BASAL LAMINA IN NEURODEGENERATIVE DISEASES

by

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(Under the Direction of YAO YAO)

ABSTRACT

The blood brain barrier (BBB) plays a key role in regulating the homeostasis of the central nervous system (CNS). BBB disruption is a hallmark of various neurodegenerative diseases, including Alzheimer's Disease (AD), Parkinson's Disease (PD), and amyotrophic lateral sclerosis (ALS). The non-cellular constituent, the basal lamina, is largely understudied compared to the cellular components due to its intrinsic complexity and lacking research tools. This thesis has two parts. In the first part, I review the pathology of neurodegenerative diseases and how the BL changes in these diseases. In the second part, I discuss my work optimizing a decellularization procedure within my laboratory. Decellularization is a clear and impartial technique utilizing complete removal of cellular matter and isolation of the BL. Here, I discuss the steps and results taken to troubleshoot and improve our protocols to fully remove cells and maintain vascular morphology, as well as future direction in the field.

INDEX WORDS: Basal lamina, Blood-Brain Barrier, Neurodegenerative Diseases, Alzheimer's Disease, Parkinson's Disease, Amyotrophic Lateral Sclerosis, Decellularization

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DEDICATION

This thesis is dedicated towards the many friends and family that have supported me along the way. Whether it was a long night of studying, grabbing a meal together, reminding me to take a break, or simply being there for me at some point the past few years, I would not be where I am if not for you.

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TABLE OF CONTENTS

Part 1. Literature Review: Roles of the Basal Lamina in Neurodegenerative Diseases

CHAPTER 1

NEURODEGENERATIVE DISEASES

Neurodegenerative diseases are a family of age-associated neurological diseases primarily characterized by degeneration of the central nervous system (CNS) in both structure and function. The three most common neurodegenerative diseases are Alzheimer's Disease (AD), Parkinson's Disease (PD), and Amyotrophic Lateral Sclerosis (ALS), which affect more than 7 million people in the US [1]. With a large aging population, the number of patients with neurodegenerative diseases is expected to increase dramatically over the next several decades. Currently, there are no available disease-modifying treatments, although some therapeutics exist to ease symptoms. Here, we mainly focus on AD, PD, and ALS.

Alzheimer's Disease

AD is the most common form of dementia, currently affecting over 6 million Americans [1]. AD is characterized by brain shrinkage in the entorhinal cortex, hippocampus, and cerebral cortex, which leads to progressive memory decline, behavioral changes, impaired mobility, hallucinations, and death [2, 3]. Pathological hallmarks of AD in the brain include the presence of extracellular β-amyloid (Aβ) peptide aggregates known as senile plaques (SPs), intracellular accumulation of hyperphosphorylated tau protein

known as neurofibrillary tangles (NFTs) [2], and Aβ deposition in the cerebral and leptomeningeal blood vessels known as cerebral amyloid angiopathy (CAA) [4].

AD is further categorized into sporadic AD (sAD), occurring with no known causes, and familial AD (fAD), linked to genetic causes. fAD may be caused by a mutation in a variety of genes, including amyloid precursor protein (APP) and presenilin-1/2 (PSEN1/2) [2]. The APOE gene is a risk factor for sAD with APOE4 heterozygotes and homozygotes having 3x and 12x higher risk, respectively [5].

Many theories have been proposed on the potential pathogenesis of AD. The amyloid cascade hypothesis (ACH) is the most popular one, which has been the basis of many translational studies [6]. It proposes that Aβ deposition and SP and NFT formation are due to genetic or environmental risk factors, which ultimately cause neuronal loss, dementia, and death [7]. Consistent with this hypothesis, Aβ is the primary constituent of SPs [8] and abnormal processing of APP occurs early in AD pathogenesis [7, 9]. However, this theory has been increasingly challenged. First, a significant percentage of geriatric people present Aβ plaques without developing clear AD symptoms [10], suggesting AD may be caused by factors other than plaques alone. Second, fAD patients only present symptoms later in life, despite having abnormal Aβ production at a young age [11]. Third, SP/NFT presence does not correlate quantitatively with cognitive impairment, age, and disease progression [12-14]. Last, no therapies targeting Aβ or based on the ACH have been successful in human trials [15].

Therefore, an alternative hypothesis, called the two-hit hypothesis, has been proposed recently. The two-hit hypothesis emphasizes the addition of vascular factors, in addition to genetic and environmental factors [16, 17]. It proposes that hypoperfusion and

blood-brain barrier (BBB) dysfunction is aggravated by vascular damage, which leads to compromised Aβ and neurotoxin clearance, and thus neurodegeneration and dementia [16, 18]. These vascular and Aβ pathways are able to act independently or synergistically of one another [16, 17]. Echoed with this hypothesis, AD mice and patients develop BBB disruption and hypoperfusion prior to Aβ accumulation and dementia [19]. Further knowledge on the relationship between BBB integrity and AD may shed light on its pathogenesis and possibly identify novel therapeutic targets.

Parkinson's Disease

PD, first described in 1817, is the second most common neurodegenerative disease [20]. Clinical symptoms of PD include resting tremor, gait disorders, and bradykinesia [21]. Various nonmotor symptoms, including olfactory dysfunction, rapid eye movement (REM) sleep disorder, depression/anxiety and possible dementia, are also present [21, 22]. Though some symptomatic treatments have been beneficial, there are no disease-modifying therapeutics or cure. Sporadic PD (sPD) cases comprise approximately 95% of all cases, while familial PD (fPD) with heritable gene mutations accounts for the rest 5% of cases [23, 24]. The genes associated with PD include α-synuclein (SNCA), leucine rich repeat kinase 2 (LRRK2), vacuolar protein sorting 35 homolog (VPS35), and glucocerebrosidase (GBA) [22, 25, 26].

PD is characterized by loss of dopaminergic (DA) neurons in the pars compacta of the substantia nigra and locus coeruleus [27]. Another hallmark of PD is the formation of Lewy bodies (LBs), which contain aggregated and post-translationally modified α-synuclein (α-syn). Decreased α-syn level is found in the CSF of PD patients [28-31],

suggesting that defective transport/clearance of α-syn may contribute to PD pathogenesis. Consistent with this hypothesis, α-syn can move bidirectionally across the BBB [28] and BBB integrity is altered in PD brains [32-35].

Amyotrophic Lateral Sclerosis

ALS is a progressive nervous system disease and one of the most prevalent neurodegenerative diseases following AD and PD. It affects approximately 2 in every 100,000 people [36]. ALS is symptomatically characterized by muscular weakness and paralysis. There are currently no disease-modifying treatments for ALS. Motor neuron degeneration in the spinal cord, motor cortex, and brain stem is the typical pathology of ALS. Similar to AD and PD, ALS cases are divided into sporadic ALS (sALS) and familial ALS (fALS). Around 15 genes, including SOD1 (Cu/Zn superoxide dismutase), TARDBP (TAR DNA-binding protein), FUS (fused in sarcoma), ANG (angiogenin precursor) and OPTN (optineurin), are linked to fALS cases [37]. Missense mutations in SOD1 cause approximately 20% of all fALS cases. Mutated SOD1 forms intracellular aggregates and alters gene expression & protein interactions, eventually leading to motor neuron death via toxic hydroxyl radicals [38]. In addition, increasing evidence shows that BBB integrity is compromised in both sALS and fALS cases. Specifically, serum protein leakage, tight junction protein reduction, pericyte loss, astrocytic endfeet detachment and degeneration, and BL component changes have been found in both postmortem human ALS samples [39-45] and ALS mouse models [44, 46-50]. The cause of BBB breakdown in ALS, however, is still unclear. Since BBB disruption occurs before motor neuron degeneration and

neuroinflammation [44, 48, 49], it is believed that BBB breakdown contributes to ALS pathogenesis.

CHAPTER 2

THE BASAL LAMINA

The BBB, a highly dynamic system in the CNS, plays a key role in maintaining CNS homeostasis. BBB breakdown is a hallmark of neurodegenerative diseases in both humans and rodents [39, 51, 52]. It is mainly composed of brain microvascular endothelial cells (BMECs), pericytes, astrocytes, and a non-cellular constituent---the BL [52, 53]. The majority of BBB research is on its cellular components, leaving the BL understudied probably due to its intrinsic complexity. However, recent studies from our laboratory and others show an active role of the BL in BBB integrity [54-56]. The role of each BL component is summarized in Table 1. It is thus logical to hypothesize that defects in the BL may lead to BBB compromise, contributing to neurodegenerative diseases.

Another common pathology in AD, PD and ALS is protein aggregation. Although the formation and function of these protein aggregates are not fully understood, reduced clearance is a contributing factor. Recent evidence highlights the glymphatic system as a major player in brain solute/waste clearance [57-65]. Specifically, cerebrospinal fluid (CSF) in the subarachnoid space moves through the periarterial space into the brain parenchyma, where it crosses the glia limitans and mixes with interstitial fluid (ISF). Then, the CSF-ISF mixture and metabolic waste move towards the perivenous and perineuronal spaces and eventually exit the CNS via deep veins, meningeal/cervical lymphatic vessels and perineural sheaths of cranial/spinal nerves. The glymphatic waste removal has been associated with aquaporin-4 (AQP4) expression/function. Reduced CSF influx and ISF

efflux have been found in four independently generated AQP4$^{-/-}$ mouse lines [57, 66], although one study failed to observe similar changes [67]. Given that the BL is a major constituent of the perivascular space [68-71] and plays a major role in AQP4 regulation [72-75], it may be also involved in the glymphatic clearance of protein and metabolic waste.

