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Role of neuroblast-derived signals in post-stroke neurovascular remodeling

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1 Introduction

1.1 The burden of ischemic stroke

Stroke is the second leading cause of death and the third leading cause of death and disability combined in the world (Feigin et al., 2021). Ischemic stroke is the most common type of stroke, constituting almost 2/3 of all strokes in 2019, followed by intracerebral hemorrhage and subarachnoid hemorrhage. Due to improvements in acute care, many patients survive the acute stroke episode but must deal with long-term consequences including physical disability, cognitive impairment, depression and anxiety (Crichton et al., 2016). Tissue plasminogen activator (tPA) remains the only FDA approved drug to treat acute stroke but is unavailable to many patients as it can only be used within a very narrow time window after ischemia. It is estimated that only 5% of patients with a stroke receive tPA acutely (Cramer, 2018). Even in patients who receive tPA in time, many patients suffer from long-term disability. It is estimated that if current trends continue, there will be more than 200 million stroke survivors and 13 million deaths from stroke annually by 2050 (Feigin et al., 2021). The total cost of stroke in Europe was estimated at EUR 60 billion in 2017: EUR 27 billion spent on healthcare, EUR 5 billion spent on nursing or residential care, EUR 16 billion spent on informal, unpaid care and EUR 12 billion attributed to lost productivity due to death and disability from stroke in working age individuals (Luengo-Fernandez et al., 2020). Despite these grave statistics, there is no effective treatment that promotes long-term recovery after stroke.

1.2 Mechanisms of brain repair

After ischemic injury, patients show some limited capacity for recovery. A plethora of preclinical and clinical studies have demonstrated that ischemic injury itself induces a wide range of neurorestorative processes, as reviewed more extensively elsewhere (Carmichael, 2016; Hermann and Chopp, 2012). One example is axonal sprouting (Li et al., 2015). Stroke induces limited axonal sprouting in the adult brain, especially in peri-infarct cortex but also from distant sites like the contra-lesional hemisphere. Behavioral activity like forced forelimb use in rodents or constraint-induced motor

therapy (CIMT) in humans modulates and enhances this endogenous response. However, stroke does not only induce signals that promote axonal sprouting but also those that inhibit axonal sprouting and motor recovery. Ephrin-A5 is expressed in reactive astrocytes after stroke and inhibits axonal sprouting (Overman et al., 2012). Blockade of ephrin-A5 in combination with forced forelimb use results in wide-spread cortical axonal-sprouting and improved functional recovery after stroke. Stroke induced peri-lesional plasticity does seem to share similarities with plasticity associated with learning and memory (Carmichael, 2016). Tonic GABAergic signaling in the hippocampus has been implicated in long-term potentiation (LTP), inhibitors of alpha5-$GABA_A$ receptors enhance memory formation (Martin et al., 2010). Tonic $GABA_A$-mediated neuronal inhibition appears to constrain plasticity in peri-infarct cortex. Pharmacological antagonism of tonic GABA signaling at a delay after stroke improves functional recovery (Clarkson et al., 2010). Positive allosteric modulators of AMPA receptors, known as ampakines, improve memory and learning. Delayed treatment with ampakines after stroke enhances motor recovery, mediated by downstream brain-derived neurotrophic factor signaling (Clarkson et al., 2011). Another example is C-C chemokine receptor type 5 (CCR5) (Joy et al., 2019). Inhibition of CCR5 has been shown to increase LTP and hippocampus-dependent learning in rodents. Stroke induces CCR5 expression in neurons; genetic or pharmacological knockdown of CCR5 promotes motor recovery. CCR5 knockdown results in upregulation of cAMP response element-binding protein and increased dual leucine zipper kinase signaling, preserved dendritic spines and enhanced axonal sprouting.

1.3 Angiogenesis

Angiogenesis refers to the sprouting of new blood vessels from pre-existing ones, a process reviewed in a recent publication by Eelen et al. (2020). Release of proangiogenic factors from the microenvironment leads to activation of endothelial cells which in turn release matrix metalloproteinases to degrade the basement membrane. Tip cells, specialized endothelial cells, start to migrate towards the angiogenic signal and are followed by stalk cells, which are primarily involved in stalk

elongation and lumen formation through cell proliferation. Once tip cells of adjacent sprouts meet, they connect in a process termed anastomosis to form a new blood vessel. Vessel perfusion is a key driver of further vessel maturation including recruitment of pericytes and basement membrane deposition. Cerebral angiogenesis is highly active during embryonic and early postnatal development but downregulated in the adult brain. However, angiogenesis can reemerge in the adult brain in pathological conditions like tumor growth or brain ischemia (Carmeliet and Storkebaum, 2002).

Studies in the early 1990s by Krupinski and colleagues on post-mortem brain tissue from stroke patients provided first evidence for post-stroke angiogenesis in humans. The authors of the study found higher microvessel densities in the penumbra with evidence for angiogenic activity, i.e. immunoreactivity for markers of activated / proliferating endothelial cells as well as angiogenic activity of tissue extract in the chorio-allantoic membrane assay. Higher vessel counts correlated with longer survival (Krupinski et al., 1994, 1993). Rodent studies suggest that pro-angiogenic genes (e.g. vascular endothelial growth factor (VEGF), fibroblast growth factor) are upregulated as early as one hour after ischemia and endothelial cells undergo cell division as early as one day after ischemia (Hayashi et al., 2003). Administration of VEGF, a major pro-angiogenic growth factor, 48 hours after ischemia promotes angiogenesis and functional recovery in rats (Zhang et al., 2000). However, administration 1 hour after stroke increases blood-brain-barrier (BBB) leakage, hemorrhagic transformation and ischemic lesion size, demonstrating the importance of timing.

1.4 Neurogenesis

The subventricular zone (SVZ) is considered the largest germinal niche in the adult human brain (Obernier and Alvarez-Buylla, 2019). B1 cells at the lateral ventricle have neural stem cell (NSC) properties. These cells have apical processes that are in contact with cerebrospinal fluid (CSF) and sit between ependymal cells. B1 cells also have a basal process which can maintain contact to blood vessels. B1 cells have

been shown to generate transient amplifying intermediate progenitors (or C cells) that can differentiate into migratory immature neurons (neuroblasts, type A cells). These neuroblasts then migrate along the rostral migratory stream (RMS) to the olfactory bulb where they differentiate into mature interneurons.

It was long believed that humans were unable to generate new neurons after birth. In a landmark study in 1998, a group around Fred H. Gage first presented evidence for neurogenesis in the adult human brain. The group looked at postmortem brain samples from cancer patients who had received 5-bromo-2'-deoxyuridine (BrdU), a thymidine analog, for diagnostic purposes. The authors identified dividing ($BrdU^+$) cells in the dentate gyrus of the hippocampus which co-localized with markers like neuronal nuclear protein (NeuN), calbindin and neuron specific enolase. The group also identified $BrdU^+$ cells in the subventricular zone, lining the lateral ventricles, which morphologically resembled progenitor cells but did not appear glial fibrillary acidic protein $(GFAP)^+$ or $NeuN^+$ (Eriksson et al., 1998).
Further evidence of adult human neurogenesis comes from studies using nuclear-bomb-test derived carbon-14 (Spalding et al., 2013). Nuclear bomb testing during the cold war lead to elevated atmospheric carbon-14 levels which reacts with oxygen to form CO_2 which can be taken up by plants during photosynthesis and enters the food chain. During cell division carbon-14 is integrated into genomic DNA at a concentration related to atmospheric carbon-14 at that particular time. The authors used this strategy to birth date hippocampal cells in adult humans. They found evidence for substantial neurogenesis throughout adulthood with only a modest decline in old age. The authors estimated that 700 new neurons were added to the hippocampus per day.

While neuroblasts from the SVZ migrate along the rostral migratory stream to the olfactory bulb to mature into neurons in rodents, no neurogenesis in detectable in the adult human olfactory bulb (Bergmann et al., 2012) even though the human SVZ shows comparable levels of neural precursor cells. One explanation may be that these cells are migrating to a different site. In a 2014 study from the Frisén lab, the group demonstrates doublecortin (DCX) expression in the striatum at similar levels as

in the hippocampus on the mRNA and protein level. In a number of cancer patients who received the thymidine analogue Iododeoxyuridine (IdU) for diagnostic purposes, the authors found striatal IdU to colocalize with neuronal markers like NeuN, microtubule-associated protein 2 (MAP2) and calretinin. Further, using carbon-14 birth dating, the group estimates that around 25% of neurons, primarily interneurons, turn over postnatally. The group hypothesized that the origin of these cells may be the SVZ (Ernst et al., 2014).

Ischemia leads to increased cell proliferation (Ki-67$^+$ cells) and an increased number of polysialylated neuronal cell adhesion molecule (PSA-NCAM)$^+$ neural progenitors near the lateral ventricular wall in humans (Macas et al., 2006; Martí-Fàbregas et al., 2010). Studies have also identified Ki67$^+$/DCX$^+$ cells in the ischemic penumbra, clustering near blood vessels in humans (Jin et al., 2006).

1.5 Neurovascular remodeling after stroke

In a number of rodent models, ischemic injury induces the proliferation of neural progenitor cells in the SVZ and the long-distance migration of neuroblasts towards sites of ischemic injury, where neuroblasts associate with the angiogenic vasculature within the first days after stroke in the neurovascular niche (Ohab et al., 2006). These neuroblasts derive from GFAP-expressing progenitor cells. Inhibition of post-stroke angiogenesis using endostatin by Ohab et al. resulted in a 10-fold reduction in the number of neuroblasts in peri-infarct cortex, 7 days after stroke. Stromal cell-derived factor 1 beta (SDF1beta) / C-X-C chemokine receptor type 4 (CXCR4) and angiopoietin 1 (Ang1) / tyrosine-protein kinase receptor 2 (Tie2) were two receptor-ligand pairs identified in that study to be involved in signaling in this neurovascular niche. SDF1beta and Ang1 expression in the vasculature was increased after stroke, while CXCR4 and Tie2 expression was elevated in neuroblasts, migrating towards ischemic injury. The authors further showed that delivery of both SDF1beta as well as Ang1 to the site of ischemic injury resulted in increased numbers of DCX$^+$ neuroblasts in peri-infarct tissue, while inhibition of CXCR4 resulted in dispersal of these cells into a greater volume. Inhibition of Tie2 reduced the volume of distribution of these cells. Both Ang1 as well as SDF1 delivery improved behavioral recovery after stroke. While

stimulation of endogenous neurogenesis enhances recovery, transgenic ablation of DCX^+ cells worsens functional outcome after stroke (Jin et al., 2010). Most neuroblasts or immature neurons that are generated after stroke do no survive long-term. Forced overuse of the paretic limb is used in rodents CIMT. In a recent study it was demonstrated that forelimb overuse promotes neural progenitor proliferation as well as migration towards and differentiation in peri-infarct cortex, enhancing long-term survival of SVZ-derived neurons. Most strikingly, synaptic integration of these SVZ-derived neurons was necessary for functional recovery (Liang et al., 2019).

1.6 Preliminary studies

Based on the above findings, the goal of the current study is to more systematically characterize the neurovascular niche in peri-infarct cortex. A better understanding of the signaling systems underlying neuroblast-vessel communication may lead to the identification of novel receptor-ligand pairs involved in the promotion of angiogenesis (without BBB disruption), neurogenesis (including differentiation, synaptic integration and long-term survival) and ultimately functional recovery after stroke.

To this end, preliminary studies in the Carmichael laboratory identified the peri-infarct neurovascular interactome. In brief, a focal cortical stroke was generated in young adult mice by permanent distal middle cerebral artery and transient bilateral common carotid artery occlusion for 15 minutes. Mice were euthanized after 7 days, brains collected, sectioned and stained for platelet endothelial cell adhesion molecule (PECAM), a marker for endothelial cells. Based on morphology, angiogenic blood vessels were identified in peri-infarct cortex and isolated using laser capture microdissection (LCM), blood vessels from contralateral cortex were selected for comparison. Total RNA was isolated from these cell populations and used for genome-wide expression profiling studies. In a second set of experiments, stroke was generated in a doublecortin promotor – red fluorescent protein (DCX-RFP) mouse line and stroke-responsive neuroblasts isolated from peri-infarct cortex and compared to neuroblasts that migrate to olfactory bulb using fluorescence activated cell sorting (FACS) purification. In a collaborative effort with the Informatics Center for

Neurogenetics and Neurogenomics (ICNN) at UCLA using advanced bioinformatic approaches, the total set of receptor-ligand pairs or receptor-extracellular matrix (ECM) signaling systems between angiogenic vessels and regenerating immature neurons after stroke (the regeneration interactome) was identified (Figure 1-1). With this data set available, we started to explore the role of neuroblast-derived signals in the neurovascular niche.

