseline levels (Figures 4-2 and 4-3). This represents a fundamental shift in how the ECM can be viewed in the context of CNS injury, which has historically been viewed as an inhibitor to axonal outgrowth and functional recovery (Baeten & Akassoglou, 2011; Galtrey & Fawcett, 2007; Regeneration beyond the Glial Scar, 2004; Soleman et al., 2013). The post-stroke ECM is not something that needs to be overcome, but is a dynamic component integral to functional recovery, similar to that seen in the developing spinal cord (Canty & Murphy, 2008; Pires-Neto et al., 1999). Unlike the guidance cue molecules seen in development, Matn2 and, to a certain extent Ucma, appear to exert stimulus for increased growth that is restricted only by other biological processes that are still unknown. Additionally, these studies indicate that, by manipulating pro-growth components of the ECM, one can achieve complete recovery from stroke in an accelerated time frame.

In the broader context of stroke, the results from these studies open the field to further directions. The differences in axonal sprouting seen between Matn2 and Ucma overexpression, combined with the variability in functional recovery, may mean that the evolution of post-stroke axonal sprouting over time can dictate the degree to which enhancing axonal sprouting can benefit functional recovery. Specifically, this can address the question in the stroke field of whether ipsilesional axonal sprouting is a mechanism through which the brain recovers

endogenously, as a recent study claimed a lack of cortico-cortical remapping in long term stroke patients (Branscheidt et al., 2022). While this study may shed doubt on cortico-cortical axonal sprouting as a mechanism for functional recovery, it leaves unanswered whether therapeutics that have beneficial effects and an observed increase in axonal sprouting exert those effects through increased axonal sprouting. Studies such as targeted inhibition of the neurons that have sprouted, such as through use of a retrogradely targeted DREADD (Roth, 2016), could answer these questions. Further, the transition from axonal sprouting to the premotor and prefrontal cortices to an increase to the somatosensory cortex observed with Matn2 overexpression compared to the sprouting patterns seen with Ucma overexpression, limited only to the somatosensory cortex, raises the question of whether the region to which axons sprout, and at what time point relative to stroke, has an impact on the recovery process. Matn2 overexpression post stroke induces axonal sprouting in a radial manner observed at one month of recovery, but this is more focused towards the somatosensory cortex at the time of full functional recovery. Sequential experiments inactivating these specific circuits, again through use of targeted inactivators, could illustrate the role these specific circuits play in progressive recovery.