Here, we summarize how BL changes in normal aging and in neurodegenerative diseases, including AD, PD and ALS. The functional significance of these changes is also discussed. BL changes in normal aging and neurodegenerative diseases are summarized in Table 2.

The Basal Lamina in Normal Aging

The BL is a 50-100nm-thick amorphous structure sandwiched between endothelial cells and astrocytic endfeet [68, 76-79]. There are two layers of BL in the brain: a parenchymal BL mainly produced by astrocytes and an endothelial BL predominantly made by BMECs, which are separated by pericytes [80-82]. The BL contains four major extracellular matrix proteins: collagen IV, laminin, nidogen, and heparan sulfate proteoglycans (HSPGs). Minor BL constituents, including fibulins, osteonectin and netrin-4, are also present [83].

Mounting evidence in rodents and humans shows that the BL thickens with age [84-89], suggesting age-associated alteration of BL composition.

The Basal Lamina in Alzheimer's Disease

Multiple studies reported BL thickening in AD brains of multiple transgenic mice models and human patients [19, 87, 90-100], although one study observed BL thinning in human brains [101]. Interestingly, BL thickening was observed in a region-specific manner, mainly in the cerebral cortex, hippocampus and thalamus [84, 95]. According to an immuno-EM study, the parenchymal BL thickens more severely than the endothelial BLM [95]. These findings established a direct association between BL thickening and AD.

How exact does BL thickening contribute to AD pathogenesis? One hypothesis is that BL thickening may compromise the glymphatic system, leading to Aβ deposition and plaque formation. It has been shown that BL thickening occurs before Aβ deposition in the vessel wall [19]. Next, disrupted perivascular Aβ drainage and brain accumulation of Aβ are observed in APOE4 transgenic mice [102]. In addition, decreased CSF influx and Aβ clearance rate was observed in both aged AD mice and young AD mice without visible Aβ plaques [103, 104]. Furthermore, intraventricular or intra-hippocampal injection of Aβ in WT mice reduced glymphatic flow [84, 104], suggesting that glymphatic flow may be disrupted by Aβ itself. Consistent with these findings, reduced CSF uptake and clearance of tau protein, which is associated with AD progression, have been found in AD patients [105, 106]. Together, these results suggest that BL thickening and disrupted glymphatic function are early events of AD, and may be targeted for early diagnosis of AD.

Though it is not yet clear what causes BL thickening in AD brains, it is believed that an imbalance of BL composition may contribute to it [107]. APOE4 is able to bind to laminin and HSPGs [108, 109], and affect BL composition [102]. All BL components have been shown to interact with Aβ and affect its biochemical properties. Specifically, the

interaction of Aβ and laminin, collagen IV, or nidogen leads to Aβ fibrillation inhibition and disruption [110-117], suggesting that BL thickening may be a reactive mechanism to protect against fibrillation. On the other hand, HSPGs may contribute to disease pathogenesis by accelerating Aβ fibrillation [118-120].

The Basal Lamina in Parkinson's Disease

Postmortem analysis of PD patient brains found capillary BL thickening [99, 121] and collapsed BL morphology [122], although there were no reports on BL changes in mouse PD models. Future studies should focus on examining BL alterations in mouse models of PD.

The functional significance of BL changes in PD remains unknown. On one hand, events in PD pathogenesis may provoke BL thickening as a protective mechanism to further prevent α-syn accumulation and aggregation. On the other hand, α-syn accumulation may be caused by increased BL thickening, further aggravating disease. Further research should distinguish these possibilities.

The Basal Lamina in Amyotrophic Lateral Sclerosis

BL thickening is present in the spinal cord and brain stem of $SOD1_{G93A}$ mice, a mouse model of ALS [46]. Additionally, multiple layers of BL and extracellular edema are also observed in these mice [46]. Collagen IV and fibrin are accumulated in the BL of sALS patients [42]. Future research should address if similar BL defects are observed in ALS patients.

Though a correlation is present between BL defects and ALS severity, whether this relationship is a cause or consequence of ALS is unclear. BL thickening and duplication occur early in the symptomatic stage of SOD1 mice [46], possibly acting as an early protective mechanism against ALS and BMEC degeneration. These defects may in turn affect BBB integrity and the glymphatic pathway. For example, BMEC detachment exposes the BL to plasma proteins, which leads to BL thickening and increased permeability in the vasculature [40, 46].

CHAPTER 3

LAMININ

Laminin is a trimeric T- or cross-shaped protein composed of α, β, and γ chains [68]. These subunits are connected via their C-terminal domains. The N-terminal domains are responsible for self-polymerization to form a sheet-like suprastructure [123]. Currently, 5 α, 4 β, and 3 γ chains have been identified, creating up to 60 different laminin isoforms [68]. Not all combinations, however, have been confirmed: only 16 laminin isoforms have been identified and 4 proposed based on *in vitro* and *in vivo* studies [68]. Importantly, different laminin isoforms have different functions (summarized in Table 1**)**. Thus, laminin functions must be studied in an isoform-specific manner.

In the brain, laminin is mainly expressed in BMECs, pericytes and astrocytes, and these cells expressed different laminin isoforms [53]. Specifically, laminin-411 and -511 are generated by BMECs [80, 124], while laminin-211 is predominantly produced by astrocytes [80, 125]. Using immunocytochemical analysis, our laboratory has shown that brain pericytes predominantly synthesize α4/α5- and γ1-containing laminins [75, 126], although laminin-α2 is found in pericytes by RNAseq analysis [127]. Therefore, the endothelial BL is primarily composed of laminin-411 and -511, while the parenchymal BL is mainly composed of laminin-211.

Loss-of-function studies were used to study the functions of different laminin isoforms in BBB integrity. However, most laminin global knockout mice (e.g. laminin-α5$^{-/-}$, -β1$^{-/-}$, and -γ1$^{-/-}$) are embryonic lethal [128-133], which hinders further investigation into

their roles in BBB integrity. Thus, various laminin conditional knockout mice have been generated.

Mice with global laminin-α4 knockout showed disrupted vascular integrity and hemorrhage at the perinatal stage, but not in adulthood [134]. Laminin-511, expressed in blood vessels at postnatal stage, is believed to compensate for the loss of -411 and thus explains the lack of phenotype in adulthood [134, 135]. To investigate the function of endothelial laminin-α5 in BBB integrity, we generated an endothelium-selective (Tie2-Cre) laminin-α5 conditional knockout mouse line. No BBB breakdown or other gross abnormalities were observed in these mutants under homeostatic conditions [136-138], suggesting that there may be mutual compensation between laminin-411 and -511. Therefore, mice with endothelium-specific deletion of laminin-411 and -511 were further generated to investigate the role of endothelial laminin in BBB integrity. We are currently characterizing the phenotypes of these mutants.

We also generated mouse lines with conditional knockout of laminin-γ1 in neural progenitor cells (Nestin-Cre) and neurons (CamK2a-Cre), respectively [73]. Out of both models, severe BBB disruption and ICH were found in the former but not latter mutants [73, 139], underlining a key role of glial cell-derived laminin in BBB regulation. Next, adenovirus expressing Cre under GFAP promoter was used to further demonstrate that BBB breakdown is due to loss of astrocytic laminin [73]. Consistent with these results, mice with global knockout of laminin-α2 demonstrate BBB disruption [73, 140]. Together, these findings suggest that astrocytic laminin plays an indispensable role in BBB maintenance.

The function of pericytic laminin in BBB integrity were investigated indirectly due to the lack of pericyte-specific markers [141]. Mice with laminin deficiency in mural cells, which include both pericytes and vascular smooth muscle cells (vSMCs), were generated using the PDGFRβ-Cre line [75]. Similarly, mice with laminin deficiency in vSMCs only were created using the SM22α-Cre line [75, 142]. Although the vSMC-specific laminin knockout mice fail to show gross abnormalities [75], the mural cell-specific laminin knockout mice display genetic background-dependent phenotypes. Specifically, these mutants show BBB breakdown and hydrocephalus with incomplete penetrance in C57/Bl6-FVB mixed background [75]. In C57Bl6 dominant background, however, these mutants develop mild BBB compromise only at old age, but are grossly normal at young ages [74]. These findings together suggest an essential role of pericytic laminin rather than vSMC-derived laminin in BBB maintenance.

Here, we summarize how laminin changes in normal aging and in neurodegenerative diseases, including AD, PD and ALS. The functional significance of these changes is also discussed. Laminin changes in normal aging and neurodegenerative diseases are summarized in Table 3.

Laminin in Normal Aging

During normal aging, most studies found decreased laminin expression in the BL in both mice [84, 103] and humans [88]. One study, however, observed increased laminin expression in retinal BL in mice [86]. This disparity may be owing to a variety of reasons, including different laminin antibodies and/or fixation protocols used in these studies. For

example, mild-moderate formaldehyde fixation reveals laminin antigen, while heavy fixation masks it [143].

Laminin in Alzheimer's Disease

How laminin changes in AD is controversial. There is evidence suggesting increased [19, 144, 145]. For instance, laminin-α1 and -γ1 expression is substantially increased in reactive astrocytes and/or senile plaques of AD human brains [144, 145]. On the other hand, there are also reports showing unaltered [91] and decreased laminin levels in AD brains [102]. One possible reason for this discrepancy is the use of pan-laminin rather than subunit-specific antibodies in some studies. A pan-laminin antibody is unable to differentiate between different laminin subunits, and thus cannot accurately reflect changes in individual laminin isoforms. Given the various functions exerted by distinct laminin isoforms [54, 68, 69, 146], laminin expression and function need to be studied in an isoform-specific manner.