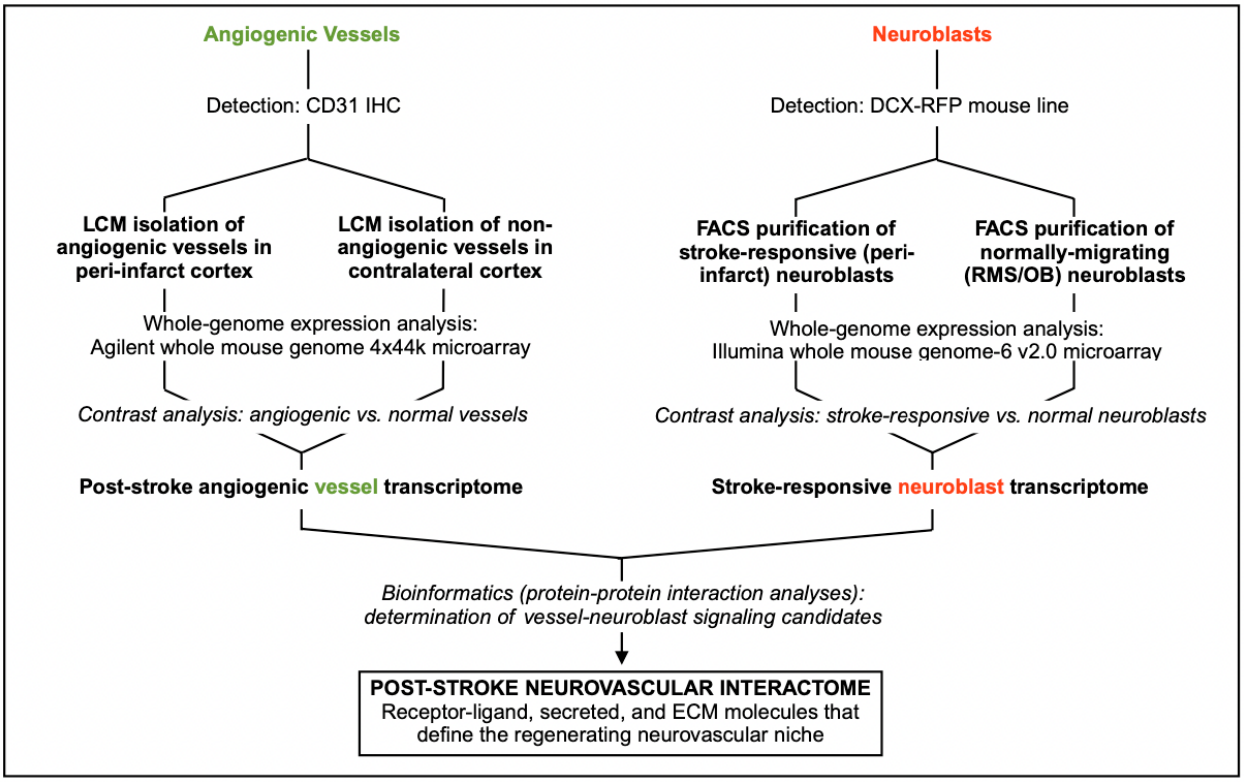

Figure 1-1 Genome-wide expression profiling studies
Angiogenic blood vessels were identified based on CD31 immunohistochemical labeling and morphology, isolated from peri-infarct cortex as well as contralateral cortex using LCM and processed for microarray studies. Neuroblasts were identified based on RFP signal and FACS purified from peri-infarct cortex as well as olfactory bulb and processed for microarray studies. Contrast analysis was performed to identify the individual transcriptome and protein-protein interaction analyses performed to identify the post-stroke neurovascular interactome. Figure adapted from Brumm (2012).

Based on gene expression change, novelty in brain regeneration and literature support for a role in angiogenesis, we selected four gene systems encoding different protein classes: Chemokine (C-C-motif) ligand 9 (CCL9), pleiotrophin (PTN), wingless-type MMTV integration site family, member 7A (Wnt7a) and Growth arrest –

specific 6 (Gas6) for further mechanistic gain- and loss-of-function studies. We found CCL9, PTN and GAS6 to be upregulated in stroke-responsive neuroblasts, while WNT7A was downregulated (Table 1-1).

Symbol	Log Ratio	Location	Type(s)	FC	↑/↓
Ccl9	1,604	Extracellular Space	cytokine	3,04	↑
GAS6	1,596	Extracellular Space	growth factor	3,02	↑
PTN	-1,599	Extracellular Space	growth factor	0,33	↓
WNT7A	-0,758	Extracellular Space	cytokine	0,59	↓

Table 1-1 Candidate gene systems
Genome-wide expression profiling studies identified upregulation of CCL9 and GAS6 as well as downregulation of PTN and WNT7A in post-stroke neuroblasts at 7d after stroke compared to neuroblasts migrating to olfactory bulb.

1.7 Chemokine (C-C-motif) ligand 9

CCL9 is a member of the macrophage inflammatory protein-1 family (MIP-1) and also referred to as MIP1-gamma (Zhao et al., 2003). Initial studies in 1995 found CCL9 to be expressed in macrophages and myeloid progenitor cells and described its chemotactic properties (Hara et al., 1995; Youn et al., 1995). Later studies described CCL9 expression in osteoclasts with a role in their motility and polarization (Lean et al., 2002). Several studies have suggested a role for CCL9 in disease processes. CCL9 was found to be upregulated in chronic kidney disease (CKD) and found to counteract kidney inflammation and fibrosis (Hemmers et al., 2022). Several studies have described a role for CCL9 in cancer biology. Colon cancer cells were found to secrete CCL9 to recruit immature myeloid cells (iMC) through CCR1, with CCR1 antagonism impairing iMC recruitment and resulting in suppressed tumor outgrowth and dissemination (Kitamura et al., 2010). In a recent study performed using a mouse model of lung tumors, CCL9 was found to drive macrophage recruitment and macrophage-derived VEGF found to drive angiogenesis (Kortlever et al., 2017). Several studies have described a role for CCL9 in the nervous system. In a streptozotocin (STZ)-induced mouse model of diabetic neuropathic pain, expression of CCL9 and its receptor CCR1 was increased in the lumbar spinal cord, predominantly in neurons. In the same study, intrathecal injection of CCL9 neutralizing antibodies resulted in delayed neuropathic pain symptoms at day 7

following STZ administration, as did injection of a CCR1 antagonist (Rojewska et al., 2018). Elevated levels of CCL9 mRNA as well as protein have been found in a model of traumatic brain injury (Ciechanowska et al., 2020). CCL23, a human CCL9 ortholog, was found to be elevated in blood samples of stroke patients within 24 hours after ischemia, however, this finding was not specific to ischemia but also found in other forms of human brain injury, i.e. in the setting of traumatic brain injury or tumors (Simats et al., 2018). Higher baseline CCL23 levels at the time of presentation were predictive for poorer outcomes after stroke in the study population. The authors of the study also found CCL9 expression acutely elevated in rodent models of cerebral ischemia and traumatic brain injury. As expression of CCL23 was maintained into the subacute phase of ischemia, the authors hypothesized that CCL23 may also have a role in neuronal repair at later stages of ischemia. In support of this hypothesis, prior studies have shown that CCL23 promotes endothelial cell proliferation and migration (Han et al., 2009).

1.8 Pleiotrophin

Initial studies around 1989 described PTN as an 18-kD heparin-binding protein that is similar in structure to fibroblast growth factors, highly expressed in perinatal rat brain and promotes neurite outgrowth in vitro (Rauvala, 1989). It was found to be highly conserved among mammals and named pleotrophin due to its diverse biological activities (Li et al., 1990). In fact, at the time of writing, a PubMed search for ‘pleotrophin’ yields well beyond 800 results. PTN has diverse roles in mammalians, including bone development, lipid metabolism, inflammation and cancer biology. An extensive discussion of all its biological activities is well beyond the scope of this thesis. The following text will focus on the role of PTN in the central nervous system and discuss findings related to angiogenesis. A number of studies have described behavioral abnormalities in PTN knockout mice. In a study published in 2014, the authors find that PTN knockout mice show evidence of cognitive inflexibility and heightened anxiety especially in novel contexts (Krellman et al., 2014). The authors describe layer-specific changes in neuronal area and density in the lateral entorhinal cortex without gross differences in cortical volume compared to wild-type animals.

Further, the authors do not find any gross pathology of the vasculature in the entorhinal cortex. PTN knockout mice also exhibit prolonged amphetamine-seeking behavior and seem to be more susceptible to amphetamine-induced neurotoxicity (Gramage et al., 2010). In a similar study, ethanol was found to upregulate PTN in the mouse prefrontal cortex and the authors reported that PTN knockout mice were more susceptible to the rewarding effects of ethanol, an effect they were able to abolish by overexpressing PTN in mouse cortex and hippocampus (Vicente-Rodríguez et al., 2014). PTN was found to be highly expressed in both mouse as well as human SVZ (Qin et al., 2017). In the same study, NPC – derived PTN was found to promote invasion of gliomas to the SVZ by activation of Rho/Rho-associated kinase signaling. A recent study (Tang et al., 2019) found NSC-derived PTN to be involved in hippocampal neuronal maturation. Loss of PTN was associated with impaired dendritic development and arborization without impacting the birth and survival of newborn neurons directly. This was mediated through anaplastic lymphoma kinase receptor activation. PTN expression and associated signaling was found to decrease with age, impairing hippocampal maturation of newborn neurons. A study in mice (Nikolakopoulou et al., 2019) has identified pericytes as a major source of PTN in the central nervous system and found that loss of pericytes is associated with neuronal loss that can be rescued by intracerebroventricular infusions of PTN. The authors of the study further showed that targeted silencing of PTN in pericytes renders neurons susceptible to ischemic and excitotoxic injury in vitro and in vivo. Initial studies (Yeh et al., 1998) found PTN mRNA and protein in cortical neurons of the adult rat brain but little or no expression in glia or endothelial cells. However, after ischemic injury, the authors found loss of PTN in injured neurons (especially in the infarct core) while expression was elevated in $GFAP^+$ astrocytes, OX42 and Griffonia simplicifolia agglutinin-IB4-positive macrophages as well as developing microvasculature. Expression remained elevated for at least 14 days in this study. In terms of angiogenesis, PTN was found to promote endothelial cell proliferation, migration and formation of tube-like structures (Souttou et al., 2001). It was also found to induce angiogenesis in the ex vivo aortic ring assay, the in vivo chicken embryo chorioallantoic membrane angiogenesis assay as well as the rabbit corneal assay (Besse et al., 2013; Mikelis et al., 2011; Choudhuri et al., 1997). PTN has been

implicated in tumor angiogenesis (Papadimitriou et al., 2016). In contrast to these findings, however, PTN was also found to negatively regulate VEGF165- and FGF2-mediated angiogenesis (Dos Santos et al., 2014; Héroult et al., 2004).

1.9 Wingless-type MMTV integration site family, member 7A

Work by Roel Nusse and Harold Varmus (1982) lead to the identification of a proto-oncogene named integration site 1 (int1), a target for the mouse mammary tumor virus (MMTV). Later studies identified its homolog in Drosophila, wingless, to be a crucial morphogen in development (Nusse and Varmus, 2012). These names were later combined and the gene renamed to Wnt1, which stands for Wingless-related integration site 1. Wnt genes are highly conserved and have diverse roles in development and disease. The Wnt family consist of 19 members, secreted glycoproteins that can signal through different pathways. In Wnt signaling, the canonical or beta-catenin dependent pathway is differentiated from non-canonical or beta-catenin independent pathways like the Ca2+ pathway or the planar cell polarity (PCP) pathway. As reviewed by Liebner and Plate (2010), the canonical pathway is activated through binding of Wnt ligands to the cysteine-rich domain (CSD) of Frizzled (Fz) receptors and low-density lipoprotein receptor-related protein 5 and 6 (LRP5/6), which results in the recruitment of Dishevelled (Dvl) and Axin to Fz, ultimately resulting in inhibition of the “destruction complex”, making it possible for beta-catenin to accumulate in the cytoplasm, translocate to the cell nucleus and modulate gene expression through binding to T cell factor/lymphoid enhancer factor.

A study conducted by a group around Ben Barres at Stanford University found canonical Wnt signaling to be necessary for CNS-specific angiogenesis in development (Daneman et al., 2009). In particular, the group identified Wnt7a and Wnt7b expression in ventral regions of the CNS to be essential for normal angiogenesis. Wnt7a was found to be involved in BBB formation by regulating expression of glucose transporter 1 (GLUT1). A recent study suggests that Wnt7a mediated canonical signaling may maintain BBB integrity after ischemia (Song et al., 2021). Wnt7a was also found to be involved in axonal remodeling and

synaptogenesis. Studies involving Wnt7a-knockout mice (Qu et al., 2013) have also identified a role in neural progenitor cell proliferation and differentiation towards a neuronal fate in the dentate gyrus of the hippocampus.

1.10 Growth-arrest – specific 6

As reviewed by Di Stasi et al. (2020) Gas6 is a vitamin K-dependent glycoprotein that was identified as a ligand to Tyro3, Mer and Axl (TAM) receptors in 1995 with the greatest binding affinity towards Axl. It is structurally related to protein S, another TAM receptor ligand. Gas6 is expressed in a wide range of tissues including endothelial cells and the CNS. Axl is a receptor tyrosine kinase, binding of Gas6 leads to receptor homodimerization, autophosphorylation of tyrosine residues and activation of different downstream signaling pathways like phosphatidylinositol-3-kinase/AKT, extracellular signal-regulated kinase 1/2 and mitogen-activated protein kinase. Gas6/Axl signaling dysregulation has been described in several cancers and is associated with cancer metastases and angiogenesis.

Both Gas6 as well as protein S have been implicated in SVZ stem cell regulation (Gely-Pernot et al., 2012). Both Gas6 and protein S as well as the TAM receptor family are expressed in SVZ. Gas6 -/- knockout mice show reduced SVZ and OB cell proliferation while protein S neutralization increases SVZ proliferation. Other studies have found a role for protein S in maintaining stem-cell quiescence and promoting differentiation towards a neuronal fate (Zelentsova et al., 2017).

Studies investigating the role of the Gas6-Axl axis in angiogenesis have shown varying results. One study (Gallicchio et al., 2005) found that Gas 6 did not promote EC chemotaxis but inhibited VEGF-A-dependent chemotaxis as well as tube formation. Gas 6 did not promote vascularization in the chorio-allantoic membrane (CAM) assay but inhibited VEGF-A dependent vascularization. The authors find that Gas6 inhibits VEGF-A mediated autophosphorylation of VEGFR-2, likely mediated by SH2 domain-containing tyrosine phosphatase 2 (SHP-2). In contrast, another study (Holland et al., 2005) found that Axl knockdown impairs human umbilical vein

endothelial cell (HUVEC) proliferation, migration as well as tube formation in vitro. The authors also found Axl necessary for the formation of functional blood vessels in a mouse model of angiogenesis. The authors hypothesize that Axl silencing may inhibit Gas6-mediated VEGFR2 inhibition. A later study (Ruan and Kazlauskas, 2012) found Axl to be necessary for angiogenesis and VEGF-A dependent activation of VEGFR2 to promote ligand-independent autophosphorylation of Axl.

In a murine model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), intracerebroventricular delivery of GAS6 reduced axonal damage, demyelination, Iba1^{+} microglia and improved the clinical course (Gruber et al., 2014). In contrast, loss of Gas6 resulted in increased inflammation, axonal damage, demyelination and impaired recovery.