5.5 Figures

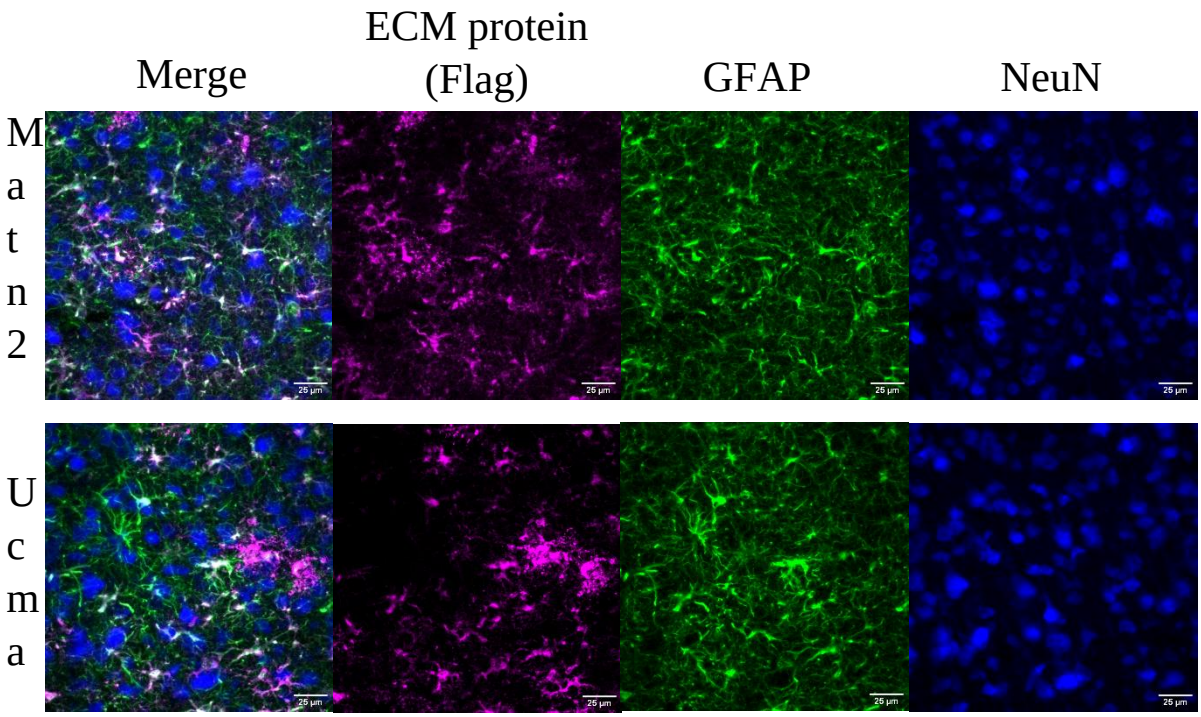


Figure 5-1. Astrocyte specific lentiviruses drive overexpression of target proteins in the peri-infarct. Viral expression was evaluated 7 days after photothrombotic stroke and virus injection. Matn2 (top panel) and Ucma (bottom panel) overexpression is restricted to GFAP positive peri-infarct astrocytes (green) and excluded from neurons (blue).