Laminin's function in AD remains mostly unclear, although evidence suggests that laminin may promote Aβ clearance in AD. For example, laminin has been reported to both inhibit Aβ40 fibril formation [110-112] and disrupt pre-formed fibrils *in vitro* [113]. High concentrations of laminin are able to disassemble the β-sheet structure and inhibit fibrillation by inducing random structural transitions of Aβ42 fibrils [114]. In addition, laminin may promote Aβ clearance through the glymphatic pathway by binding to the Aβ-APOE complex [147, 148]. APOE4 displays diminished binding affinity to laminin *ex vivo* and thus diminished Aβ clearance ability compared to APOE3 [149], which may explain the high risk of APOE4 in AD. The functions of individual isoforms of laminin in AD

pathogenesis should be investigated *in vivo* using conditional knockout mouse lines in future studies.

Laminin in Parkinson's Disease

Currently, there are no compositional studies in the BL of PD brains. Future research should focus on determining the expression of individual laminin isoforms in PD brains using subunit-specific antibodies.

The role of laminin in PD brains is not entirely clear, although evidence suggests that laminin may have a neuroprotective role in PD. Laminin acts as a neurite-outgrowth promoting factor in various *in vitro* models [150]. The laminin-HSPG complex is able to enhance neurite outgrowth *in vitro* [151]. Likewise, *in vitro* studies also showed enhanced neurite outgrowth from peptide nanofibers with HS mimetic and laminin-derived epitopes [152]. Consistent with these *in vitro* studies, the KDI domain of laminin-γ1 demonstrated a protective role in the 6-hydroxydopamine-induced PD model in mice [153]. Similarly, laminin-mimetic peptide amphiphile nanofibers substantially enhanced dopamine and tyrosine hydroxylase levels, reduced cleaved-Cas-3 levels, and improved function and tissue integrity in this PD model [154]. These results highlight a therapeutic potential of laminin in PD.

It remains unknown which laminin isoforms mediate the neuroprotective function in PD. Some studies suggest that this beneficial effect may be partially mediated by laminin-511. For example, laminin-511 has been shown to induce mi-R-130a and suppress PTEN protein, promoting survival and differentiation of dopaminergic neurons [155, 156]. Since BMECs undergo degeneration in PD [122] and laminin-511 is generated by BMECs

[80, 124], it is hypothesize that loss of laminin-511 through BMEC degeneration may contribute to PD. This hypothesis, however, needs further investigation.

Laminin in Amyotrophic Lateral Sclerosis

How laminin changes in ALS is inconsistent. Using a laminin-α1/β1 antibody, it was reported that laminin expression was reduced in the spinal cord of symptomatic $SOD1_{G93A}$ mice [47]. Using a pan-laminin antibody, it was found that laminin expression in the muscular basement membrane was unaltered [157]. Interestingly, laminin-α2 and -β2 expression is decreased and laminin-α4 is absent in limb muscles but not in extraocular muscles in ALS patients [158]. On the other hand, astrocytes in white matter strongly express laminin γ1 along the cervical and thoracic spinal cords in ALS patients, which correlates with disease severity [159]. Laminin-111 expression is also increased in the skin basement membrane of ALS patients [160]. The different results may be explained by the use of different antibodies. Additionally, changes in laminin levels may also be caused by degeneration of pericyte [161], BMECs, and astrocytes [46], which are all able to synthesize laminin. Future studies should focus on elucidating subunit-specific expression of laminin in ALS.

The substantially altered laminin levels in ALS suggests a crucial role of laminin in this disorder. It is hypothesize that laminin-γ1 may play a beneficial role in ALS, given that it is highly upregulated in astrocytes [159] and that its KDI domain has a protective role in neurons in both glutamate-induced excitotoxicity [162] and 6-hydroxydopamine-induced neuronal death models [153]. Further investigation is needed to test this hypothesis and elucidate the functions of other laminin isoforms in ALS.

CHAPTER 4

COLLAGEN IV

Collagen IV, the most abundant component of the BL, is mainly produced by endothelial cells, astrocytes and pericytes [126, 163-166]. Structurally, collagen IV is a trimeric protein containing three α-chains. Six different collagen IV α-chains have been recognized so far (COL4A1-6) [167-169]. Collagen IV is able to form sheet-like suprastructures by dimerizing at its NC1 domain and tetramerizing at its 7s domain [170, 171].

Loss-of-function studies show that collagen IV plays crucial roles in embryonic development and vascular integrity. Genetic knockout of COL4A1/2 causes structural deficiencies in the BL and embryonic lethality at E10.5 - E11.5, although development in earlier stages is unaffected [55]. Exon 41 deletion in COL4A1 in both alleles results in embryonic lethality and ICH [172]. Interestingly, mice with exon 41 deletion in only one allele show ~50% perinatal lethality with ICH and porencephaly in a small percentage of survivors [173, 174]. To investigate the function of collagen IV in different cells, mice with cell-specific abrogation of COL4A1 were generated. Deletion of BMEC- or pericyte-derived COL4A1 leads to ICH, increased retinal vascular branching, porencephaly, and macroangiopathy [175]. Loss of astrocytic COL4A1, on the contrary, results in very mild ICH without defects in retinal vascular branching [175]. Consistent with these findings, varying magnitudes of vascular defects and/or brain damage are found in mice with missense mutations in COL4A1 and COL4A2 [172, 176, 177]. These studies collectively

suggest that collagen IV is an important player in BL maintenance and vascular integrity, but does not significantly contribute to initial BL assembly [53].

Here, we summarize how collagen IV changes in normal aging and in neurodegenerative diseases, including AD, PD and ALS. The functional significance of these changes is also discussed. Collagen IV changes in normal aging and neurodegenerative diseases are summarized in Table 4.

Collagen IV in Normal Aging

Controversial results exist on how collagen IV changes during normal aging. On one hand, both rodent and human studies reported upregulated collagen IV levels during aging [86-88]. One study, however, found decreased collagen IV in aged mice [84]. It should be noted that there is also evidence supporting unaltered collagen IV expression during aging in both mice and humans [89, 103]. This discrepancy may be due to distinct experimental methods and conditions. Future research should determine how exactly collagen IV changes during normal aging.

Collagen IV in Alzheimer's Disease

Different AD models show inconsistent alterations of collagen IV. 3xTG (APP_{swe}/$PSEN1_{M146V}$/Tau_{P301L}) transgenic mice show increased expression of collagen IV [90, 92], while the APP_{swe} ($APP_{K670N,\ M671L}$) [178] and APOE4 [102] models exhibit decreased levels of collagen IV, and $PSEN1_{P117L}$ mice display unaltered collagen IV levels [91]. Unlike mouse studies, postmortem human studies consistently reported increased collagen IV levels in AD brains [87, 93, 95-97, 99, 179, 180], although one study found

unchanged collagen IV expression [89]. Interestingly, these collagen IV changes are region-specific. Specifically, collagen IV levels appear normal in the neocerebellum [181], but are dramatically increased in the frontal and temporal cortex in both subclinical (Braak stage 3-4) and AD (Braak stage 5-6) patients [93, 99]. It is thus important to specify the genetic models and brain regions in AD research.

How collagen IV functions and contributes to AD pathogenesis is unknown. Collagen IV has been shown to bind to APP with high affinity *in vitro* [182, 183], prevent the formation of β-sheet-structured Aβ40 aggregates [116], and induce Aβ42 fibril disassembly [114]. These findings suggest that collagen IV may have a protective mechanism against disease progression, and that collagen IV may have a therapeutic potential in AD. The functional significance of collagen IV in AD needs to be investigated *in vivo* in future studies.

Collagen IV in Parkinson's Disease

Most studies consistently showed increased collagen IV expression in PD in both mice [184] and patients [99, 121]. It should be noted that one study failed to observe any changes in total length and density of collagen IV^+ blood vessels in PD patients, although collagen IV intensity was not measured [122]. Since several different fPD mouse models exist, it is important to illuminate how collagen IV changes in PD using multiple models.

There are several hypotheses on the functional significance of collagen IV in PD. One claims that abnormal collagen IV expression may aggravate PD pathogenesis by inducing ER stress [185]. This hypothesis is supported by a strong association between altered Golgi morphology and COL4A2 upregulation in PD models [184]. Collagen IV

upregulation may also alter BL morphology and function, affecting BBB integrity and glymphatic flow. In addition, collagen IV may also play a role in regulating α-syn aggregation and PD progression. The exact role of collagen IV in PD needs further investigation.

Collagen IV in Amyotrophic Lateral Sclerosis

How collagen IV changes in ALS is controversial. $SOD1_{G93A}$ mice show increased collagen IV in the spinal cord at 18 weeks of age [44]. Collagen IV-expressing microglia are observed in the anterior horn of these mice at 15 weeks [44]. In the medulla and spinal cords of ALS patients, collagen accumulation has been found in capillary BL [42]. On the contrary, studies in ALS patients have also reported decreased perivascular collagen IV [44, 186]. Reduced or unaltered expression of collagen IV has also been noted in non-neural tissues in ALS patients, such as the skin, serum and muscle [157, 187]. Since collagen IV is increased in glial cells but reduced in other tissues [44], it is hypothesized that this upregulation may be a compensatory mechanism in response to downregulation of collagen IV or other BL components. This hypothesis, however, needs further investigation.

Collagen IV changes in the CNS may play a significant role in the pathogenesis of ALS. Increased expression of collagen IV may be due to upregulation in glial cells, implying a neuroprotective role of collagen IV in ALS. High collagen IV expression in glial cells could also be due to increased uptake, implying a detrimental role of collagen in ALS. Determining the relationship between glial cells and collagen IV may help elucidate the functional significance of collagen IV in ALS.