Gas6 expression was found to be decreased 24 hrs after MCAo in rats (Wu et al., 2018). In the same study, the authors further describe reduced infarct volume as well as higher neurological function scores after treatment with rGas6, dependent upon inhibition of proinflammatory signaling.
In a mouse model of intracranial hemorrhage, intranasal delivery of Gas6 was found to reduce brain edema and improve behavioral outcomes, dependent upon increased expression of suppressor of cytokine signaling 1 and 3 (Tong et al., 2017).
A recent study (Guo et al., 2021) found that in patients with acute ischemic stroke undergoing intravenous thrombolysis, lower levels of serum Axl are associated with higher rates of hemorrhagic transformation. In a set of rodent studies, the authors also found that treatment with rGAS6 resulted in less BBB disruption and hemorrhagic transformation after MCAo with reduced infarction volumes and less pronounced neurological deficits.

1.11 Study design and specific aims

We hypothesized that candidate genes dysregulated in stroke-responsive neuroblasts are involved in post-stroke angiogenesis and neurogenesis. To test this hypothesis, we performed mechanistic gain- and loss-of-function studies in two widely established

models of ischemic stroke: middle cerebral artery occlusion and photothrombosis (PT). In our gain-of-function studies, we employed either lentivirus mediated gene overexpression or sustained protein release from a hyaluronan based hydrogel sitting in the infarct cavity. Loss-of-function was achieved through lentivirus delivered microRNA mediated gene knockdown. We used different thymidine analogues to track cell proliferation after stroke. We looked at both short (14d) as well as long-term (42d) outcomes to assess both immediate as well as prolonged effects. More specifically, we hypothesized that:

1. Candidate gene overexpression or delivery will enhance post-stroke endothelial cell proliferation and increase overall vessel density in peri-infarct tissue short- and long-term.
2. Knockdown of candidate genes will impair post-stroke endothelial cell proliferation and decrease overall vessel density in peri-infarct tissue short- and long-term.
3. Candidate gene overexpression will enhance post-stroke neurogenesis, based on $NeuN^{+}EdU^{+}$ cells in peri-infarct tissue after long-term survival.
4. Knockdown of candidate genes will impair post-stroke neurogenesis, as assessed by $NeuN^{+}EdU^{+}$ cells in peri-infarct tissue after long-term survival.

2 Materials and Methods

2.1 Mice

All studies were performed on young adult (2-4 months old) C57BL/6 mice obtained from Jackson Laboratories, housed under 12:12 hours light:dark cycles, at 4 mice per cage with food and water provided ad libitum. Experiments were performed in accordance with the National Institutes of Health Animal Protection Guidelines and approved by the University of California, Los Angeles Animal Research Committee.

2.2 Lentiviral injections and photothrombotic model of ischemic stroke

For the gain-of-function studies, EF1alpha-driven lentiviral constructs expressing mouse CCL9, WNT7A, GAS6 or PTN were used. For the loss-of-function studies, EF1alpha-driven constructs expressing microRNA sequences designed using the Block-iT system (Thermo Fisher) targeting mouse CCL9, WNT7A, GAS6 or PTN were used. Two viral injections of 1.5 µl were performed (AP 0, ML 0.5 and 2.5, DV 0.7mm) using a 33G syringe (Hamilton Company) at 200 nl/min using a small volume syringe pump (Chemyx) attached to a stereotaxic frame (Figure 2-1).

14 days after viral injections, a focal cortical stroke was generated in young adult (2-4 months old) C57BL/6 mice using photothrombosis as described previously (Clarkson et al., 2010). In brief, animals were anesthetized using 5% isoflurane in 100% O_2 and maintained at 1.5 – 2% isoflurane throughout the procedure. Body temperature was maintained at 37.0°C ± 0.5 °C using a heating pad. Mice were placed in a stereotactic apparatus, the skull exposed through a midline incision and the surface cleared of connective tissue and dried. Rose Bengal at a concentration of 10mg/ml in saline was administered intraperitoneally. After 5 minutes, a cold light source (KL1500 LCD, Carl Zeiss) attached to a 40x objective was positioned above the forelimb motor cortex (coordinates AP 0.0mm, ML 1.5) and the brain illuminated through the intact skull for 18 min. After illumination, the wound was closed using tissue adhesive and the

animal returned to its cage for recovery (Figure 2-2). This approach generates a well-defined ischemic lesion in the forelimb region of the motor cortex (Figure 2-3). Sample size was 4-7 per group.

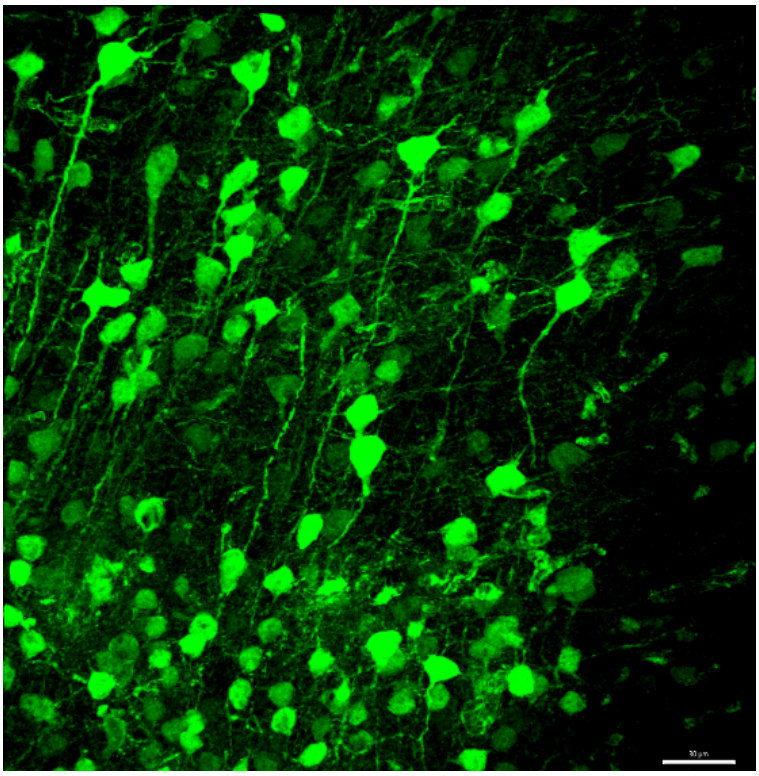

Figure 2-1 Lentiviral transduction of neurons in peri-infarct cortex
Representative confocal micrograph showing neuronal expression of reporter gene (eGFP) at the time of analysis. Scale bar: 30 μm.

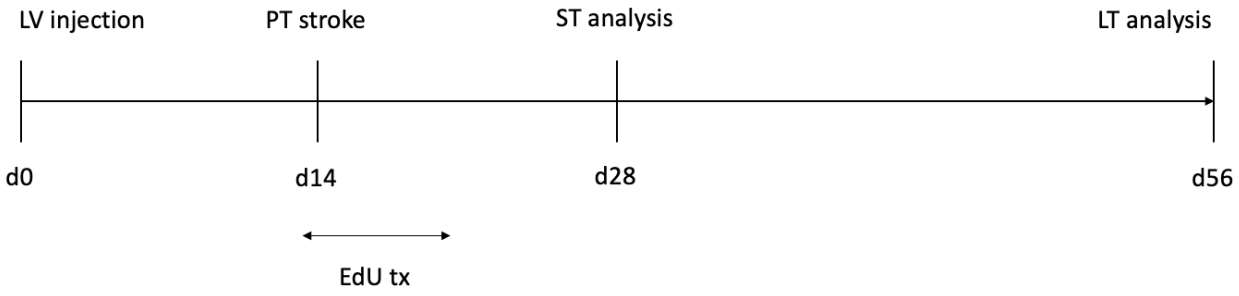

Figure 2-2 Experimental time course (virus)
Brief overview of time course in lentiviral studies. Lentiviral injection is performed on day 0, photothrombotic stroke generated on day 14. Starting 24 hrs prior to stroke

and continuing for 7 days, mice receive EdU. Mice are sacrificed for analysis on day 28 (short-term) or 56 (long-term) for tissue analysis.

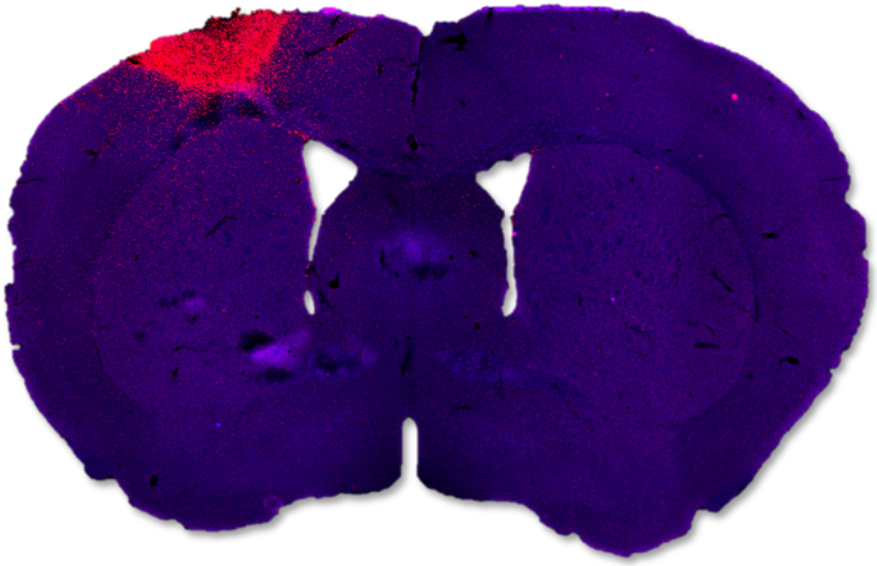

Figure 2-3 Model of photothrombotic stroke
Low magnification micrograph showing typical infarct size (red) in our model of ischemic stroke targeting forelimb motor cortex.

2.3 Middle cerebral artery occlusion model and hydrogel implantation

For hydrogel studies, young adult (2-4 months old) C57BL/6 mice underwent permanent distal middle cerebral artery occlusion as well as permanent occlusion of the ipsilateral common carotid artery. The procedure was performed under isoflurane anesthesia and body temperature maintained at 37.0°C ± 0.5 °C using a heating pad. In brief, an incision was made between the left eye and ear and the temporal muscle bisected. The middle cerebral artery was visualized through the intact skull and a craniotomy performed above the site of occlusion using a small surgical drill. The middle cerebral artery was thermocoagulated at the distal trunk proximal to the site of bifurcation into the frontal and posterior MCA branches. The temporal muscle was put back in place and the skin closed. The animal is then repositioned to expose the ventral surface of the neck, a midline incision made and the left common carotid artery visualized and separated from the vagus nerve. The CCA is thermocoagulated

proximal to its bifurcation and wounds are closed. This results in an infarct in barrel field somatosensory cortex (Figure 2-4). At three days post-stroke, a hyaluronan/heparin sulfate proteoglycan biopolymer hydrogel impregnated with either PBS or CCL9 protein (at a concentration of 600 ul/ml) was injected at two locations directly into the infarct cavity: 1. AP 0.7, ML 3.00, DV 1.2mm 2. AP -0.46, ML 3.25 and DV 0.85 mm). A volume of 6 µl was injected per site at a rate of 500 nl / minute with a 2 min delay using a small volume syringe pump (Chemyx) attached to a stereotaxic frame (Figure 2-5).

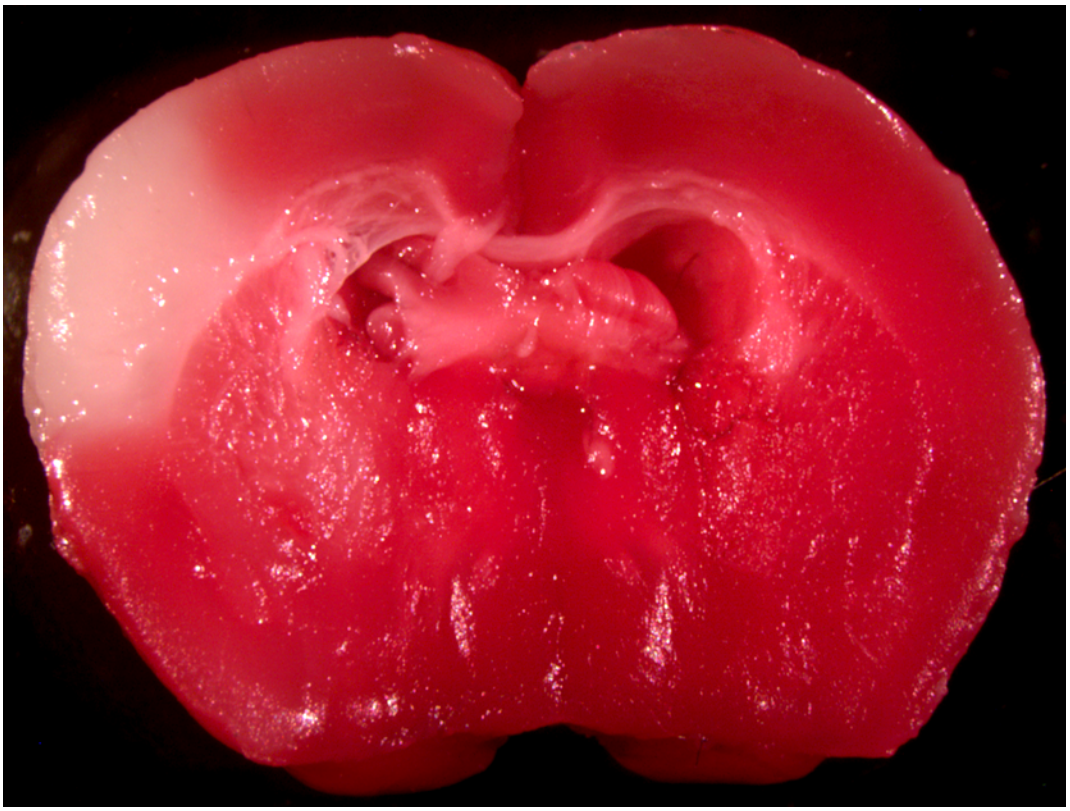

Figure 2-4 MCAo model of ischemic stroke
Low-magnification picture showing brain section after 2,3,5-Triphenyltetrazolium chloride (TTC) treatment, 24 hours after MCAo. Infarct is visible in white.