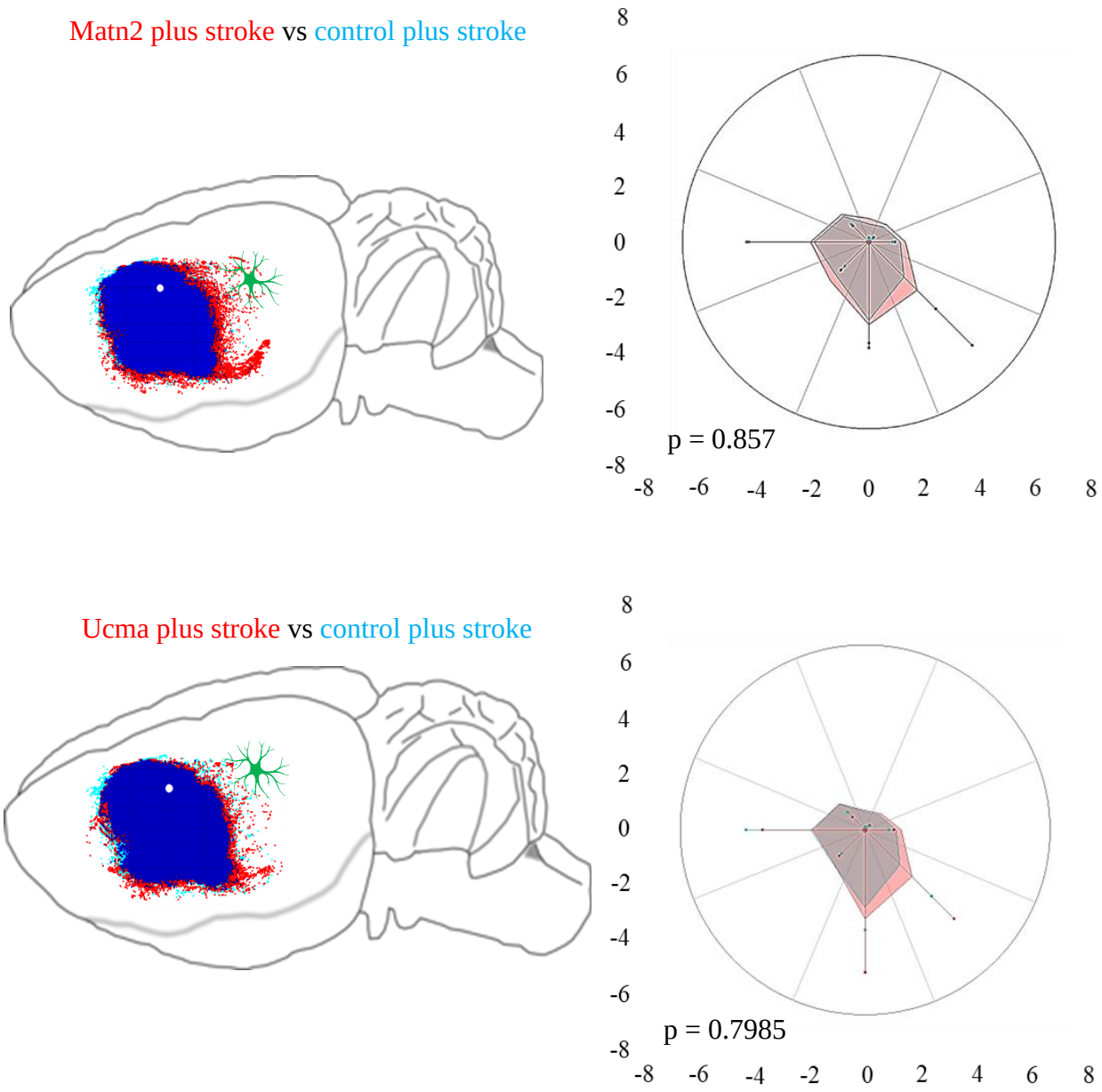


Figure 5-2. Matn2 and Ucma do not exert guidance cue effects in the post-stroke brain. Matn2 and Ucma were overexpressed in the barrel cortex following photothrombotic stroke in the motor cortex, and axons traced from the peri-infarct 28 days later via biotinylated dextran amine labeling. A green astrocyte indicates the location of protein overexpression. No significant differences in axonal sprouting patterns were observed.