CHAPTER 5

NIDOGEN

Nidogen, or entactin, is a glycoprotein attached to three globular and rod-like domains. Nidogen cannot self-polymerize to form superstructures. Instead, it acts as a crosslinker between collagen IV and laminin. It is hypothesized that nidogen functions to stabilize collagen IV and laminin networks.

Two nidogen isoforms, nidogen-1 and -2, have been identified in mammals [188]. Loss-of-function studies show normal phenotype in mice lacking either nidogen-1 or nidogen-2 [188-192], indicating mutual compensation between these two isoforms. Further studies demonstrate redistribution and upregulation of nidogen-2 in nidogen-1 knockout mice, while there is no change in nidogen-1 expression in nidogen-2 knockout mice [192, 193]. Knockout of both isoforms simultaneously leads to early perinatal death and severe BL defects [56, 194, 195]. Altogether, these results suggest that there is functional compensation between nidogen isoforms, which prevents the use of these single knockouts to study nidogen's function in BBB integrity. Conditional nidogen-1/2 double knockout mice may overcome the early perinatal lethality of the global double knockout mice and enable study of its roles at later stages.

Here, we summarize how nidogen changes in normal aging and in neurodegenerative diseases, including AD, PD and ALS. The functional significance of these changes is also discussed. Nidogen changes in normal aging and neurodegenerative diseases are summarized in Table 5.

Nidogen in Normal Aging

During normal aging, both reduced [84] and increased expression of nidogen have been observed in mouse brains [86]. In postmortem human brains, however, only increased nidogen expression was found during normal aging [89]. Future research should clarify how exactly nidogen changes during normal aging.

Nidogen in Alzheimer's Disease

There is limited research on how nidogen changes in AD. One study reported decreased expression of nidogen in postmortem human brains [89]. Future studies should validate this finding and determine if this change can be replicated in AD mouse models.

Our understanding of nidogen function mainly comes from *in vitro* studies. Like laminin and collagen IV, nidogen can inhibit Aβ40 fibrillation in a dose-dependent manner and induce Aβ40 and/or Aβ42 fibril disassembly [114], suggesting a beneficial role of nidogen in AD. Elucidating nidogen's function in AD *in vivo* relies on loss-of-function studies.

Although nidogen single knockout mice fail to show gross abnormalities in homeostatic conditions due to functional compensation [188-192], AD may aggravate vascular and/or neurological damage, revealing phenotypes in these mice. In addition, conditional double knockout mice may overcome perinatal lethality of the global double knockout mice [68], enabling investigation of nidogen's function in AD.

Nidogen in Parkinson's Disease

How nidogen changes in PD brains and the functional significance of this alteration remain unknown. Novel mouse models may help answer these important questions.

Nidogen in Amyotrophic Lateral Sclerosis

Similarly, it is unclear how nidogen changes in ALS and what the functional significance of this alteration is. This is an interesting area for further research.

CHAPTER 6

HEPARAN SULFATE PROTEOGLYCANS

Heparan sulfate proteoglycans (HSPGs) are glycoproteins covalently attached to heparan sulfate (HS) chains. Two major types of HSPGs exist in the BL: agrin and perlecan.

Perlecan: Perlecan is a large HSPG comprised of five domains (I-V) and three glycosaminoglycan chains. It is unable to self-polymerize or form sheet-like suprastructures [196]. Instead, perlecan binds to other BL components and/or heparin-binding growth factors [196-199]. Perlecan global knockout mice die around E10 - E12 and exhibit severe developmental defects in the heart and brain [200-202]. The BL is deteriorated in areas that experience mechanical stress, but interestingly BL formation at early stages is unaffected in these mutants [201], suggesting an important role of perlecan in BL maintenance but not assembly. Perlecan mutant mouse lines have been generated to overcome embryonic lethality. For instance, a C-to-Y mutation at residue 1532 and the neomycin cassette (termed C1532Yneo) induce reduced perlecan secretion and a skeletal phenotype similar to Schwartz-Jampel syndrome [203]. It is unclear whether these C1532Yneo mice have BBB disruption. Interestingly, normal perlecan levels and no abnormalities are found in mice harboring only the mutation without the neo cassette (termed C1532Y) [203]. In addition, transgenic mice expressing perlecan under the Col2α1 promoter and cartilage-specific enhancer showed no BBB defects under homeostatic conditions [204]. These mutants, however, demonstrated exacerbated BBB damage and

attenuated pericyte accumulation after ischemic stroke [204], suggesting a dispensable role of perlecan in homeostatic but not pathological conditions. Subsequent mechanistic study showed that perlecan promoted PDGFRβ signaling and pericyte migration [204]. Together, these results suggest that perlecan repairs BBB integrity by recruiting pericytes after ischemic stroke.

Agrin: Agrin is a multidomain HSPG that is found in all BMs in the body [205, 206]. Agrin exists in two isoforms: z+ and z0, and only the z0 isoform is present in the BM/BL. A correlation study reported that agrin accumulates in the BL during development [207], highlighting an possible role of agrin in early BBB development. A loss-of-function study shows that agrin knockout mice are embryonic lethal [208], preventing investigation of agrin's function at later stages. Conditional ablation of agrin in endothelial cells (Tie2-Cre) leads to reduced AQP4 expression but intact BBB structure [72], suggesting that agrin is important for biochemical but not structural maintenance of the BBB. The exact function of agrin in BBB integrity requires further studies.

Here, we summarize how HSPGs change in normal aging and in neurodegenerative diseases, including AD, PD and ALS. The functional significance of these changes is also discussed. HSPG changes in normal aging and neurodegenerative diseases are summarized in Table 6.

HSPGs in Normal Aging

Mouse studies have shown increased perlecan expression in normal aging [84, 86, 103]. However, there are no human studies regarding perlecan changes in normal aging. It

is critical to determine whether perlecan levels also increase in human brains during normal aging in the future.

Few studies examined agrin changes during normal aging. One study reported increased agrin expression in aged human brains [88]. This finding should be validated in both mouse and human brains in future studies.

HSPGs in Alzheimer's Disease

HSPG expression is increased in a region-specific manner in AD brains. Specifically, it showed a 9.3-fold and 6.6-fold increase in the hippocampus and superior frontal gyrus in AD brains [209]. Functional studies suggest that HSPGs may have detrimental role in AD. First, HSPGs can bind Aβ via the HS chains and accelerate Aβ fibril formation [118-120, 210]. The increased Aβ binding affinity and fibrillation capability of HSPGs have been associated with the sulfation pattern of HS [211, 212]. Specifically, sulfation of HS increases Aβ affinity, while desulfated HS almost entirely loses Aβ binding capability [211]. Removal of O-sulfate on heparin partially inhibits aggregation of Aβ, while removal of all sulfate groups prevents Aβ fibril formation completely [212]. Next, multiple *in vitro* studies suggest that Aβ-HS interaction mutually protects each other from degradation [213]. It has been shown that HSPGs block the proteolytic degradation of fibrillar but not non-fibrillar Aβ *in vitro* [214], while Aβ inhibits heparanase activity and HS degradation, preventing proteolytic breakdown of HSPGs [215]. Consistent with this hypothesis, heparanase overexpression lowers amyloid burden in APP mouse models [216]. Further mechanistic studies show that heparanase either releases Aβ from the plaques or inhibits tau fibril formation and propagation [213]. These

results altogether support a detrimental role of HSPGs at nearly every stage of AD progression. Decreasing HSPGs or increasing heparanase activity may have a therapeutic potential in AD.

Perlecan expression is unchanged in APOE4 mice [102], but increased in human AD patients [93]. There is controversy on whether SPs, NFT, and CAA contain perlecan. One study found perlecan in these lesions [217], while several other studies found the opposite [218-220]. Different antibodies and/or experimental approaches may explain this discrepancy. How perlecan changes in AD mouse models and human patients should be elucidated in future research. Contrary to general HSPG data, perlecan exhibits a neuroprotective role against Aβ toxicity. The domain V of perlecan inhibits neurotoxic signaling cascades by negatively regulating Aβ-integrin-α2β1 interaction *in vitro* [221, 222]. Furthermore, perlecan domain V is able to restore angiogenesis and reverse Aβ toxicity in BMECs *in vitro* [223]. Together, these results suggest a potential therapeutic potential of perlecan in AD, although more research is needed to determine how perlecan regulates Aβ *in vivo*. Perlecan hypomorphic (C1532Yneo) mice and/or perlecan-knockout rescued mutants may be useful in answering this important question.

Similarly, controversial findings exist on how agrin is altered in AD brains. Agrin has been found to be unaltered in APOE4 mice [102]. However, both decreased [101] and increased [93, 148] expression of agrin have been observed in AD patients. Agrin is detected in SPs, NFTs, and CAA in AD brains [118, 119, 148, 218, 219]. Interestingly, agrin in normal brains is soluble in 1%SDS, while agrin from AD brains is not [224]. Since fibrillar Aβ, like agrin from AD brains, is also insoluble in 1% SDS [224], it is speculated that agrin may interact with Aβ. Future studies should clarify how exactly agrin changes

in AD brains in both mice and humans. Agrin has been shown to inhibit Aβ deposition. For example, agrin overexpression is associated with decreased Aβ level [72]. In APPswe/PSEN1$_{dE9}$ mice, deletion of agrin in endothelium but not neurons results in increased Aβ40 deposition [72]. In addition, agrin may also play a role in the glymphatic system to clear Aβ. Glymphatic clearance has been reported to be AQP4-dependent [225-227]. Consistent with these findings, reduced astrocytic AQP4 expression was found in APPswe/PSEN1$_{dE9}$ mice without endothelial agrin [72], and loss of AQP4 significantly increases Aβ accumulation without affecting Aβ proteases in AD mice [228]. These findings suggest a negative role of agrin in AD pathogenesis. The exact function and therapeutic potential of agrin in AD need further investigation.