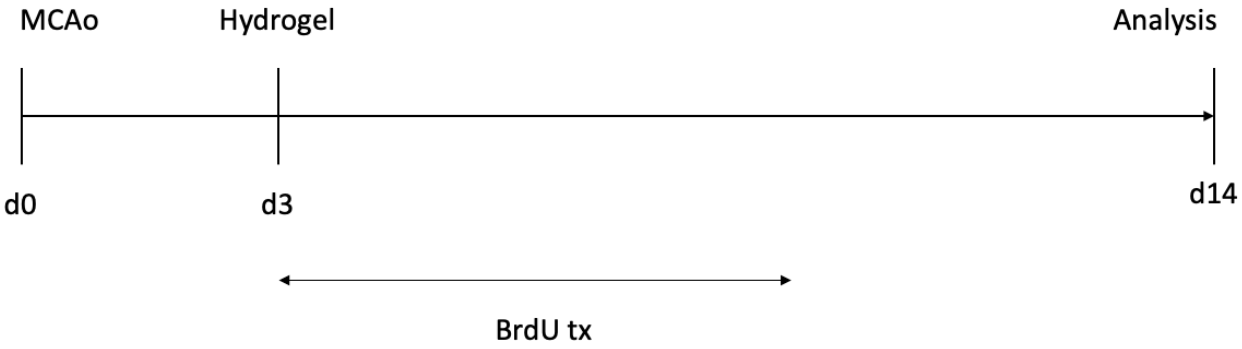

Figure 2-5 Experimental time course (hydrogel)
Brief overview of time course in hydrogel studies. MCAo was performed on day 0, hydrogel implanted on day 3 and mice sacrificed for tissue analysis on day 14. BrdU was injected bi-daily from days three to nine post-stroke.

2.4 Administration of thymidine analogues

To track cell proliferation, mice were given 5-Ethyl-2′-deoxyuridine (EdU, Carbosynth, NE08701), a thymidine analogue, from day 13 to day 20 (i.e. starting 24 hours before stroke and continuing for seven consecutive days post-stroke) at a concentration of 200 µg/ml in drinking water with recommended dose cherry-flavored TMS antibiotic. Water was changed every 48 hours. To develop EdU, a protocol modified from the Click-iT EdU Imaging Kit (Thermo Fisher) was used. Briefly, during immunohistochemistry, tissue was incubated in 100 mM tris-buffered saline (TBS) pH 7.6 with 4 mM CuSo4, 100 mM sodium ascorbate and a fluorophore-azide at a concentration of 2µM (i.e. Sulfo-Cyanine3 azide, Lumiprobe D1330) for 30 minutes at room temperature.

For hydrogel studies, mice received bi-daily intraperitoneal injections of the thymidine analogue BrdU from days three to nine after stroke. For histological visualization of BrdU, sections were pretreated with 2N HCl for 30min at 37°C and neutralized with sodium tetraborate buffer, pH 8.5 at room temperature before incubation in primary antibody (Tsai et al., 2006).

2.5 Tissue processing

Mice were sacrificed either 14 or 42 days after stroke and transcardially perfused with periodate-lysine-paraformaldehyde fixative (containing 2% paraformaldehyde) as previously described (McLean and Nakane, 1974). Brain tissue was cryoprotected in 30% sucrose, flashfrozen and sectioned using a cryostat (Leica Biosystems, CM0530) at a thickness of 40 µm per section. For further analysis, serial sections through the infarct, 160 µm apart, were selected. For immunohistochemistry, sections were rinsed in 0.02M KPBS for 3x 5 minutes, blocked in 0.02M KPBS, 5% normal donkey serum (NDS) and 0.3% Triton-X for 30 to 60 min and incubated in 0.02M KPBS, 2% NDS, 0.1% Triton-X and primary antibodies overnight. The following day, sections were again rinsed in 0.02M KPBS 3x5 minutes, incubated in 0.02M KPBS, 2% NDS, 0.1% Triton-X and secondary antibodies for 60 minutes, rinsed in 0.02M KPBS 3x5 minutes, mounted on slides, air-dried, dehydrated in ascending ethanol washes and washed in xylenes (2x 1 minute) prior to application of DPX mounting medium and coverslipping. Following primary antibodies were used: rabbit anti-NeuN (Abcam), rat anti-CD31 (BD Biosciences). Imaging was performed using a confocal laser scanning microscope (Nikon) at 40x magnification, two fields of view medial to the infarct per section were used for analysis.

Tissue processing was performed in an analogous fashion for the hydrogel studies. As a fixative, 4% PFA was used. Endothelial cells were visualized by using primary antibodies against Glucose Transporter 1 (rabbit anti-GLUT1, Abcam).

2.6 Imaging

Image analysis was performed using Imaris (Bitplane). Z-Stacks were converted from .nd2 to .ims prior to analysis. Image analysis was performed in an automated fashion using a Matlab script and the Imaris extension EasyXT. In brief, all images underwent baseline subtraction and gamma correction prior to analysis. To identify $CD31^+/EdU^+$ cells, a 3D surface object was generated using the CD31 channel as a source. In a second step, a mask was generated for the CD31 surface object, setting all voxels

outside the surface to 0. Another surface object was then created using the EdU channel as a source and filtered based on overlap with the previously generated CD31 mask so that at least 90% of the voxels overlapped. Identification of $NeuN^+/EdU^+$ cells was performed in a similar fashion. Vessel volume was estimated based on the properties of the generated surface objects.

For hydrogel studies, double-positive cells ($BrdU^+/GLUT1^+$) were counted manually with the investigator blinded to treatment conditions. 6 serial sections through the infarct were used per animal and five 60x z-stacks acquired per section, 2 medial and 2 lateral to the infarct as well as one image in subcortical white matter.

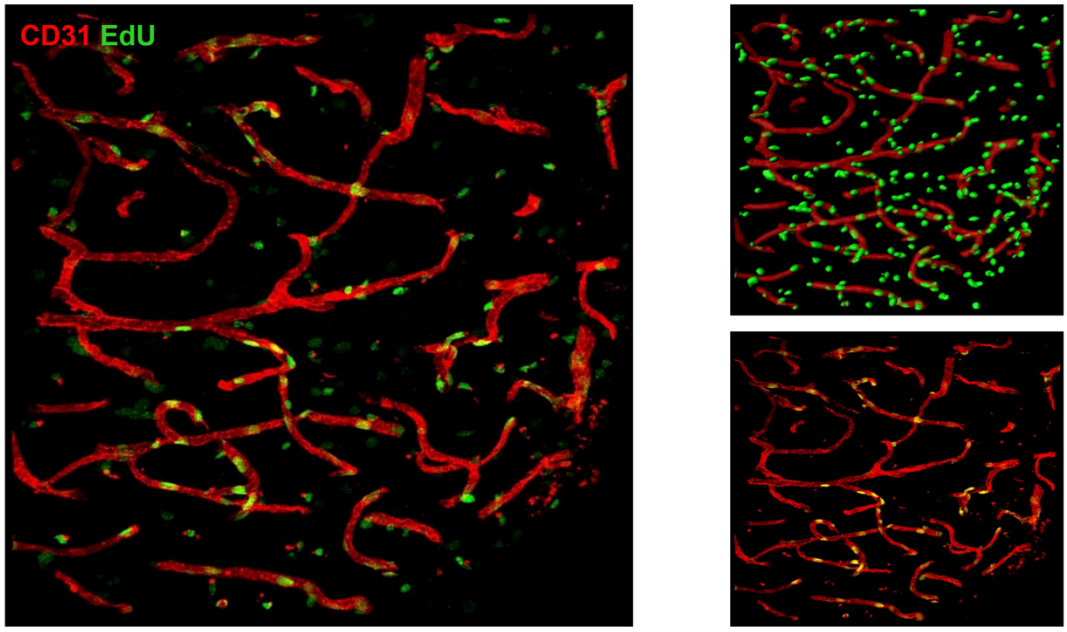

Figure 2-6 Vessel segmentation approach
Pictures showing z-stack images of CD31 and EdU with all cells prior to segmentation (left), during segmentation in Imaris (top right) and with only double-positive cells at 40x magnification (bottom right).

2.7 Infarct volume analysis

To estimate the dimensions of the infarct, serial sections, 160 μm apart, through the entire infarct were selected from the angiogenesis studies. 4x overview images were captured and the area of infarction measured using ImageJ. Total infarct volume was calculated by multiplying measured areas and intersection distances.

2.8 Statistical Analysis

Sample sizes for experiments are based on similar previously published studies using a significance level of a = 0.05 with 80% power to detect differences. Data are mean ± SD. Statistical testing was either ordinary one-way ANOVA followed by Dunnett's multiple comparisons test or unpaired two-tailed Student's t-test. All data available to the author at the time of writing was included in this thesis.

3 Results

3.1 Ischemic injury results in post-stroke neurovascular remodeling

Based on previously published results, we hypothesized that ischemic injury by itself would induce post-ischemic neurovascular remodeling processes like angiogenesis and neurogenesis (Ohab et al., 2006). Consistent with these findings, mice receiving lentiviral injections with a control sequence, receiving a photothrombotic stroke at day 14, showed significant increases in endothelial cell proliferation, inmigration of neuroblasts as well as close association of blood vessels and neuroblasts in the peri-infarct region (Figure 3-1).

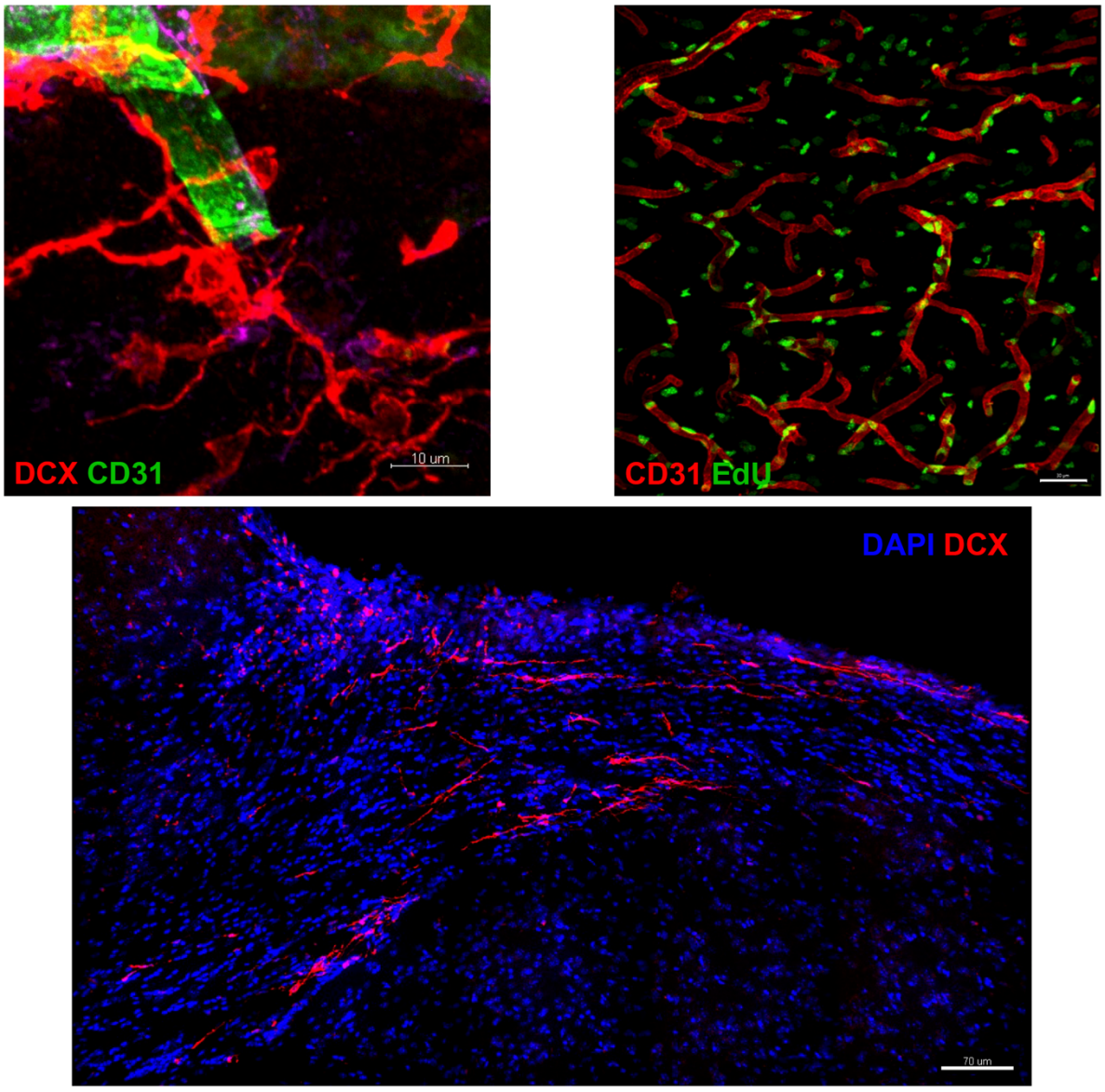

Figure 3-1 Neurovascular remodeling after stroke
Bottom: Stroke resulted in the in-migration of large numbers of neuroblasts (red) to the site of ischemic injury. Top-right: In peri-infarct tissue, we found high numbers of angiogenic blood vessels (CD31 in red, EdU in green). Top-left: After stroke, in-migrating neuroblasts (red) closely associated with the local vasculature (green).