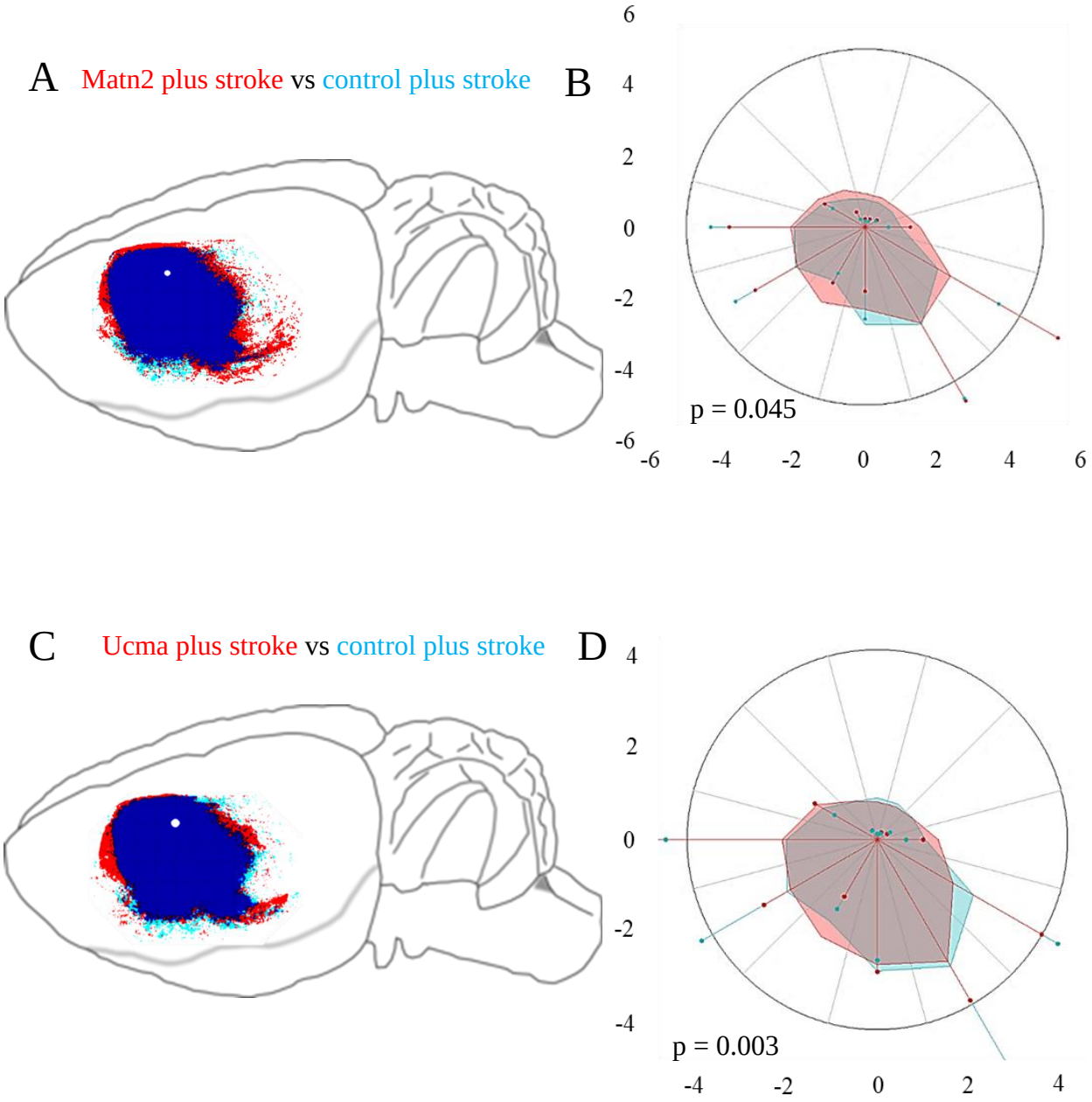


Figure 5-3 Limiting overexpression of Matn2 and Ucma to the peri-infarct results in changed patterns of axonal sprouting. Matn2 and Ucma overexpression was limited to the peri-infarct through use of the astrocyte specific GFAP promoter. Data are presented as in Chapter 3 Figure 3-5 A,B) Limiting Matn2 overexpression to the peri-infarct significantly increases axonal sprouting, targeted primarily towards the somatosensory cortex (p=0.045, Hotelling's T2 test for spatial distribution). C,D) Limiting Ucma overexpression to the peri-infarct significantly decreases axonal sprouting (p=0.003, Hotelling's T2 test for spatial distribution)

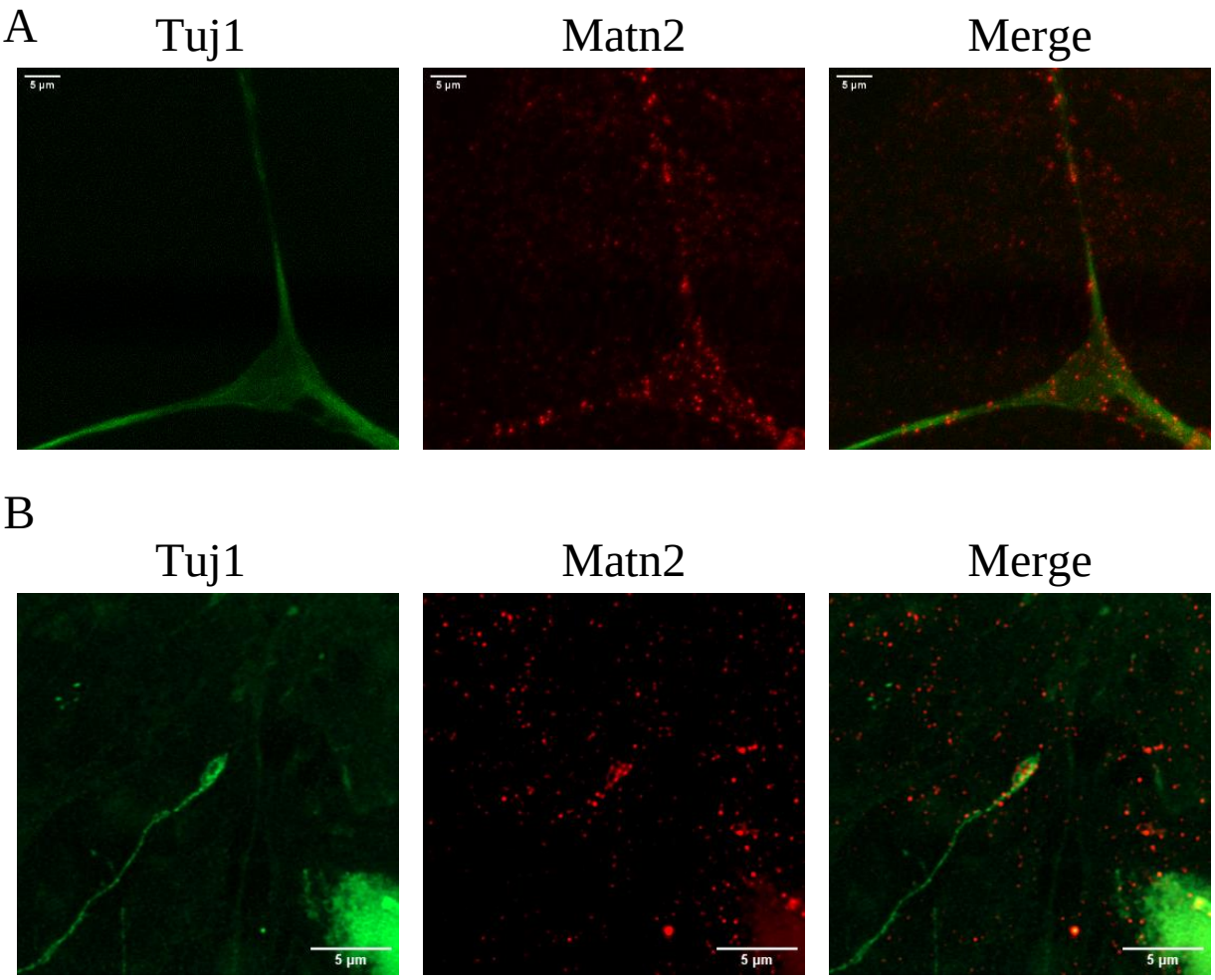


Figure 5-4 Matn2 is localized outside of the cell body. A) Surface staining of living cells shows Matn2 expression on the cell body in addition to extending neurites, indicating extracellular deposition by extending neurites. B) Super-resolution imaging of Matn2 shows it is present in the proximal portion of an extending neurite.