HSPGs in Parkinson's Disease

How HSPGs change in PD is currently unknown. Future studies should answer this important question. Studies have reported a beneficial role of a laminin-HSPG complex in PD. This laminin-HSPG complex is able to promote neurite outgrowth *in vitro* [151]. Peptide nanofibers containing HS mimetic and laminin-derived epitopes are able to induce neurite outgrowth *in vitro* [152], and reduce brain injury and enhance functional recovery *in vivo* [154]. It is unclear, however, whether this beneficial effect is from HS, laminin, or both. Since HSPGs are absent in LBs and do not affect LB fibrillation and stabilization [229], laminin is more likely to play the neuroprotective role. This hypothesis, however, requires further study.

It remains unknown how perlecan changes in ALS. This is an interesting area for future research.

Although no studies have examined agrin changes in the CNS of ALS patients [157], agrin is significantly reduced in the spinal cord [49] and neurouscular junctions [230] in $SOD1_{G93A}$ mice at the symptomatic but not presymptomatic state. This finding suggests that agrin may be involved in symptom onset in ALS. Consistent with this hypothesis, agrin has been shown to regulate AChR clustering and other postsynaptic membrane features in muscle [231]. It is possible that decreased agrin affects receptor clustering and postsynaptic function, aggravating symptomatic onset. This hypothesis requires further investigation.

CHAPTER 7

KEY CHALLENGES AND FUTURE DIRECTIONS

Significant progress has been made in BL research and neurodegenerative diseases, thanks to evolving strides in genetic and biochemical technology. Nonetheless, several key challenges and questions need to be addressed in future research.

Firstly, how exactly the BL and its components are altered in neurodegenerative diseases is inconsistent. This is due to the use of different antibodies and/or detection methods. For example, pan-laminin antibodies are unable to determine changes of individual laminin isoforms. In addition, different mouse models and types/stages of diseases may also cause disparities. To address these issues, subunit-specific antibodies, standard protocols, and various disease models/types/stages should be used in future studies.

Secondly, the functions of the BL and its components in the pathogenesis of neurodegenerative diseases are unknown. This is mainly because many knockout mouse lines are embryonic lethal, preventing further studies in adulthood. This may be overcome by generating conditional knockout mice. In addition, functional compensation may cover the phenotypes of some knockout mouse lines. This may be overcome by generating compound conditional knockout mice, which contain abrogation of several isoforms simultaneously. Novel tools will help address these problems and allow investigation of the functions of the BL and its components in neurodegeneration.

Lastly, the causes of many neurodegenerative diseases are not fully understood. Many genetic mouse models have been generated to mimic pathological features of these diseases. However, these genetic models are unable to accurately represent the majority sporadic cases. Additionally, multiple genes have been linked to AD, PD, or ALS. Thus, key findings should be validated in multiple models containing different genes. It is also essential to validate these findings in postmortem human samples.

Part 2. Experimental Data: Optimization of Brain Decellularization Protocol

CHAPTER 8

BACKGROUND

The BL and its components are often understudied due to its intrinsic complexity and unique features. For example, the BL is heavily cross-linked with low solubility and BL components are highly glycosylated. To accurately determine BL composition in normal and diseased conditions, high-purity BL devoid of cellular components may be needed. Decellularization, a process that removes all cellular components from a tissue and thus enriches the BL, has been developed. This technique has enabled many challenging studies and significantly moved the field forward. For example, decellularization, which removes soluble cellular species, has allowed accurate visualization of the BL in mouse kidney, heart and liver [232]. It also makes further studies into organization, composition, protein-protein interaction and quantification possible. Given the essential role of decellularization in BL research, I aimed to optimize the brain decellularization protocol for future use. I examined a few published brain decellularization protocols and successfully optimized one that may be used in future studies.

CHAPTER 9

MATERIALS AND METHODS

Animals

Wild-type mice of 48-56 weeks old and both genders were maintained in C57B16 dominant background. All mice were maintained in the animal facilities at the University of Georgia with free access to water and food. Experimental procedures were conducted in accordance with the NIH guide for care and use of animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Georgia.

Decellularization Protocols

The process of decellularization optimization is summarized in Figure 1.

Protocol-1

Protocol-1 was adapted from a previous study [233]. Briefly, mouse brains were immediately harvested, cut into 1mm sections and flash frozen at -80C. Samples were then incubated in 4% sodium deoxycholate in dH_2O (2hr, 105rpm), washed with 1% Pen/Strep in PBS to prevent contamination. Next, the samples were incubated in 3% Triton X-100 in PBS (1hr, 105rpm) and washed with 1% Pen/Strep in PBS. This process was repeated two more times. Samples were then incubated in PBS containing DNase I (25 μg/mL) (Worthington #LS002007) and $MgCl_2$ (5 mM) overnight, followed by three washes in PBS. The decellularized brain sections were stored at 4°C.

Modified Protocol-1

I modified Protocol-1 in the following aspects. First, brain sections with different thickness (300μm, 500μm, and 1000μm) were used. Next, distinct incubation time in DNase (12hr vs 24hr) was used. Third, prolonged incubation time (1x and 2x) in detergent was used. Fourth, different repeating cycles (3x and 6x) were used.

Protocol-2

Protocol-2 was adapted from a previous study [234]. Briefly, mouse brains were harvested in cold 1x PBS and cut into 500μm sections. Samples were immediately flash frozen 4 times before incubated in 1% Pen/Strep in dH_2O (72hr). Then, the samples were treated in 1% Triton X-100 in dH_2O (60min, 60rpm), water (30min, 60rpm), 4% sodium deoxycholate in dH_2O (60min, 60rpm), water (30min, 60rpm), PBS containing DNase (25μg/mL) and $MgCl_2$ (5mM) (60min, 60rpm), and water (30min, 60rpm). This process was repeated one more time and decellularized samples were stored at 4°C.

Modified Protocol-2

Protocol-2 was modified to better maintain vascular morphology. Specifically, the incubation time in 1% Pen/Strep in water was reduced from 72hr to 0hr and 24hr.

Validation of Decellularization Efficacy

The efficacy of decellularization was determined by immunohistochemical analysis. The decellularized brain samples were embedded into OCT and cut into 30μm sections using the cryostat. Sections from superficial, middle, and deep layers from the samples were collected on slides and subjected to immunostaining. Some decellularized thick sections were subjected to immunostaining without sectioning. Briefly, brain sections

were fixed in 4% PFA for 15min and washed in PBS 3 times. Sections were blocked in blocking buffer (5% normal donkey serum in PBS, 1% BSA, 0.3% Triton X-100) for 2hr at room temperature. Then, sections were incubated with anti-laminin-111 (1:1000, Sigma L9393) antibody overnight at room temperature. Sections were then incubated with either Alexa Fluor-488 conjugated donkey anti-rabbit (1:1000, Invitrogen A21206) or Alexa Fluor-594 conjugated donkey anti-rabbit (1:1000, Invitrogen A21207) secondary antibody for 2hr at room temperature. Sections were then mounted with Fluoromount-G with DAPI. The stained sections were then imaged under a Nikon Eclipse Ti microscope.

CHAPTER 10

RESULTS

We observed poor decellularization efficacy using protocol-1 (Figure 2). Specifically, DAPI-positive cells (blue) remained visible and pan-laminin (green) showed a fragmented vascular pattern, indicating incomplete cell lysis.

Stressed conditions were then used to test individual variables for further optimization. This includes decreasing tissue thickness for decellularization (Figure 3), increasing the incubation time in DNase solution (Figure 4), increasing incubation time in detergent (Figure 5), or increasing the cycle number in detergent (Figure 6). All modifications to protocol-1 yielded incomplete decellularization. Specifically, all tissues had visible DAPI-positive cells (blue) and fragmented or absence of pan-laminin (red) (Figure 2-6), showing incomplete cell lysis and BL damage.

Using Protocol-2, we found decreased pan-laminin (red) and absence of DAPI (blue) staining in tissue (Figure 7), suggesting complete decellularization of the tissue but degradation of the BL.

It was suspected that BL degradation was due to excessive exposure to water. Thus, incubation times in 1% Pen/Strep in water were reduced to 0 hours and 24 hours. We found abundance of laminin signals (red) with typical vascular patterns and no DAPI (blue) staining in the decellularized tissue in both conditions (Figure 8), indicating successful decellularization. Notably, tissues with 0 hour incubation in water showed much better

vascular morphology than tissues with 24 hour incubation, suggesting that prolonged exposure to water indeed caused laminin degradation.

CHAPTER 11

DISCUSSION

Multiple decellularization protocols have been developed. Some utilize detergents to disrupt cell membrane [233][235]. For example, sodium dodecyl sulfate (SDS) and Triton X-100 have been used in decellularization. The concentration of detergent is critical in this process, since low concentration will cause incomplete decellularization, while high concentration may disrupt interactions among BL components, leading to biased results. It should be noted that different types (ionic vs. nonionic) of detergents may have distinct ability to disrupt plasma membranes. In addition, DNA fragments are usually observed in tissues using detergent-based protocols, even with the addition of DNase [240].

There are also methods that employ acids and bases [236] or physical methods [237, 238] to decellularize tissues. Protocols that utilize acid and/or base methods usually use peracetic acid and/or reversible alkaline to disrupt cells. Although effective in lysing cells, these methods often alter the mechanical properties of the BL in the tissue [236, 239]. Physical methods, such as freeze-thaw cycles [238] or high hydrostatic pressure [237], are in general successful in lysing cells and maintaining mechanical strength, but less effective in removing DNA remnants.