3.2 CCL9 and WNT7A promote endothelial cell proliferation after stroke

We hypothesized that candidate genes dysregulated in stroke-response neuroblasts have a role in post-stroke neurovascular remodeling. More specifically, the aim of the current study was to assess whether general overexpression of candidate genes in peri-infarct cortex can promote angiogenesis. To this end, young adult C57BL/6 mice received lentiviral injections with a construct driving expression of candidate genes under the ubiquitous EF1a promoter, with either eGFP or TagBFP2 as a reporter gene. In a set of time course experiments we established robust reporter gene expression by day 14. Mice received a photothrombotic stroke in the forelimb motor cortex at day 14 and were followed for an additional 6 weeks. Mice received EdU, a thymidine analogue, in drinking water from day 13 to day 20 to label dividing cells. Mice were sacrificed 6 weeks post-stroke, tissue fixed, sectioned and stained for CD31 and EdU. A set of four 40x confocal micrographs were generated for analysis and the number of double-positive $CD31^+/EdU^+$ cells counted using Bitplane Imaris. We found that stroke by itself results in a significant increase in endothelial cell proliferation in peri-infarct tissue (data not shown). Among the candidate genes tested, WNT7a (161.5 ± 17.60 versus 578.0 ± 328.9 cells / mm^2, $p = 0.0244$) and CCL9 (161.5 ± 17.60 versus 1013 ± 120.6 cells / mm^2, $p < 0.0001$) overexpression resulted in a significant increase in post-stroke endothelial cell proliferation compared to controls (Figure 3-2, Figure 3-3) while overexpression of GAS6 (161.5 ± 17.60 vs 418.6 ± 289.4 cells / mm^2, $p = 0.2233$) and PTN (161.5 ± 17.60 vs 314.3 ± 129.9 cells / mm^2, $p = 0.5816$) did not.

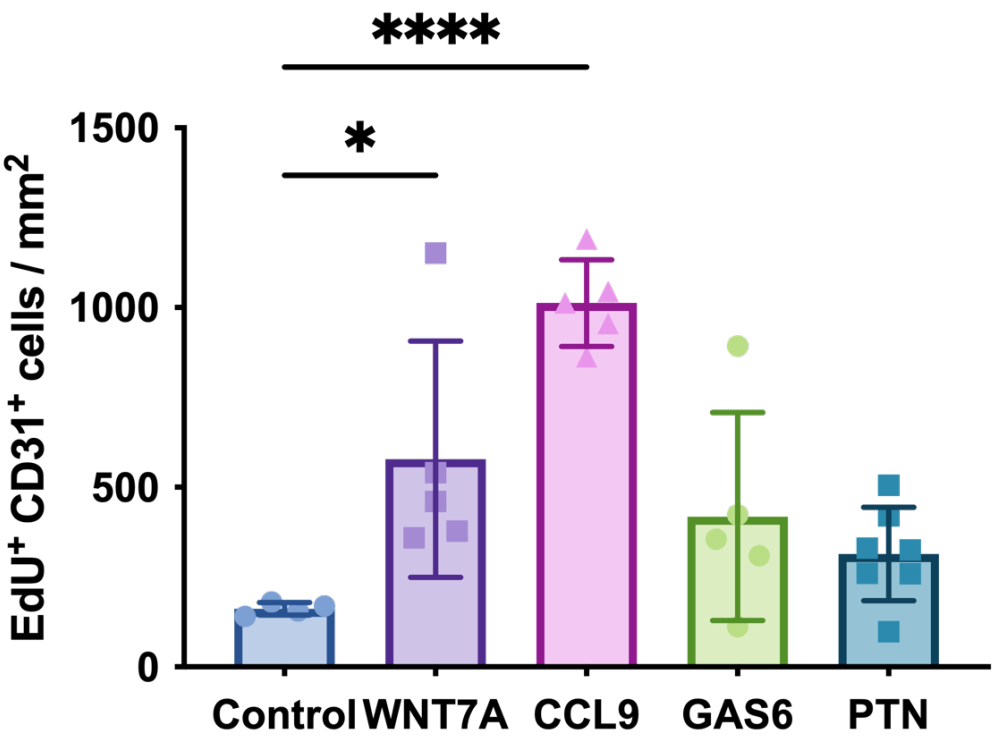

Figure 3-2 Effect of candidate gene overexpression on EC proliferation
Number of EdU^+ $CD31^+$ cells per mm^2 in peri-infarct cortex 8 weeks after lentiviral overexpression of candidate genes, 6 weeks post-stroke. Both WNT7A as well as CCL9 overexpression significantly increased the number of double-positive cells. Data are mean ± SD. Statistical testing was ordinary one-way ANOVA followed by Dunnett's multiple comparisons test. **** = $p < 0.0001$, * = $p < 0.05$, n = 4-7 per group.

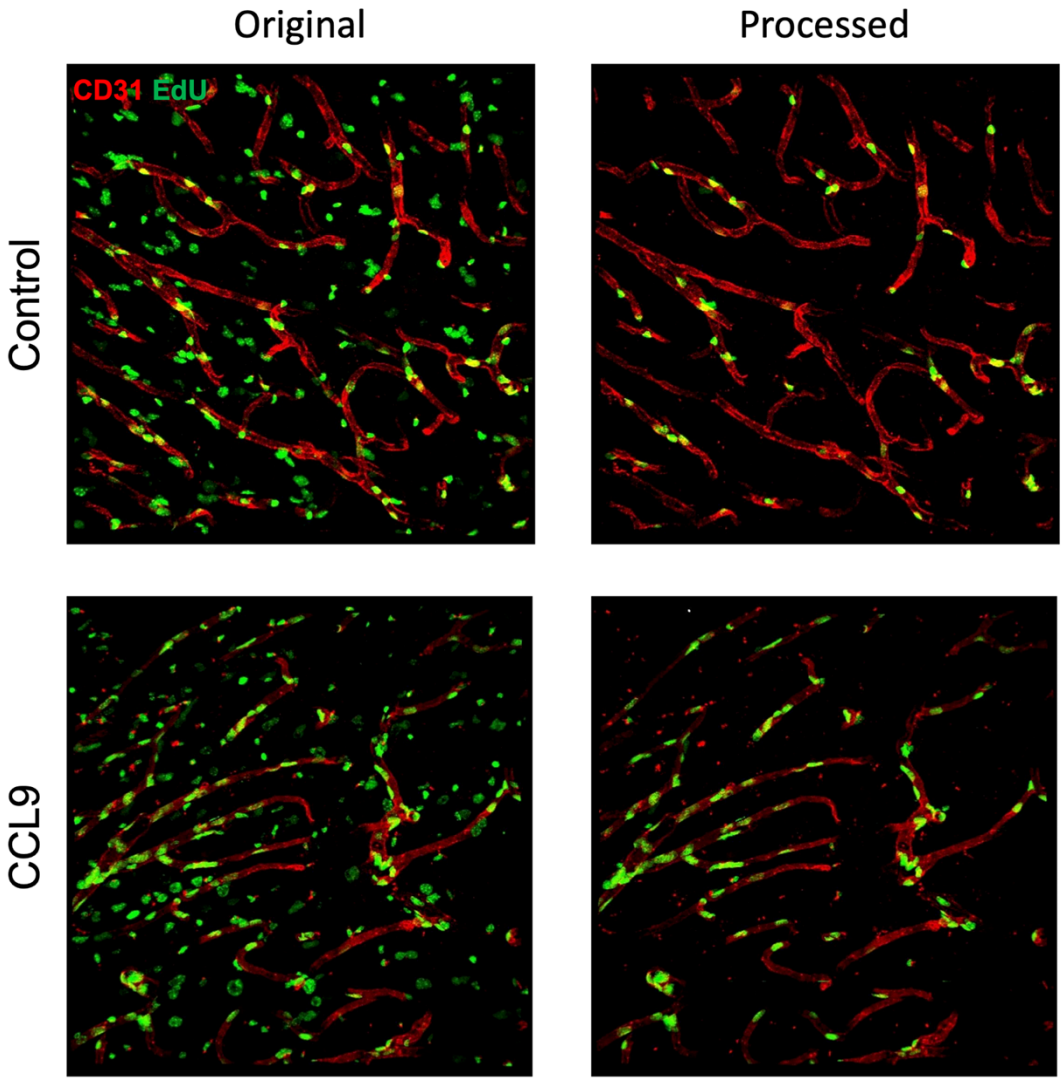

Figure 3-3 CCL9 overexpression increases EC proliferation after stroke
CCL9 overexpression increased endothelial cell proliferation in peri-infarct cortex. 40x confocal micrographs showing EdU in green, CD31 in red. Representative images for each condition are shown with all nuclei visible (left) or only double-positive nuclei (right).

3.3 Loss of CCL9 attenuates EC proliferation after stroke

At this point we had established that overexpression of CCL9 and WNT7A can promote endothelial cell proliferation, a key step in angiogenesis, in our model of ischemic stroke. However, this does not answer the question, whether these genes are necessary for the angiogenic response seen after ischemia. We hypothesized that loss of candidate genes that are upregulated after stroke in neuroblasts will

impair endothelial cell proliferation and thereby at least partially impair the angiogenic response. To test this hypothesis, we used lentiviral constructs expressing microRNA sequences to achieve knockdown of candidate genes. We found robust lentiviral expression by day 14 and analogous to our gain-of-function studies, a stroke was generated at this time, EdU given in drinking water from day 13 to 20 and animals followed for 6 weeks before sacrifice, tissue processing, imaging and analysis. We found that knockdown of CCL9 resulted in a significant reduction of endothelial cell proliferation after stroke (678.8 ± 268.0 vs 166.0 ± 50.57, p = 0.002) while knockdown of PTN did not (678.8 ± 268.0 vs 937.0 ± 179.6, p = 0.1166), see Figure 3-4 and Figure 3-5 for comparison.

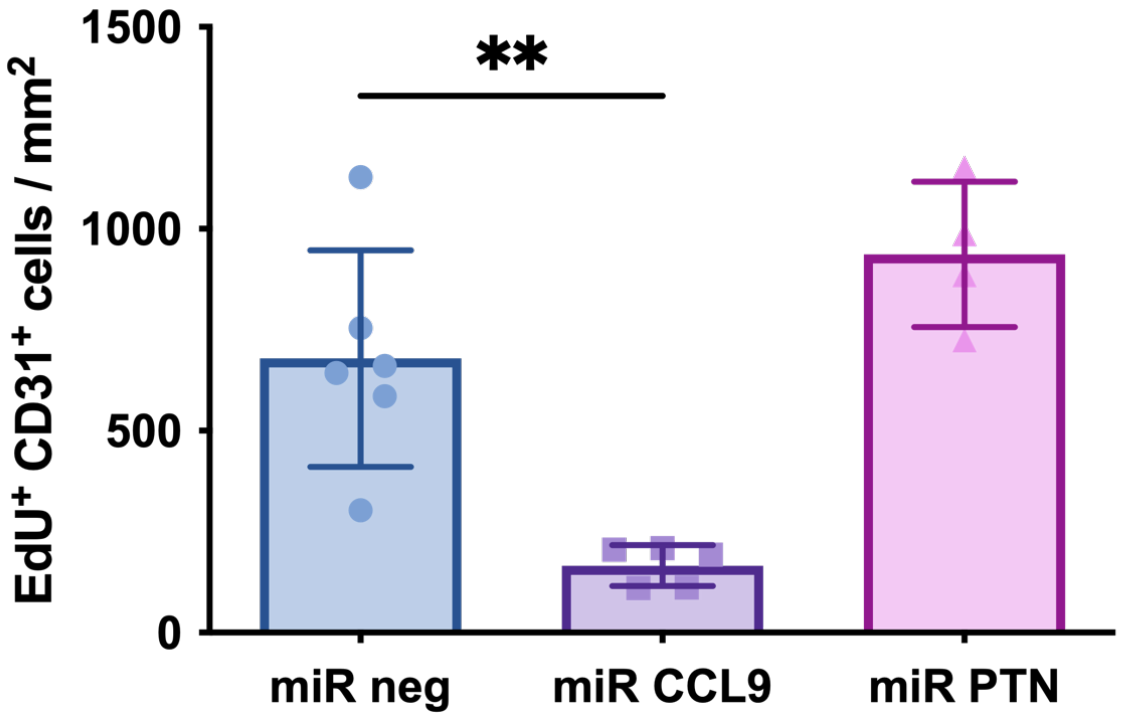

Figure 3-4 Loss of CCL9 reduces EC proliferation after stroke
Number of EdU^+ $CD31^+$ cells / mm^2 in peri-infarct cortex 8 weeks after lentiviral microRNA mediated knockdown of candidate genes, 6 weeks post-stroke. CCL9 knockdown significantly decreased the number of double-positive cells. Data are mean ± SD. Statistical testing was ordinary one-way ANOVA followed by Dunnett's multiple comparisons test. ** = p <0.01, n = 4-6 per group.

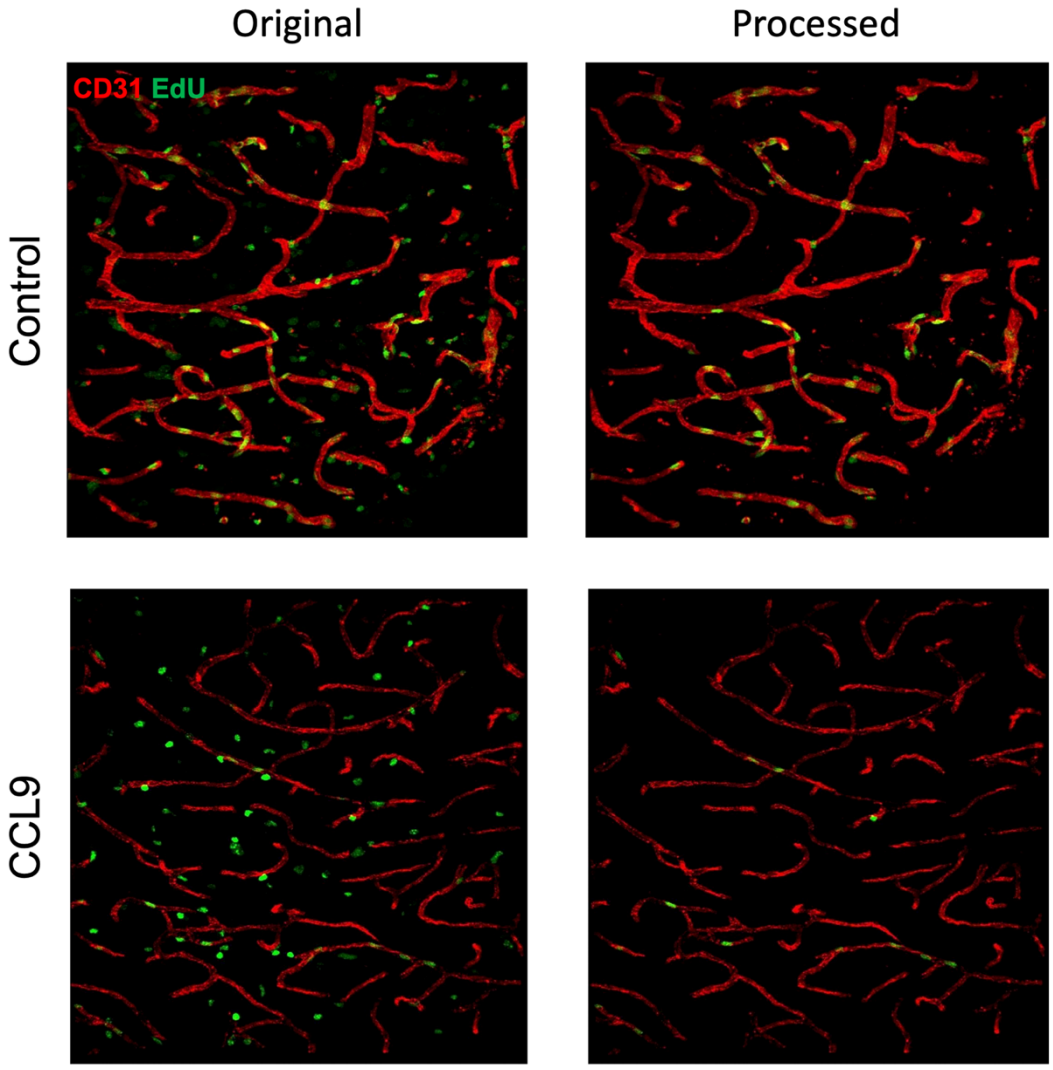

Figure 3-5 Loss of CCL9 impairs angiogenic response after stroke
CCL9 knockdown decreases endothelial cell proliferation in peri-infarct cortex. 40x confocal micrographs showing EdU in green, CD31 in red. Representative images for each condition are shown with all nuclei visible (left) or only double-positive nuclei (right) visible.