5.6 References

Baeten, K. M., & Akassoglou, K. (2011). Extracellular matrix and matrix receptors in blood-brain barrier formation and stroke. *Developmental Neurobiology*, *71*(11), 1018–1039. https://doi.org/10.1002/dneu.20954

Ballester, B. R., Maier, M., Duff, A., Cameirão, M., Bermúdez, S., Duarte, E., Cuxart, A., Rodríguez, S., Mozo, R. M. S. S., & Verschure, P. F. M. J. (2019). A critical time window for recovery extends beyond one-year post-stroke. *Journal of Neurophysiology*, *122*(1), 350–357. https://doi.org/10.1152/JN.00762.2018/ASSET/IMAGES/LARGE/Z9K0071951220003.JPEG

Barreto, G. E., Sun, X., Xu, L., & Giffard, R. G. (2011). Astrocyte Proliferation Following Stroke in the Mouse Depends on Distance from the Infarct. *PLOS ONE*, *6*(11), e27881. https://doi.org/10.1371/JOURNAL.PONE.0027881

Benarroch, E. E. (2015). Extracellular matrix in the CNS. *Neurology*, *85*(16), 1417–1427. https://doi.org/10.1212/WNL.0000000000002044

Biernaskie, J., Chernenko, G., & Corbett, D. (2004). Efficacy of Rehabilitative Experience Declines with Time after Focal Ischemic Brain Injury. *Journal of Neuroscience*, *24*(5), 1245–1254. https://doi.org/10.1523/JNEUROSCI.3834-03.2004

Branon, T. C., Bosch, J. A., Sanchez, A. D., Udeshi, N. D., Svinkina, T., Carr, S. A., Feldman, J. L., Perrimon, N., & Ting, A. Y. (2018). Efficient proximity labeling in living cells and organisms with TurboID. *Nature Biotechnology*, *36*(9), 880–887. https://doi.org/10.1038/nbt.4201

Branscheidt, M., Ejaz, N., Xu, J., Widmer, M., Harran, M. D., Cortés, J. C., Kitago, T., Celnik, P., Hernandez-Castillo, C., Diedrichsen, J., Luft, A., & Krakauer, J. W. (2022). No evidence for motor-recovery-related cortical connectivity changes after stroke using resting-state fMRI. *Journal of Neurophysiology*, *127*(3), 637–650. https://doi.org/10.1152/JN.00148.2021/ASSET/IMAGES/LARGE/JN.00148.2021_F006.JPEG

Canty, A. J., & Murphy, M. (2008). Molecular mechanisms of axon guidance in the developing corticospinal tract. *Progress in Neurobiology*, *85*(2), 214–235. https://doi.org/10.1016/J.PNEUROBIO.2008.02.001

Carmichael, S. T., Kathirvelu, B., Schweppe, C. A., & Nie, E. H. (2017). Molecular, Cellular and Functional Events in Axonal Sprouting after Stroke. *Experimental Neurology*, *287*(Pt 3), 384. https://doi.org/10.1016/J.EXPNEUROL.2016.02.007

Cook, D. J., Nguyen, C., Chun, H. N., L Llorente, I., Chiu, A. S., Machnicki, M., Zarembinski, T. I., & Carmichael, S. T. (2017). Hydrogel-delivered brain-derived neurotrophic factor promotes tissue repair and recovery after stroke. *Journal of Cerebral Blood Flow & Metabolism*, *37*(3), 1030–1045. https://doi.org/10.1177/0271678X16649964

Deepa, S. S., Carulli, D., Galtrey, C., Rhodes, K., Fukuda, J., Mikami, T., Sugahara, K., & Fawcett, J. W. (2006). Composition of perineuronal net extracellular matrix in rat brain: a different disaccharide composition for the net-associated proteoglycans. *The Journal of Biological Chemistry*, *281*(26), 17789–17800. https://doi.org/10.1074/JBC.M600544200