Our optimized protocol employs a combination of multiple techniques. It may be useful in the field of BL research. For example, it may be used to decellularize brains from various neurodegenerative diseases, generating high-purity BL in these conditions. The enriched BL will enable accurate and unbiased quantification of BL composition in various

neurodegenerative diseases, which has been controversial due to limitations in detect methods. The optimized brain decellularization protocol will significantly advance our knowledge in BL and open windows for new research by providing a unique tool to study a previously understudied yet important field.

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TABLES AND FIGURES

Table 1: Loss-of-function studies on major BL components

Targets	**Mutations**	**Cre lines**	**Phenotypes**	**References**
Collagen 4A1/2	Global KO	-	Embryonic lethality (E10.5-11.5), BM structural deficiencies	[55]
	Missense mutations	-	Vascular defects, brain damage of differing severity	[172, 176, 177]
Collagen 4A1	Loss of exon 41 in both alleles	-	Embryonic lethality, ICH	[172]
	Loss of exon 41 in one allele	-	Perinatal lethality with ICH, Porencephaly	[173, 174]
	Conditional knockout	Tie2-Cre	ICH, Increased retinal vascular branching, Porencephaly, Macroangiopathy	[175]

		PDGFRβ-Cre	ICH, Increased retinal vascular branching, Porencephaly, Macroangiopathy	[175]
		GFAP-Cre	Very mild ICH, No defects in retinal branching	[175]
Laminin α2	Global knockout	-	BBB disruption	[73, 140]
Laminin α4	Global knockout	-	Disrupted vascular integrity, Hemorrhage at perinatal stage	[134]
Laminin α5	Global knockout	-	Embryonic lethality (~E17)	[128-130]
	Conditional knockout	Tie2-Cre	No gross abnormalities	[136-138]
Laminin β1	Global knockout	-	Embryonic lethality (E5.5-6.5)	[131]
Laminin γ1	Global knockout	-	Embryonic lethality (E5.5-6.5)	[131-133]

	Conditional knockout	Nestin-Cre	BBB breakdown, ICH	[73, 139]
		CamK2a-Cre	No BBB breakdown or ICH	[73, 139]
		PDGFRβ-Cre	BBB breakdown and hydrocephalus in C57Bl6/FVB mixed background	[75]
			Age-dependent mild BBB breakdown without hydrocephalus in C57Bl6 dominant background	[74]
		SM22α-Cre	No gross abnormalities	[75, 142]
Nidogen 1	Global knockout	-	Grossly normal, Upregulation of nidogen 2	[188-191, 193]
Nidogen 2	Global knockout	-	Grossly normal	[192]
Nidogen 1/2	Global knockout	-	Perinatal lethality, BM defects	[56, 194, 195]

Agrin	Global knockout	-	Embryonic lethality	[208]
	Conditional knockout	Tie2-Cre	Intact BBB structure, Reduced AQP4 expression	[72]
Perlecan	Global knockout	-	Embryonic lethality (E10-12), Developmental defects, BM deterioration in areas with high mechanical stress	[200-202]
	Hypomorph (C1532Yneo)	-	Reduced perlecan secretion, skeletal phenotype similar to Schwartz-Jampel syndrome	[203]
	C1532Y		Normal perlecan secretion, grossly normal	[203]
	Knockout rescued	-	Viable and intact BBB under homeostatic conditions, exacerbated BBB damage and attenuated pericyte	[204]

			accumulation after ischemic stroke	

Table 2: Summary of BL changes in normal aging and neurodegenerative diseases

		BL	Model	Reference
Normal aging	**Rodents**	Thickening		[84]
		Thickening		[85]
		Thickening		[86]
	Humans	Thickening		[87]
		Thickening		[88]
		Thickening		[89]
AD	**Rodents**	Thickening	3xTG	[90]
		Thickening	$PSEN1_{P117L}$	[91]
		Thickening	3xTG	[92]
		Thickening	$APP_{swe/E693G}$	[19]
	Humans	Thinning		[101]
		Thickening		[93]
		Thickening		[94]
		Thickening		[95]
		Thickening		[96]
		Thickening		[97]
		Thickening		[87]

		Thickening		[98]
		Thickening		[99]
		Thickening		[100]
		Thickening		[89]
PD	**PD Rodents**	-	-	-
	PD Humans	Thickening		[99]
		Thickening		[121]
		Morphological Change		[122]
ALS	**ALS Rodents**	Thickening, Duplication	$SOD1_{G93A}$	[46]
	ALS Humans	Exposed to plasma proteins		[42]

Table 3: Summary of laminin changes in normal aging and neurodegenerative diseases

		Laminin	Model	Reference
Normal aging	**Aged Rodents**	↓		[103]
		↓		[84]
		↑		[86]
	Aged Humans	↓		[88]
AD	**AD Rodents**	↔	$PSEN1_{P117L}$	[91]
		↑	$APP_{swe/E693G}$	[102]
		↓	APOE4	[178]
	AD Humans	↑		[144]
		↑		[145]
PD	**PD Rodents**	-	-	-
	PD Humans	-	-	-
ALS	**ALS Rodents**	↓	$SOD1_{G93A}$	[47]
	ALS Humans	↓ (muscle BM and limb muscles)		[158]
		↑		[159]
		↑ (skin)		[160]

Table 4: Summary of collagen IV changes in normal aging and neurodegenerative diseases

		Collagen IV	Model	Reference
Normal aging	**Rodents**	↔		[103]
		↓		[84]
		↑		[86]
	Humans	↑		[87]
		↑		[88]
		↔		[89]
AD	**Rodents**	↑	3xTG	[90]
		↔	$PSEN1_{P117L}$	[91]
		↑	3xTG	[92]
		↓	APOE4	[102]
		↓	APP_{swe}	[178]
	Humans	↑		[93]
		↑		[95]
		↑		[96]
		↑		[97]
		↑		[87]
		↑		[99]
		↔		[89]
		↑		[179]
		↑		[180]

PD	**Rodents**	↑	α-synuclein$_{A30P}$	[184]
	Humans	↑		[99]
		↑		[121]
ALS	**Rodents**	↑	SOD1$_{G93A}$	[44]
	Humans	↑		[42]
		↓		[44]
		↓		[186]
		↓ (skin and serum)		[187]
		↔ (muscle)		[157]

Table 5: Summary of nidogen changes in normal aging and neurodegenerative diseases

		Nidogen	**Model**	**Reference**
Normal Aging	**Rodents**	↓		[84]
		↑		[86]
	Humans	↑		[89]
AD	**Rodents**	-	-	-
	Humans	↓		[89]
PD	**Rodents**	-	-	-
	Humans	-	-	-
ALS	**Rodents**	-	-	-
	Humans	-	-	-

Table 6: Summary of HSPG changes in normal aging and neurodegenerative diseases

		Perlecan	Agrin	Model	Reference
Normal Aging	**Rodents**	↑			[103]
		↑			[84]
		↑			[86]
	Humans	↑			[88]
AD	**Rodents**	↔	↔	APOE4	[102]
	Humans	↓			[101]
		↑	↑		[93]
PD	**Rodents**	-	-	-	-
	Humans	-	-	-	-
ALS	**Rodents**		↓	$SOD1_{G93A}$	[49]
			↓ (neuromuscular junctions)	$SOD1_{G93A}$	[230]
	Humans		↔ (neuromuscular junctions)		[157]

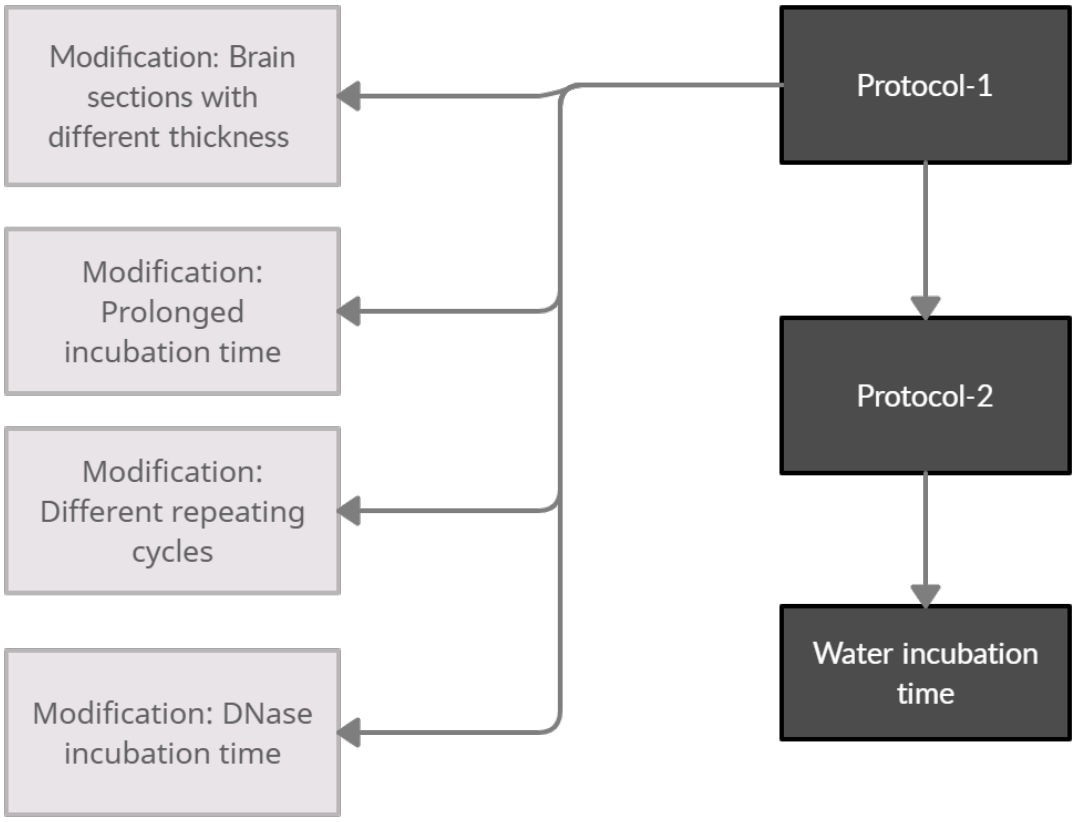

Figure 1: Summary of protocol optimization steps.