3.4 CCL9 modulation alters peri-infarct vessel density

With initial results indicating that candidate genes can modulate early steps of angiogenesis like endothelial cell proliferation we next tested whether this translates to macroscopic changes, i.e. vessel density in peri-infarct tissue. Only overexpression of CCL9 resulted in a significant increase in peri-infarct vessel density (199539 ±

67065 vs 519826 ± 85942 μm^3 / section, p = 0.01109) while overexpression of WNT7A (199539 ± 67065 vs 436590 ±209668 μm^3 / section, p = 0.0695), GAS6 (199539 ± 67065 vs 386818 ± 210244 μm^3 / section, p = 0.1835) and PTN (199539 ± 67065 vs 208119 ± 81858 μm^3 / section p = 0.9999) did not (Figure 3-6).

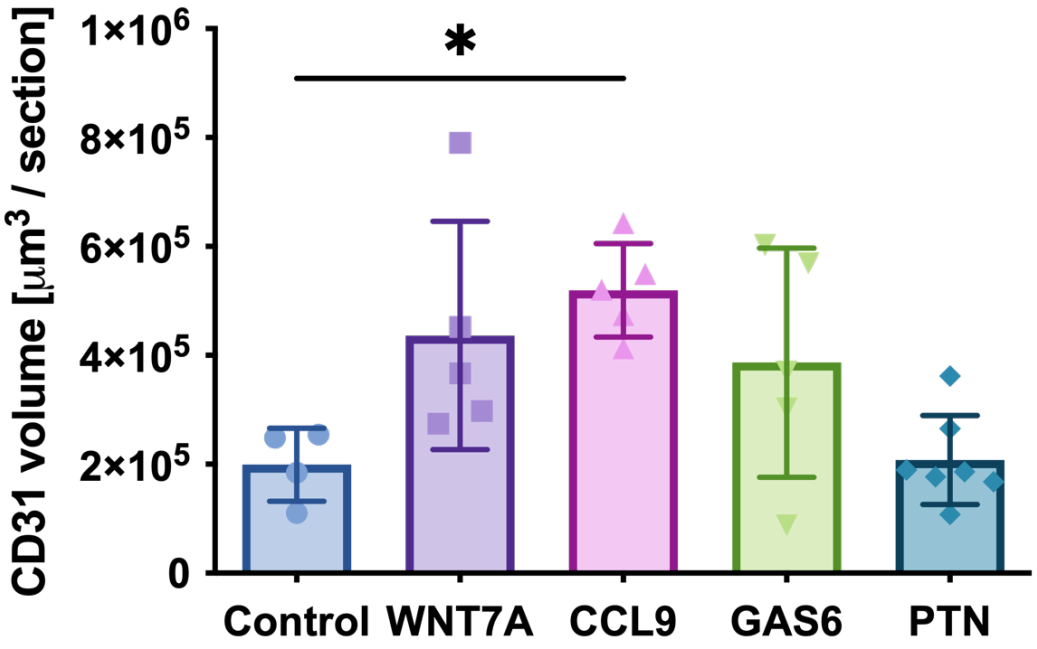

Figure 3-6 Overexpression of CCL9 increases vessel density post-stroke
Volume of CD31 in µm3 per section in peri-infarct cortex 8 weeks after lentiviral mediated overexpression of candidate genes, 6 weeks post-stroke. CCL9 overexpression significantly increased $CD31^+$ volume. Data are mean ± SD. Statistical testing was ordinary one-way ANOVA followed by Dunnett's multiple comparisons test. * = $p < 0.05$, n = 4-7 per group.

Knockdown of CCL9 resulted in a significant decrease in peri-infarct vessel density (439373 ± 98151 vs 135195 ± 33994 μm^3 / section, p = 0.0019) while PTN knockdown resulted in increased $CD31^+$ volume (439373 ± 98151 vs 661028 ± 191626 μm^3 / section, p = 0.0228) (Figure 3-7).

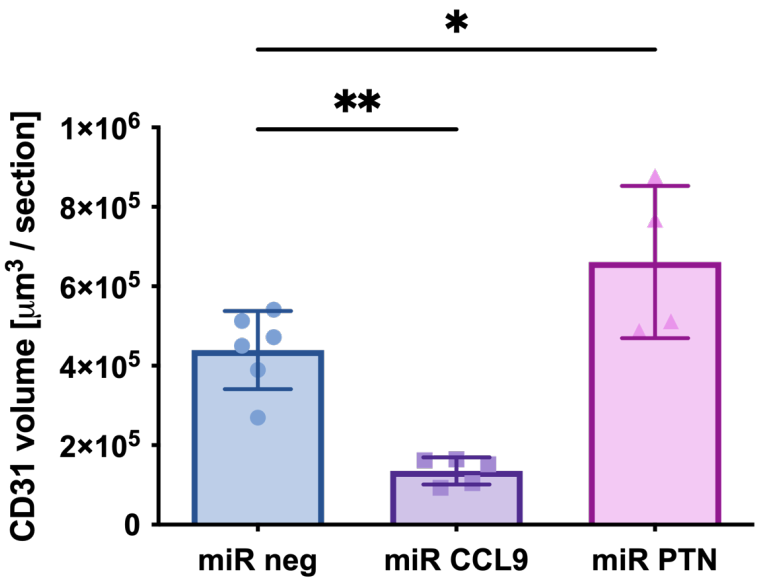

Figure 3-7 Loss of candidate genes modules vessel density after stroke
Volume of CD31 in µm^3 per section in peri-infarct cortex 8 weeks after lentiviral microRNA mediated knockdown of candidate genes, 6 weeks post-stroke. CCL9 knockdown significantly decreased CD31 volume, while PTN knockdown increased CD31 volume. Data are mean ± SD. Statistical testing was ordinary one-way ANOVA followed by Dunnett's multiple comparisons test. * = $p < 0.05$, ** = $p < 0.01$, n = 4-6 per group.

3.5 Loss of candidate genes impairs post-stroke neurogenesis

As discussed earlier, stroke induces a neurogenic response with a small fraction of DCX$^+$ cells differentiating into mature neurons expressing the neuronal marker NeuN. To investigate whether candidate genes have a role in this endogenous neurogenic response, we employed lentiviral mircoRNA mediated knockdown of candidate genes with expression driven by the ubiquitous promotor ef1alpha. We found that knockdown of CCL9, WNT7A as well as PTN impaired the neurogenic response, based on the NeuN$^+$/EdU+ cell population at 6 weeks after stroke (15.29 ± 4.866 vs 1.187 ± 0.1266 cells/mm^2, p=0.0066 for CCL9, vs 4.742 ± 1.270 cells/mm^2, p=0.0233 for WNT7A and vs 3.559 ±1.130 cells/mm^2, p= 0.0157 for PTN).

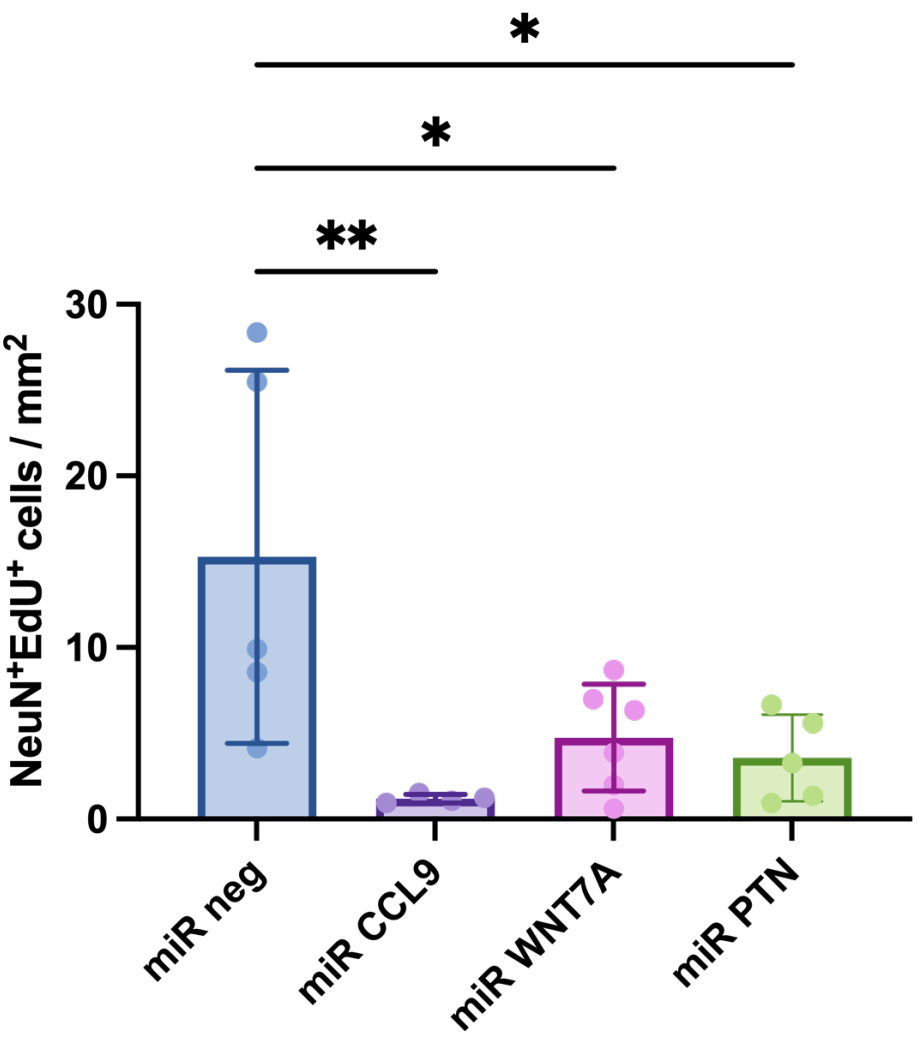

Figure 3-8 Loss of candidate genes impairs post-stroke neurogenesis
Number of $NeuN^+EdU^+$ cells per mm^2 in peri-infarct cortex 8 weeks after lentiviral microRNA mediated knockdown of candidate genes, 6 weeks post-stroke. Knockdown of CCL9, WNT7A as well as PTN reduced post-stroke neurogenesis. Data are mean ± SD. Statistical testing was ordinary one-way ANOVA followed by Dunnett's multiple comparisons test, n = 5-7 per group.

3.6 WNT7A and CCL9 promote post-stroke neurogenesis

To test whether candidate genes can enhance the endogenous neurogenic response, we performed lentiviral overexpression of candidate genes in peri-infarct tissue and assessed the number of newborn neurons, i.e., $NeuN^+EdU^+$ cells in peri-infarct cortex, 6 weeks after ischemia. We found WNT7A (1.672 ± 1.074 vs 5.137 ± 2.191 cells per mm^2, $p = 0.008$) as well as CCL9 (1.672 ± 1.074 vs 8.484 ±1.521 cells per mm^2, $p < 0.0001$) to significantly increase the number of newborn neurons while PTN did not (1.672 ± 1.074 vs 1.133 ±1.339 cells per mm^2, $p = 0.9153$) (Figure 3-9, Figure 3-10).