Dzyubenko, E., Manrique-Castano, D., Kleinschnitz, C., Faissner, A., & Hermann, D. M. (2018). Topological remodeling of cortical perineuronal nets in focal cerebral ischemia and mild

hypoperfusion. *Matrix Biology*, *74*, 121–132. https://doi.org/10.1016/J.MATBIO.2018.08.001

Fleetwood, A., Lawrence, T., Hamilton, J., Cook, A., Kiss, I., Aszódi, A., Hagemeier, K., Laverick, L., Perreau, V., Jokubaitis, V., Emery, B., Kilpatrick, T., Butzkueven, H., & Gresle, M. (2014). Axonally derived matrilin-2 induces proinflammatory responses that exacerbate autoimmune neuroinflammation. *J Immunol.*, *178*(8), 5245–5252. https://doi.org/10.1172/JCI71385

Galtrey, C. M., & Fawcett, J. W. (2007). The role of chondroitin sulfate proteoglycans in regeneration and plasticity in the central nervous system. *Brain Research Reviews*, *54*(1), 1–18. https://doi.org/10.1016/J.BRAINRESREV.2006.09.006

Ghuman, H., Massensini, A. R., Donnelly, J., Kim, S.-M., Medberry, C. J., Badylak, S. F., & Modo, M. (2016). ECM hydrogel for the treatment of stroke: Characterization of the host cell infiltrate. *Biomaterials*, *91*, 166–181. https://doi.org/10.1016/j.biomaterials.2016.03.014

Giger, R. J., Hollis, E. R., & Tuszynski, M. H. (2010). Guidance molecules in axon regeneration. In *Cold Spring Harbor perspectives in biology* (Vol. 2, Issue 7, p. a001867). Cold Spring Harbor Laboratory Press. https://doi.org/10.1101/cshperspect.a001867

Ginsberg, M. H. (2014). Integrin activation. *BMB Reports*, *47*(12), 655. https://doi.org/10.5483/BMBREP.2014.47.12.241

Hira, K., Ueno, Y., Tanaka, R., Miyamoto, N., Yamashiro, K., Inaba, T., Urabe, T., Okano, H., & Hattori, N. (2018). Astrocyte-derived exosomes treated with a semaphorin 3A inhibitor enhance stroke recovery via prostaglandin D2 synthase. *Stroke*, *49*(10), 2483–2494. https://doi.org/10.1161/STROKEAHA.118.021272

Jonas, A., Thiem, S., Kuhlmann, T., Wagener, R., Aszodi, A., Nowell, C., Hagemeier, K., Laverick, L., Perreau, V., Jokubaitis, V., Emery, B., Kilpatrick, T., Butzkueven, H., & Gresle, M. (2014). Axonally derived matrilin-2 induces proinflammatory responses that exacerbate autoimmune neuroinflammation. *The Journal of Clinical Investigation*, *124*(11), 5042–5056. https://doi.org/10.1172/JCI71385

Kaprielian, Z., Imondi, R., & Runko, E. (2000). Axon guidance at the midline of the developing CNS. *The Anatomical Record*, *261*(5), 176–197. https://doi.org/10.1002/1097-0185(20001015)261:5<176::AID-AR7>3.0.CO;2-R

Kennedy, M. J., & Hanus, C. (2019). Architecture and Dynamics of the Neuronal Secretory Network. *Annual Review of Cell and Developmental Biology*, *35*, 543. https://doi.org/10.1146/ANNUREV-CELLBIO-100818-125418

Laabs, T., Carulli, D., Geller, H. M., & Fawcett, J. W. (2005). Chondroitin sulfate proteoglycans in neural development and regeneration. In *Current Opinion in Neurobiology* (Vol. 15, Issue 1, pp. 116–120). Elsevier Ltd. https://doi.org/10.1016/j.conb.2005.01.014

Li, S., Nie, E. H., Yin, Y., Benowitz, L. I., Tung, S., Vinters, H. V, Bahjat, F. R., Stenzel-Poore, M. P., Kawaguchi, R., Coppola, G., & Carmichael, S. T. (2015). GDF10 is a signal for axonal sprouting and functional recovery after stroke. *Nature Neuroscience*, *18*(12), 1737–1745. https://doi.org/10.1038/nn.4146