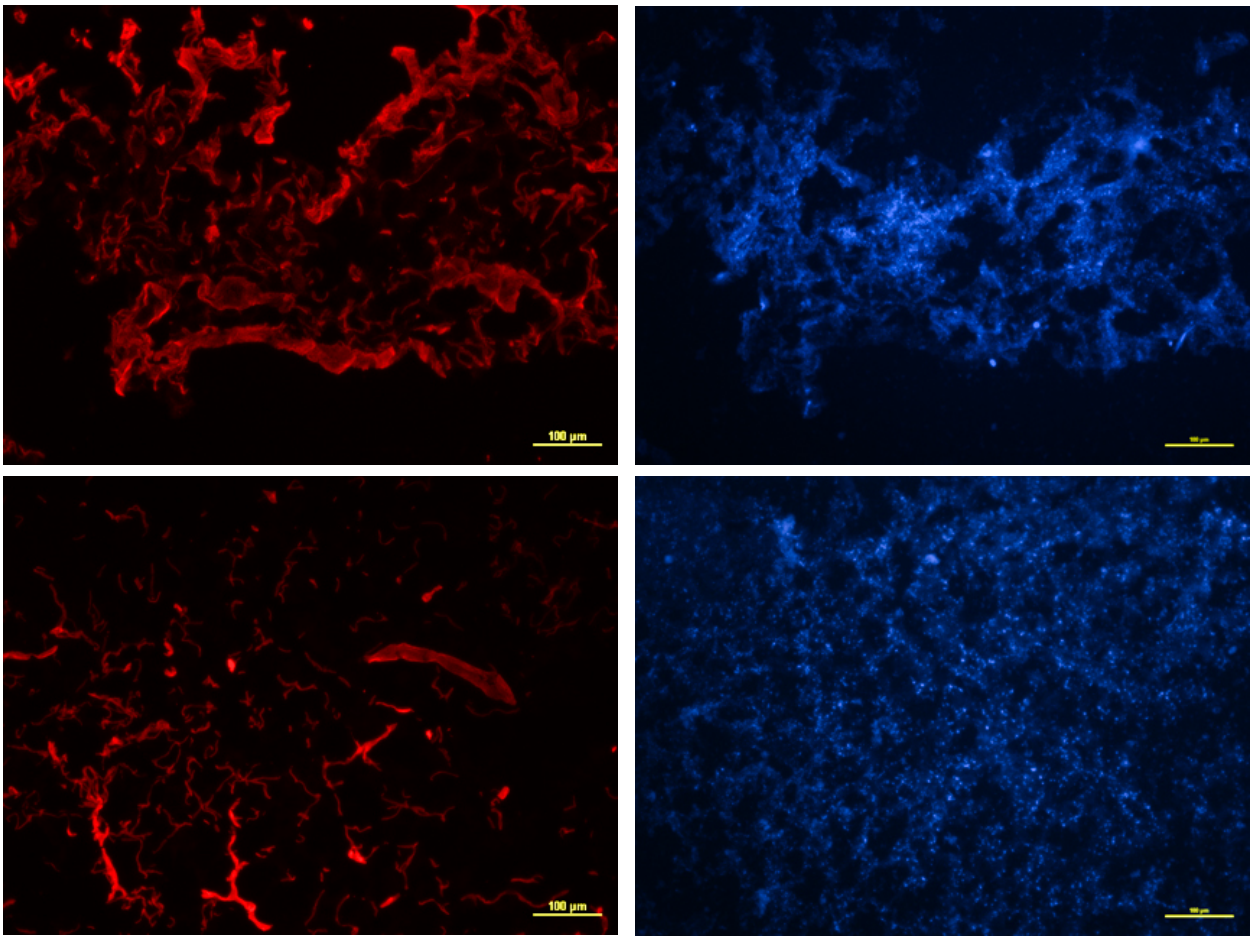

Figure 3: IHC staining of modified protocol-1 with surface layer section (top) and 500µm inner layer section (bottom) samples using the initial adapted protocol. Representative images showing laminin (red) and DAPI (blue) from 30µm sections at 20x.

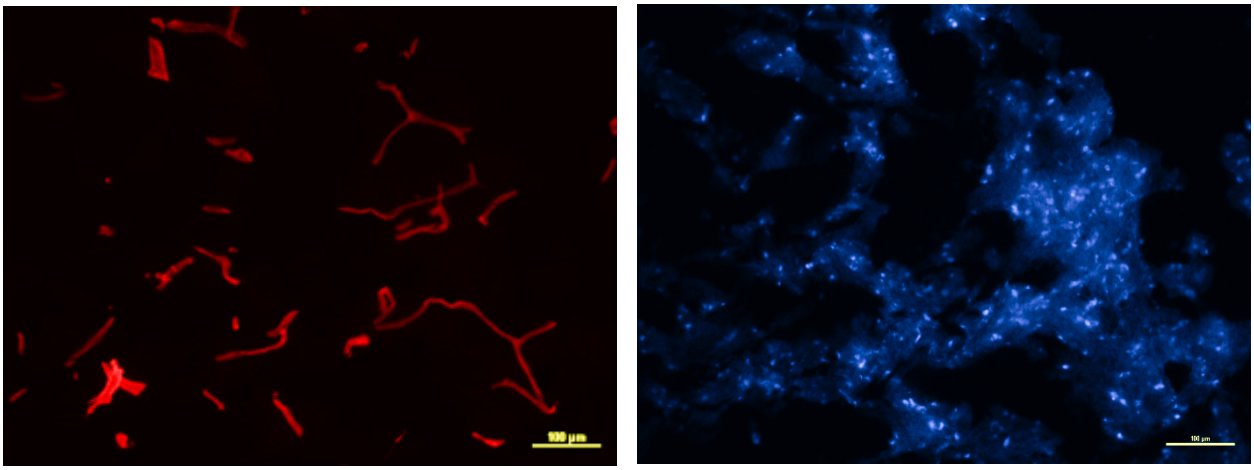

Figure 2: IHC staining of 500µm (top) and 300µm (bottom) samples using the protocol-1. Representative images showing laminin (left) and DAPI (right) from 30µm sections at 20x.

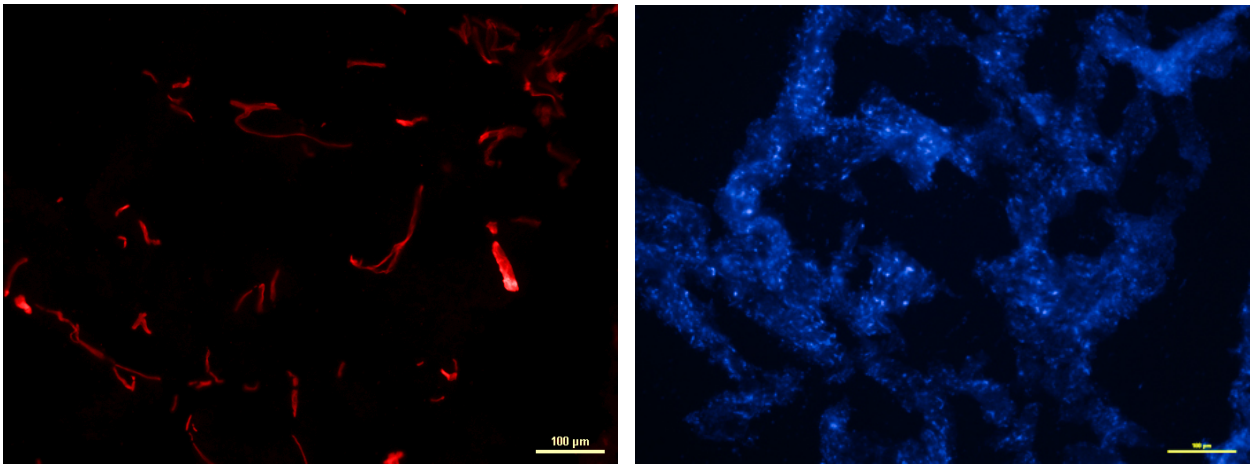

Figure 4: IHC staining of modified protocol-1 with 24hr in DNase. Representative images showing laminin (red) and DAPI (blue) of 30µm sections at 20x.