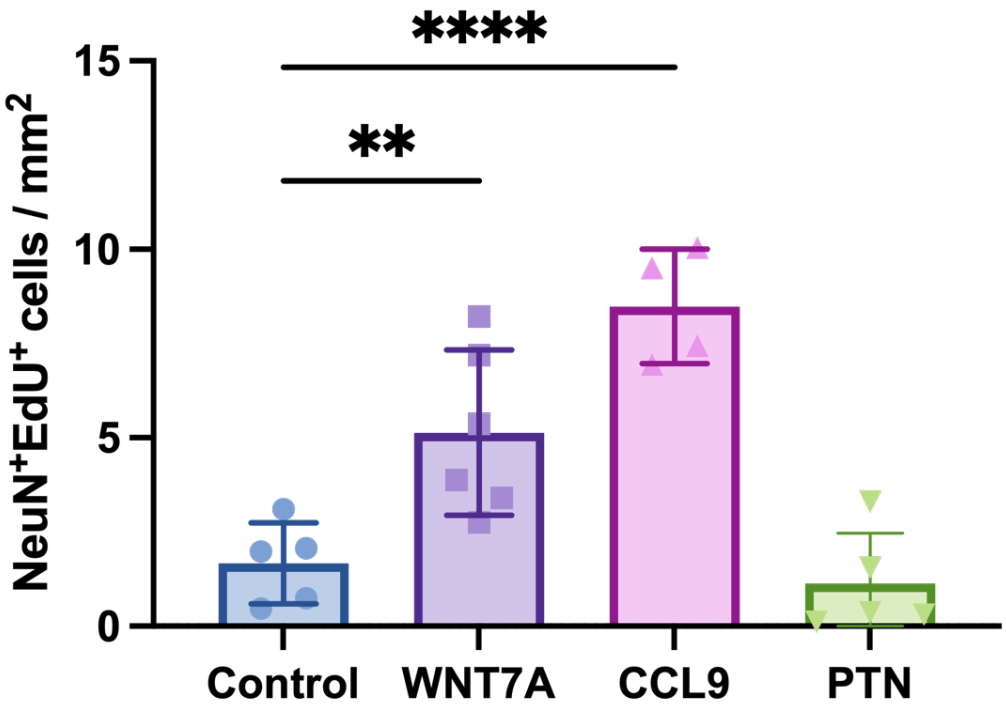

Figure 3-9 Overexpression of WNT7A and CCL9 promotes neurogenesis
Number of $NeuN^+EdU^+$ cells per mm^2 in peri-infarct cortex 8 weeks after lentiviral overexpression of candidate genes, 6 weeks post-stroke. Overexpression of WNT7A as well as CCL9 increased post-stroke neurogenesis. Data are mean ± SD. Statistical testing was ordinary one-way ANOVA followed by Dunnett's multiple comparisons test, n = 4-6 per group.

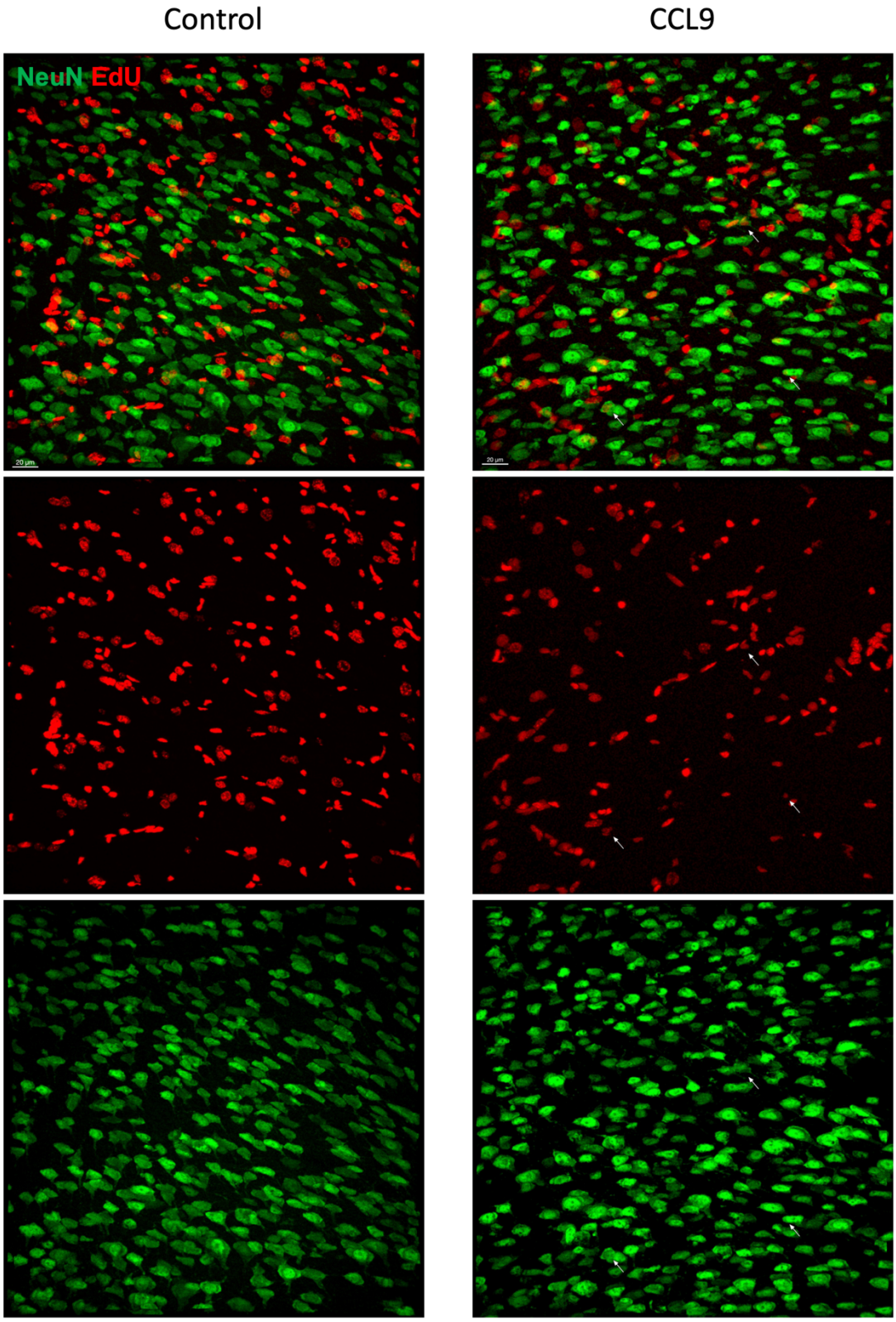
Control
CCL9
NeuN EdU
20 μm
20 μm

Figure 3-10 CCL9 overexpression promotes neurogenesis
CCL9 overexpression increases post-stroke neurogenesis. Image shows NeuN labeling mature neurons (in green) as well as EdU (in red) incorporated into dividing cells. Representative co-labeled newborn neurons highlighted (arrow).

3.7 CCL9 does not alter volume of infarction long-term

As stable expression or knockdown of CCL9 could affect infarct size both through initial neuroprotective as well as later regenerative processes, we estimated infarct size at 6 weeks after stroke but did not find any significant differences (Figure 3-11).

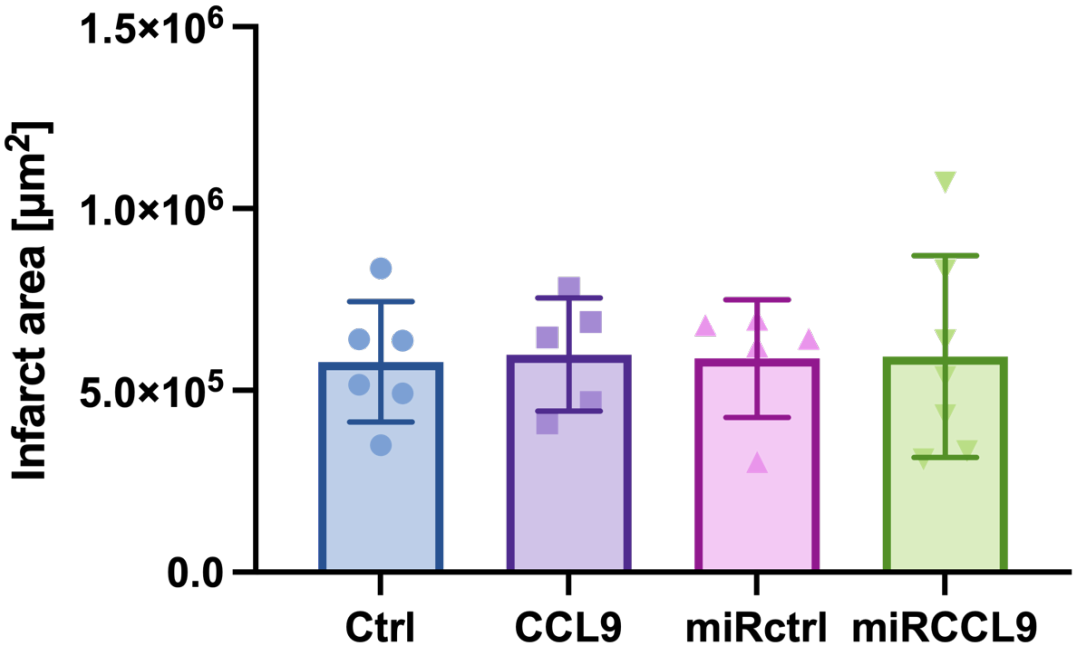

Figure 3-11 Effect of candidate genes on long-term infarct volume
Infarct size analysis. Region of infarction was identified at 4x magnification at 6 weeks post-stroke based on autofluorescence signal. No significant differences in infarct area were found between groups. Data are mean ± SD. Statistical testing was ordinary one-way ANOVA followed by Dunnett's multiple comparisons test, n = 5-7 per group.

3.8 Hydrogel delivery of CCL9 promotes angiogenesis

In a separate set of studies, we investigated whether implantation of a hyaluronan/heparin sulfate proteoglycan biopolymer hydrogel impregnated with CCL9 protein at a concentration of 200 µg/ml three days after stroke into the infarct core results in increased endothelial cell proliferation based on GLUT1/BrdU co-labeling.

We found that 14 days after stroke, CCL9 delivery through the hydrogel resulted in a significant increase in double positive cells compared to PBS controls (13.00 ± 4.967 vs 23.63 ± 7.596 cells, p=0.0309).

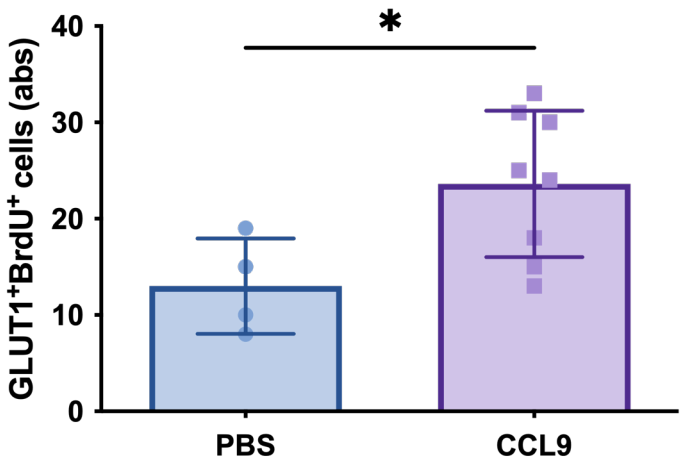

Figure 3-12 CCL9 delivery increases EC proliferation after stroke
Number of GLUT1$^+$BrdU$^+$ cells per animal in peri-infarct tissue 2 weeks after stroke with hydrogel delivery of CCL9 protein starting day three post-stroke. Data are mean ± SD. Statistical testing was unpaired two-tailed t test, n = 4-8 per group.

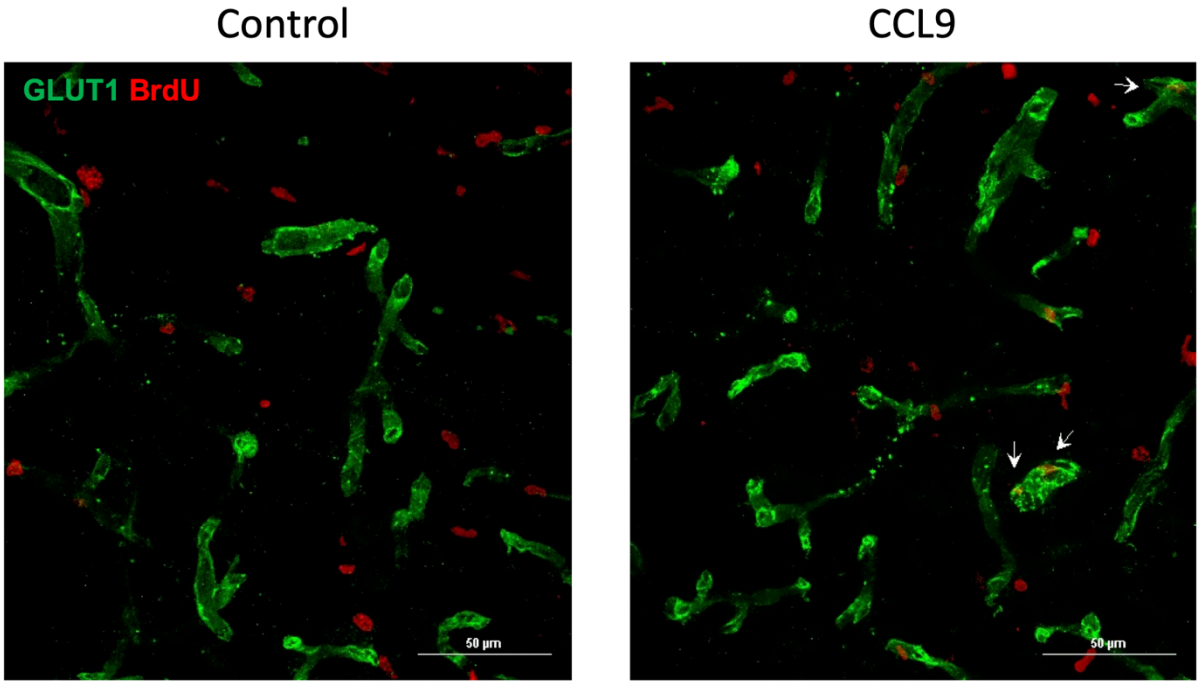

Figure 3-13 Hydrogel delivery of CCL9 promotes EC proliferation after stroke
Hydrogel delivery of CCL9 protein starting day 3 after MCAo resulted in increased numbers of GLUT1$^+$ (green) BrdU$^+$ (red) cells at 14 days post-stroke.

4 Discussion

Stroke is one of the leading causes of death and disability in the world yet remains without an effective treatment that promotes long-term functional recovery (Cramer, 2018; Feigin et al., 2021). Previous studies have demonstrated that stroke itself induces a wide range of plasticity mechanisms both in animal models as well as humans (Carmichael, 2016). Ischemic stroke has been shown to induce neural progenitor proliferation and migration of these cells towards sites of injury (Ohab et al., 2006). In peri-infarct cortex, these in-migrating neuroblasts have been shown to associate with the angiogenic vasculature in the neurovascular niche. Inhibition of post-stroke angiogenesis impairs this neurogenic response, establishing a causal link between angiogenesis and neurogenesis. Promotion of post-stroke angiogenesis as well as neurogenesis has been shown to improve functional recovery after stroke. The signaling systems involved in post-stroke neurovascular remodeling are just partially characterized.