Li, S., Overman, J. J., Katsman, D., Kozlov, S. V., Donnelly, C. J., Twiss, J. L., Giger, R. J., Coppola, G., Geschwind, D. H., & Carmichael, S. T. (2010a). An age-related sprouting transcriptome provides molecular control of axonal sprouting after stroke. *Nature Neuroscience*, *13*(12), 1496–1506. https://doi.org/10.1038/nn.2674

Li, S., Overman, J. J., Katsman, D., Kozlov, S. V., Donnelly, C. J., Twiss, J. L., Giger, R. J., Coppola, G., Geschwind, D. H., & Carmichael, S. T. (2010b). An age-related sprouting transcriptome provides molecular control of axonal sprouting after stroke. *Nature Neuroscience*, *13*(12), 1496–1504. https://doi.org/10.1038/nn.2674

Li, X., Li, Y., Li, S., Li, H., Yang, C., & Lin, J. (2021). The role of Shh signalling pathway in central nervous system development and related diseases. *Cell Biochemistry and Function*, *39*(2), 180–189. https://doi.org/10.1002/CBF.3582

Malin, D., Sonnenberg-Riethmacher, E., Guseva, D., Wagener, R., Aszódi, A., Irintchev, A. A., Riethmacher, D., Irintchev, A. A., & Riethmacher, D. (2009). The extracellular-matrix protein matrilin 2 participates in peripheral nerve regeneration. *Journal of Cell Science*, *122*(Pt 7), 995–1004. https://doi.org/10.1242/jcs.040378

Onesto, M. M., Short, C. A., Rempel, S. K., Catlett, T. S., & Gomez, T. M. (2021). Growth Factors as Axon Guidance Molecules: Lessons From in vitro Studies. *Frontiers in Neuroscience*, *15*. https://doi.org/10.3389/FNINS.2021.678454

Overman, J. J., Clarkson, A. N., Wanner, I. B., Overman, W. T., Eckstein, I., Maguire, J. L., Dinov, I. D., Toga, A. W., & Carmichael, S. T. (2012). A role for ephrin-A5 in axonal sprouting, recovery, and activity-dependent plasticity after stroke. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(33). https://doi.org/10.1073/PNAS.1204386109/SUPPL_FILE/PNAS.201204386SI.PDF

Piecha, D., Muratoglu, S., Morgelin, M., Hauser, N., Studer, D., Kiss, I., Paulsson, M., & Deak, F. (1999). Matrilin-2, a Large, Oligomeric Matrix Protein, Is Expressed by a Great Variety of Cells and Forms Fibrillar Networks. *Journal of Biological Chemistry*, *274*(19), 13353–

13361. https://doi.org/10.1074/jbc.274.19.13353

Pires-Neto, M. A., Braga-De-Souza, S., & Lent, R. (1999). Extracellular matrix molecules play diverse roles in the growth and guidance of central nervous system axons. *Brazilian Journal of Medical and Biological Research*, *32*(5), 633–638. https://doi.org/10.1590/S0100-879X1999000500017

Poplawski, G. H. D., Kawaguchi, R., Van Niekerk, E., Lu, P., Mehta, N., Canete, P., Lie, R., Dragatsis, I., Meves, J. M., Zheng, B., Coppola, G., & Tuszynski, M. H. (2020). Injured adult neurons regress to an embryonic transcriptional growth state. *Nature*, 1–6. https://doi.org/10.1038/s41586-020-2200-5

Qin, J. Y., Zhang, L., Clift, K. L., Hulur, I., Xiang, A. P., Ren, B.-Z., Lahn, B. T., Pasleau, F., Tocci, M., Leung, F., Kopchick, J., Martin-Gallardo, A., Montoya-Zavala, M., Kelder, B., Taylor, J., Chen, H., Oellig, C., Seliger, B., Manthorpe, M., … Cherbas, L. (2010). Systematic Comparison of Constitutive Promoters and the Doxycycline-Inducible Promoter. *PLoS ONE*, *5*(5), e10611. https://doi.org/10.1371/journal.pone.0010611

Roth, B. L. (2016). DREADDs for Neuroscientists. *Neuron*, *89*(4), 683. https://doi.org/10.1016/J.NEURON.2016.01.040

Sarkar, A., LeVine, D. N., Kuzmina, N., Zhao, Y., & Wang, X. (2020). Cell Migration Driven by Self-Generated Integrin Ligand Gradient on Ligand-Labile Surfaces. *Current Biology*, *30*(20), 4022-4032.e5. https://doi.org/10.1016/J.CUB.2020.08.020