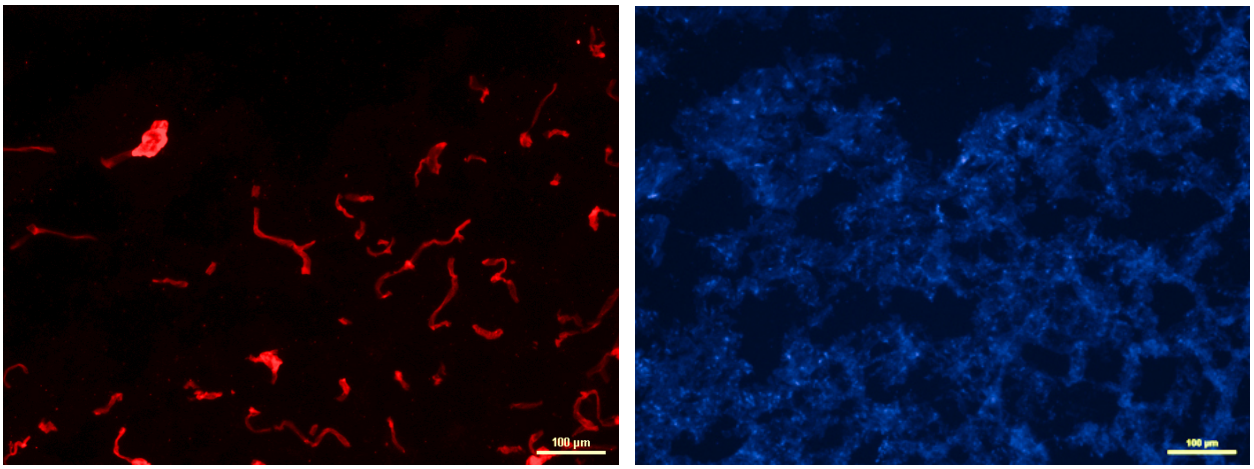

Figure 5: IHC staining of modified protocol-1 with prolonged detergent incubation cycles. Representative images showing laminin (red) and DAPI (blue) of 30µm sections at 20x.

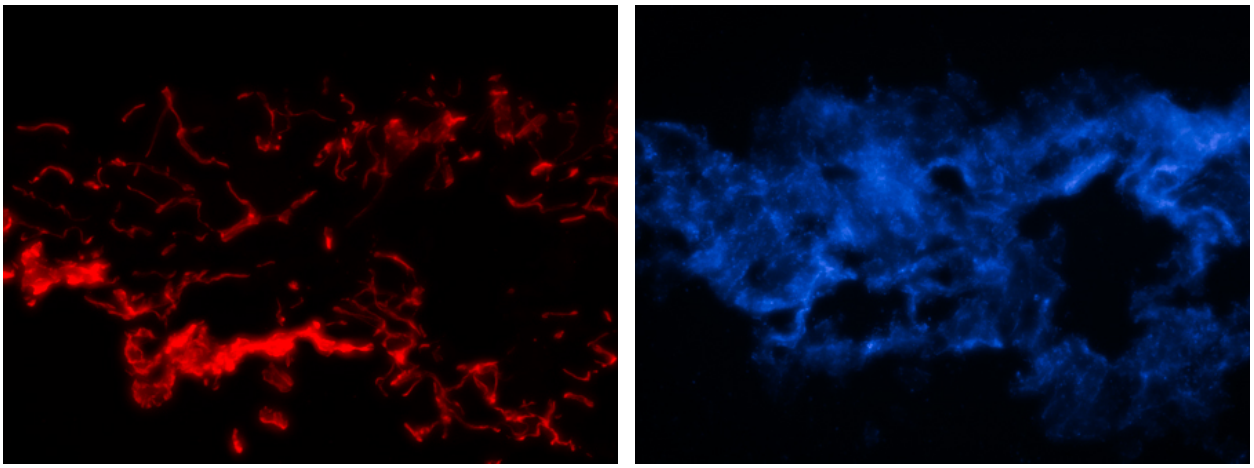

Figure 6: IHC staining of modified protocol-1 with repeated protocol cycles. Representative images showing laminin (red) and DAPI (blue) of 30µm sections at 20x.

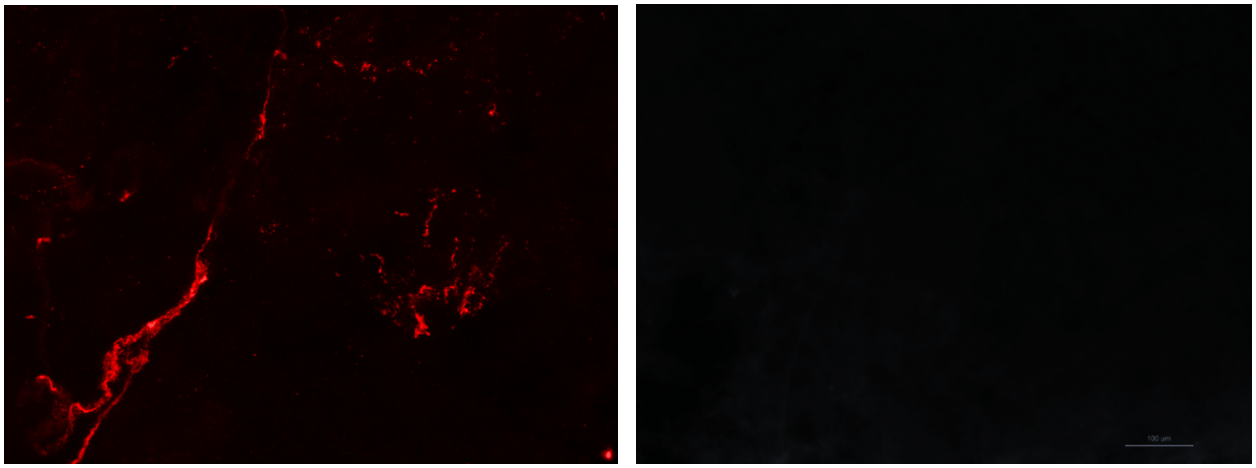

Figure 7: IHC staining of protocol-2. Representative images showing laminin (red) and DAPI (blue) of 500µm tissues at 20x.

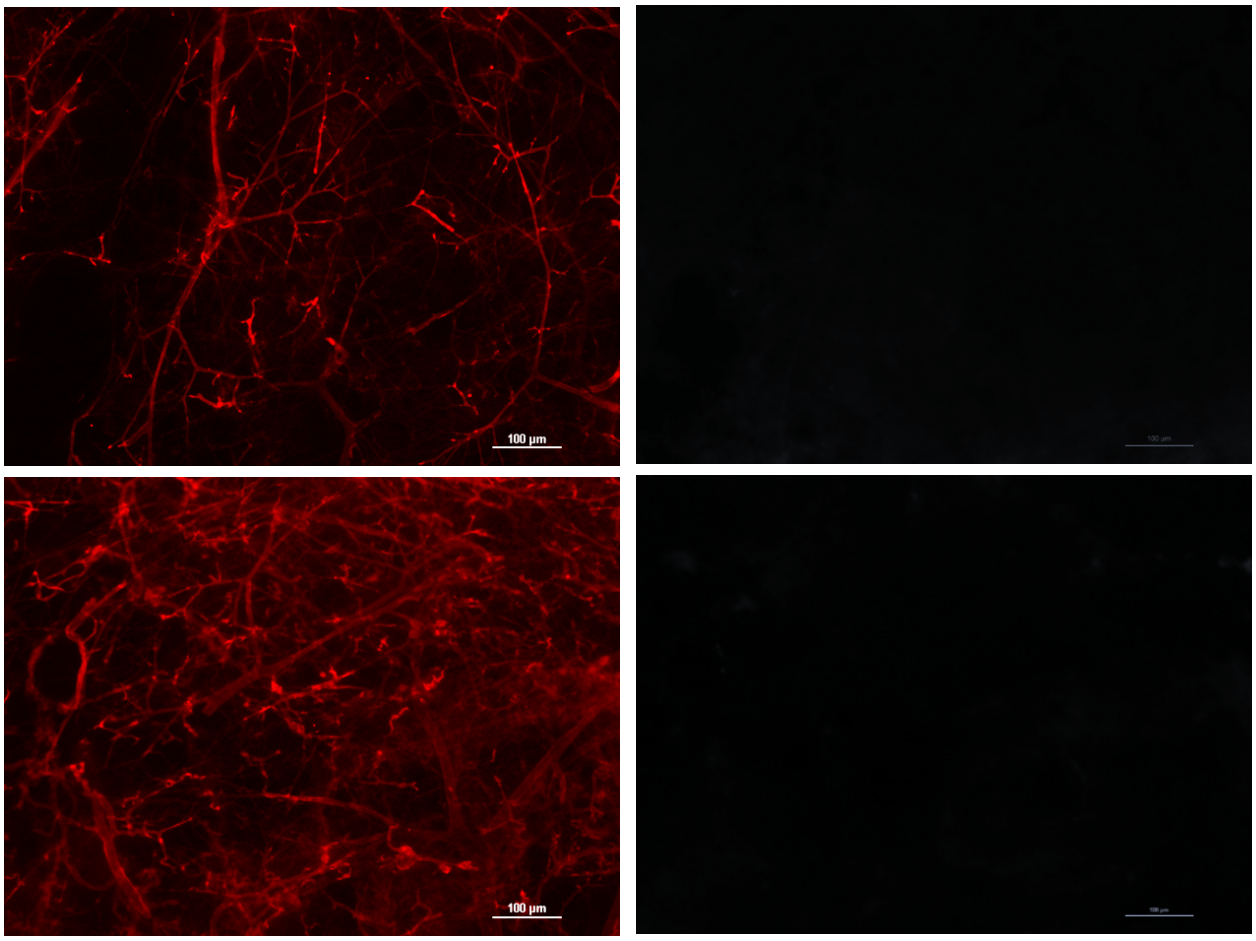

Figure 8: IHC staining of the protocol-2 modifications with 0hr incubation of water (top) and 24hr incubation (bottom). Representative images showing laminin (red) and DAPI (blue) of 500µm tissues at 20x.