To systemically investigate the signaling systems underlying angiogenic vessel – neuroblast communication, previous studies in the Carmichael laboratory, based on DNA microarray data, identified the neurovascular interactome. The aim of this study was to select a set of gene systems specifically dysregulated in stroke-response neuroblasts for further, mechanistic gain- and loss-of-function studies in two well-established models of ischemic stroke. Based on degree of dysregulation and novelty in post-ischemic brain repair, we selected WNT7A, PTN, GAS6 and CCL9 for further studies. We found these genes to be either downregulated (WNT7A, PTN) or upregulated (GAS6, CCL9) after ischemia in stroke-responsive neuroblasts.

To overexpress candidate genes, we used lentiviral constructs encoding candidate genes under the control of the ef1α promotor. Lentiviral injections were performed into cortical areas adjacent (medial and lateral) to the targeted site of ischemic injury on the left hemisphere. We found robust reporter expression at 2 weeks after lentivirus injection. At this point, we generated a photothrombotic ischemic lesion localized to forelimb motor cortex. Tissue analysis was performed after a short (2 weeks) or long

(6 weeks) survival period. Consistent with previous reports (Ohab et al., 2006), we found DCX^+ neuroblasts migrating towards the site of ischemic injury at 2 weeks after stroke even in the control conditions. In peri-infarct cortex, we found high numbers of proliferating (i.e., EdU^+CD31^+) endothelial cells. Looking at long-term effects, we found that overexpression of both WNT7A and CCL9 resulted in a significant increase in proliferating endothelial cells, while overexpression of GAS6 and PTN did not. In a second step, we looked at whether candidate gene overexpression increased vessel density in peri-infarct cortex. Among the genes tested, only CCL9 significantly increased vessel density in peri-infarct cortex at 6 weeks post-stroke.

To investigate whether candidate genes are involved in the endogenous post-stroke angiogenic response, we performed lentivirus-delivered microRNA-mediated ubiquitous knockdown of candidate genes. The data available at the time of writing is presented in this thesis (CCL9 and PTN). CCL9 knockdown significantly reduced post-stroke endothelial cell proliferation and vessel density in peri-infarct cortex. Loss of PTN did not result in significant changes in endothelial cell proliferation but did significantly increase vessel density at 6 weeks post-stroke.

In a separate set of experiments, we investigated whether delivery of CCL9 protein starting on day three post-stroke would promote angiogenesis in a MCAo model of ischemic stroke. To this end, we injected a hyaluronan-based hydrogel impregnated with CCL9 into the infarct cavity for sustained protein release over several weeks. We used BrdU to track cell proliferation and measured the number of proliferating endothelial cells ($GLUT1^+BrdU^+$) at 14 days after stroke. We found CCL9 protein to significantly increase endothelial cell proliferation.

Taken together, this study provides first evidence that CCL9 has a role in the post-ischemic angiogenic response and both lentiviral overexpression as well as hydrogel delivery can promote angiogenesis as tested in two well established models of ischemic stroke. Our findings regarding CCL9 are consistent with previous findings by other groups that have identified a role for CCL9 in non-CNS angiogenesis (Han et al., 2009; Kortlever et al., 2017) and add to this body of knowledge by providing

evidence for a role in CNS angiogenesis in the setting of ischemic stroke. Wnt7a did increase endothelial cell proliferation but not vascular density at 6 weeks after stroke. However, studies in the Carmichael laboratory found Wnt7a to increase GLUT1 expression (data not shown), consistent with the idea that Wnt7a may primarily be involved in BBB maturation (Daneman et al., 2009; Song et al., 2021). Both PTN and GAS6 have been implicated in non-CNS angiogenesis with ambiguous results (Dos Santos et al., 2014; Gallicchio et al., 2005; Holland et al., 2005; Papadimitriou et al., 2016; Ruan and Kazlauskas, 2012). However, to our knowledge, there is no evidence for a role in CNS angiogenesis and in our study, we did not find these genes to have a clear role in post-stroke angiogenesis.

As discussed earlier, stroke itself induces the birth of new neurons, a process termed neurogenesis. To test whether candidate genes are involved in post-stroke neurogenesis, we analyzed the number of $NeuN^{+}EdU^{+}$ cells (i.e., cells proliferating after the time of ischemic injury, expressing a marker for mature neurons) at 6 weeks post-stroke after lentiviral mircoRNA-mediated knockdown of candidate genes. We found that loss of CCL9, WNT7A and PTN significantly reduced post-stroke neurogenesis. In contrast, overexpression of only CCL9 and WNT7A increased the number of $NeuN^{+}EdU^{+}$ cells at 6 weeks after stroke.

To our knowledge, our study is the first to find a role for CCL9 in post-stroke neurogenesis. Wnt7a has been implicated in CNS neurogenesis (Qu et al., 2013), however, we are not aware of any studies providing evidence for a role in post-stroke neurogenesis. PTN has been shown to have a role in neuronal maturation and post-ischemic neuroprotection (Nikolakopoulou et al., 2019). Our study adds to this body of knowledge by providing evidence for a role in post-stroke neurogenesis.

As lesion size itself can impact the neurovascular response, we looked at whether infarct area was altered with overexpression or knockdown of CCL9 at 6 weeks post-stroke. We did not find any significant differences.

Our study has several limitations that should be addressed in future investigations. Our lentiviral as well as hydrogel delivery approaches are not neuroblast-specific, leaving unanswered how specifically targeting expression of these gene systems in neuroblasts modulates the neurovascular response after stroke. It remains unclear, whether the effects seen in our study are due to direct effects of the candidate gene systems on endothelial cells and neural progenitor cells or whether these are mediated by other gene systems and cell types (like microglia in the case of CCL9). While we provide evidence for angiogenesis, it remains unclear, whether some of the candidate genes may cause BBB disruption which would adversely affect outcome after stroke. Are these blood vessels functional, i.e., is there blood flow? While we measured the number of mature newborn neurons, future studies should investigate, whether these neurons are functional and integrated into the local circuitry. The image analysis approach in this study using Bitplane Imaris was conducted in an automated fashion with fixed settings using a large number of images. Variations in image quality may especially interfere with the segmentation of small structures like nuclear antigens, potentially resulting in false positives or negatives. While part of the angiogenesis experiments were replicated in experiments using manual quantification, this has not yet been performed for the parts of the study exploring post-stroke neurogenesis. And ultimately, while we provided evidence for a role of some of these candidate genes in neurovascular remodeling, future studies should address whether this affects functional recovery.

Taken together, in a set of mechanistic gain- and loss-of-function studies, we were able to identify novel regulators of post-stroke neurovascular remodeling. CCL9 generated the most robust and reproducible phenotype. Loss of CCL9 impaired post-stroke angiogenesis and neurogenesis. Overexpression of CCL9 enhanced both the angiogenic and neurogenic response. Finally, using a local drug delivery approach, we were able to enhance the pro-angiogenic response using CCL9 protein and reproduce our findings in a second model of ischemic stroke.

5 Abstract

Stroke is one of the leading causes of death and disability yet remains without an effective treatment. Stroke itself induces a wide range of repair mechanisms that allow for limited recovery, including angiogenesis and neurogenesis. Previous studies have demonstrated that neuroblasts migrate to sites of ischemic injury and preferentially associate with the angiogenic vasculature. The signaling systems underlying neuroblast-vessel communication are incompletely understood. A better understanding of these endogenous repair mechanisms may lead to novel therapeutic approaches. To this end, previous studies in the Carmichael laboratory, using genome-wide expression profiling studies, identified the post-stroke neurovascular interactome. In this study, we selected four genes dysregulated in stroke-responsive neuroblasts for mechanistic gain-and loss-of-function studies: CCL9, WNT7A, GAS6 and PTN. We hypothesized that candidate gene overexpression will enhance the angiogenic and neurogenic response, while gene knockdown will impair post-stroke neurogenesis and angiogenesis. To test this hypothesis, we employed lentiviral gene overexpression or miRNA mediated gene knockdown in a photothrombotic model of ischemic stroke and quantified the number of proliferating endothelial cells (ECs), peri-infarct vessel density and the number of newborn neurons. We found CCL9 and WNT7A overexpression to significantly increase EC proliferation after stroke, while knockdown of CCL9 reduced the number of proliferating ECs. Overexpression of CCL9 increased peri-infarct vessel density, while loss of CCL9 lead to a significant reduction in vessel density. Loss of PTN increased vessel density without showing any effects on EC proliferation. Both WNT7A as well as CCL9 overexpression increased post-stroke neurogenesis ($NeuN^+/EdU^+$ cells) while loss of CCL9, WNT7A and PTN impaired the neurogenic response. In a second study, we delivered CCL9 protein through a hydrogel sitting in the infarct cavity in a MCAo model of ischemic stroke and also found EC proliferation to be enhanced. Taken together, we identified several novel regulators of post-stroke neurovascular remodeling. We found CCL9 to produce the most robust effects on neurovascular remodeling, which we were able to reproduce in two different models of ischemic stroke with two different delivery approaches.

6 Zusammenfassung

Beim Schlaganfall handelt es sich um eine der führenden Ursachen für Morbidität und Mortalität, jedoch fehlt eine effektive Behandlung. Die Ischämie selbst induziert eine Reihe an Reparaturmechanismen, welche eine begrenzte Regeneration erlauben und u.a. die Angiogenese und Neurogenese umfassen. Studien haben gezeigt, dass neuronale Vorläuferzellen (Neuroblasten) zu ischämischen Regionen migrieren und sich hier bevorzugt in die Nähe neu gebildeter Gefäße begeben. Die Signalsysteme, die für die Gefäß-Neuroblasten-Kommunikation zuständig sind, sind nur unzureichend verstanden. Ein besseres Verständnis dieser endogenen Reparaturmechanismen könnte zu neuen Therapieansätzen führen. Mit diesem Ziel wurde im Carmichael Labor, mittels genom-weiter Expressionsanalysen sowie bioinformatischer Interaktionsuntersuchungen das post-ischämische neurovaskuläre Interaktom identifiziert. In dieser Studie wählten wir vier Gene, welche in ischämie-responsiven Neuroblasten dysreguliert waren, für weitere Experimente aus: CCL9, WNT7A, GAS6 und PTN. Unsere Hypothese war, dass eine Überexpression dieser Gene die postischämische angiogenetische und neurogenetische Antwort verstärken würde, während ein Knockdown diese Antwort abschwächen würde. Um diese Hypothese zu testen verwendeten wir Lentiviren zur Überexpression oder zum microRNA vermittelten Gen-Knockdown in einem photothrombotischen Modell des ischämischen Schlaganfalls. Wir quantifizierten die Endothelzellenproliferation, die Gefäßdichte im Periinfarkt Gewebe und die Anzahl neugebildeter Neurone. In unseren Untersuchungen zeigte sich durch die Überexpression von CCL9 und WNT7A ein signifikanter Anstieg der Endothelzellenproliferation nach Schlaganfall, während ein Knockdown von CCL9 zu einer signifikanten Reduktion führte. Ein PTN Knockdown führte zu einem Anstieg der Gefäßdichte, ohne dass PTN einen signifikanten Effekt auf die Endothelzellenproliferation zeigte. Eine Überexpression von WNT7A als auch CCL9 führte zu einer post-ischämischen Zunahme der Neurogenese ($NeuN^+/EdU^+$-Zellen), während ein Knockdown von CCL9, WNT7A und PTN die post-ischämische Neurogenese signifikant reduzierte. In einem weiteren Experiment setzten wir ein Hydrogel ein, um das Peri-Infarktgewebe über mehrere Tage mit CCL9 Protein zu behandeln. Dieses Hydrogel wurde direkt in den

Infarktkern im MCAo-Model des Schlaganfalls implantiert. Auch hier zeigte sich eine Zunahme der Endothelzellproliferation. Zusammenfassend gelang es uns, mehrere neue Regulatoren der post-ischämischen neurovaskulären Regeneration zu identifizieren. CCL9 zeigte den ausgeprägtesten Effekt auf die neurovaskuläre Regeneration, wobei sich die Effekte in zwei unterschiedlichen Modellen des ischämischen Schlaganfalls mit zwei unterschiedlichen Interventionsansätzen reproduzieren ließen.

7 Abbreviations

ANOVA	Analysis of variance
BBB	Blood-brain-barrier
BrdU	5-bromo-2'-deoxyuridine
CAM	Chorio-allantoic membrane
CCL9	Chemokine (C-C motif) ligand 9
CCR5	C-C chemokine receptor type 5,
CIMT	Constraint-induced motor therapy
CSD	Cysteine-rich-domain
CSF	Cerebrospinal fluid
DCX	Doublecortin
DCX-RFP	Doublecortin - red fluorescent protein
Dvl	Dishevelled
EAE	Experimental autoimmune encephalomyelitis
EC	Endothelial cell
ECM	Extracellular matrix
EdU	5-Ethynyl-2′-deoxyuridine
eGFP	Enhanced green fluorescent protein
FACS	Fluorescence activated cell Sorting
GAS6	Growth-arrest-specific gene-6
GFAP	Glial fibrillary acidic protein
GLUT1	Glucose transporter 1
HUVEC	Human umbilical vein endothelial cell
IdU	Iododeoxyuridine
iMC	Immature myeloid cells
int1	Integration site 1
LCM	Laser capture microdissection
LTP	Long-term potentiation
MAP2	Microtubule-associated protein 2

MCAo	Middle cerebral artery occlusion
NeuN	Neuronal nuclear protein
NPC	Neural progenitor cell
NSC	Neural stem cell
PBS	Phosphate buffered saline
PCP	Planar cell polarity
PECAM	Platelet/endothelial cell adhesion molecule-1
PSA-NCAM	Polysialylated neuronal cell adhesion molecule
PT	Photothrombosis
PTN	Pleiotrophin
RMS	Rostral migratory stream
SHP-2	SH2 domain-containing tyrosine phosphatase 2
STZ	Streptozotocin
SVZ	Subventricular zone
TAM	Tyro3, Mer, Axl
tPA	Tissue plasminogen activator
TTC	2,3,5-Triphenyltetrazolium chloride
VEGF	Vascular endothelial growth factor
WNT7A	Wingless-Type MMTV Integration Site Family, Member 7A

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10 Curriculum vitae

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