Seuffert, F., Weidner, D., Baum, W., Schett, G., & Stock, M. (2018). Upper zone of growth plate and cartilage matrix associated protein protects cartilage during inflammatory arthritis. *Arthritis Research & Therapy*, *20*(1), 88. https://arthritis-

research.biomedcentral.com/articles/10.1186/s13075-018-1583-2

Regeneration beyond the glial scar, 5 Nature Reviews Neuroscience 146 (2004). https://doi.org/10.1038/nrn1326

Soleman, S., Filippov, M. A. A., Dityatev, A., & Fawcett, J. W. W. (2013). Targeting the neural extracellular matrix in neurological disorders. *Neuroscience*, *253*, 194–213. https://doi.org/10.1016/j.neuroscience.2013.08.050

Takano, T., Wallace, J. T., Baldwin, K. T., Purkey, A. M., Uezu, A., Courtland, J. L., Soderblom, E. J., Shimogori, T., Maness, P. F., Eroglu, C., & Soderling, S. H. (2020). Chemico-genetic discovery of astrocytic control of inhibition in vivo. *Nature*, *588*(7837), 296–302. https://doi.org/10.1038/s41586-020-2926-0

Taschenberger, G., Tereshchenko, J., & Kügler, S. (2017). A MicroRNA124 Target Sequence Restores Astrocyte Specificity of gfaABC1D-Driven Transgene Expression in AAV-Mediated Gene Transfer. *Molecular Therapy. Nucleic Acids*, *8*, 13. https://doi.org/10.1016/J.OMTN.2017.03.009

Van Vactor, D. (1998). Adhesion and signaling in axonal fasciculation. *Current Opinion in Neurobiology*, *8*(1), 80–86. https://doi.org/10.1016/S0959-4388(98)80011-1

Varadarajan, S. G., & Butler, S. J. (2017). Netrin1 establishes multiple boundaries for axon growth in the developing spinal cord. *Developmental Biology*, *430*(1), 177. https://doi.org/10.1016/J.YDBIO.2017.08.001

Varadarajan, S. G., Kong, J. H., Phan, K. D., Kao, T. J., Panaitof, S. C., Cardin, J., Eltzschig, H., Kania, A., Novitch, B. G., & Butler, S. J. (2017). Netrin1 Produced by Neural Progenitors,

Not Floor Plate Cells, Is Required for Axon Guidance in the Spinal Cord. *Neuron*, *94*(4), 790-799.e3. https://doi.org/10.1016/J.NEURON.2017.03.007

Vecino, E., & Kwok, J. C. F. (2016). The Extracellular Matrix in the Nervous System: The Good and the Bad Aspects. *Composition and Function of the Extracellular Matrix in the Human Body*. https://doi.org/10.5772/62527

Wahl, A. S., Omlor, W., Rubio, J. C., Chen, J. L., Zheng, H., Schröter, A., Gullo, M., Weinmann, O., Kobayashi, K., Helmchen, F., Ommer, B., & Schwab, M. E. (2014). Asynchronous therapy restores motor control by rewiring of the rat corticospinal tract after stroke. *Science*, *344*(6189), 1250–1255. https://doi.org/10.1126/SCIENCE.1253050/SUPPL_FILE/WAHL.SM.PDF

Wu, Z., Makihara, S., Yam, P. T., Teo, S., Renier, N., Balekoglu, N., Moreno-Bravo, J. A., Olsen, O., Chédotal, A., Charron, F., & Tessier-Lavigne, M. (2019). Long-Range Guidance of Spinal Commissural Axons by Netrin1 and Sonic Hedgehog from Midline Floor Plate Cells. *Neuron*, *0*(0). https://doi.org/10.1016/j.neuron.2018.12.025

Xiao, Y., Petrucco, L., Hoodless, L. J., Portugues, R., & Czopka, T. (2022). Oligodendrocyte precursor cells sculpt the visual system by regulating axonal remodeling. *Nature Neuroscience 2022*, 1–5. https://doi.org/10.1038/s41593-022-01023-7

Yaguchi, M., Ohashi, Y., Tsubota, T., Sato, A., Koyano, K. W., Wang, N., & Miyashita, Y. (2013). Characterization of the properties of seven promoters in the motor cortex of rats and monkeys after lentiviral vector-mediated gene transfer. *Human Gene Therapy Methods*, *24*(6), 333–344. https://doi.org/10.1089/hgtb.2012.238

Yu, T. W., & Bargmann, C. I. (2001). Dynamic regulation of axon guidance. *Nature*

Neuroscience 2001 4:11, *4*(11), 1169–1176. https://doi.org/10.1038/nn748