SAHLGRENSKA ACADEMY

# The role of reactive gliosis in post-stroke secondary neurodegeneration in mice

Degree Project in Medicine

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## Abstract

The consequences of stroke include not only the loss of function due to neuronal death in the infarcted area but also secondary neurodegeneration in the adjacent and remote parts of brain. To better understand the role of astrocytes in stroke-related neurodegeneration we studied the deposition of the neurodegeneration marker amyloid-β peptide (Aβ), neuroinflammation in terms of microglia activation and neuronal density in the thalamus of the genetically ablated *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice (a model of attenuated reactive gliosis) 7 weeks after the photothrombotic cortical stroke induction. *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice showed an increased Aβ deposition in the ipsilateral lateral dorsal (LD) thalamic nucleus (versus the contralateral LD nucleus) compared to wildtype (WT) mice, as assessed with immunohistochemistry. The number of microglia cells in the same nucleus did also increase in the *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice to a greater level than in the WT mice, while the neuronal density showed comparable decrease in both groups after stroke. These results suggest that reactive gliosis may play a protective role by preventing neurodegeneration at later stages after stroke, namely by decreasing Aβ deposition in the thalamus 7 weeks after induced cortical infarct.

**List of abbreviations**

Aβ = Amyloid Beta
Iba1=Allograft inflammatory factor, protein expressed only in activated macrophages
NeuN=A neuronal nuclear antigen
*GFAP*$^{-/-}$*Vim*$^{-/-}$=Mice with no functioning genetic encoding for the GFAP or vimentin proteins
GFAP= Glial fibrillary acidic protein; cytoskeletal protein needed for reactive gliosis.
WT=Wild Type mice, with functional alleles encoding GFAP and vimentin.
LD=Lateral dorsal nucleus of the thalamus
VPL= Ventral posterolateral nucleus of the thalamus
ABC=Avidin-biotin complex
HRP= Streptavidin-horseradish peroxidase
DAB=Diaminobenzidine
SEM= Standard error of the mean

# 1 Introduction

## 1.1 Basic characteristics of stroke.

Stroke is one of the leading causes of death and disability worldwide (Feigin et al., 2009; Lozano et al, 2012). Ischemic stroke, which is caused by embolism or thrombosis of the brain arteries, accounts for 75-80% of all strokes. Haemorrhagic stroke, another important type of stroke, occurs due to intracerebral or subarachnoid bleeding (van Asch et al, 2010). Both ischemic and haemorrhagic stroke are associated with necrosis (infarction) of the brain tissue leading to the consequences depending on the location and extensiveness of the lesion. The area surrounding necrosis (penumbra) experience less pronounced damage that can be partially reversible (Hossmann, 2009; Liu et al., 2012).

The consequences of stroke include not only the loss of function of the affected area but also disturbances in the remote parts of brain connected with the infarcted region (reviewed by Pekny et al., 2019). Stroke often results in cognitive dysfunction, and since many stroke patients have advanced age, it is often difficult to distinguish between stroke complications and age-related neurodegenerative cognitive dysfunction (Amtul et al., 2014; Yasuno et al., 2019). Alzheimer disease, the most common cause of senile dementia, is a neurodegenerative disease associated with progressive accumulation of amyloid-beta peptide (Aβ) in the form of extracellular senile plaques (Breijyeh et al., 2020). Several studies have demonstrated the association between stroke and neurodegeneration, including Aβ deposition, so called secondary neurodegeneration (Pendlebury, 2012). Both clinical observations using positron emission tomography (Pendlebury, 2012; Yasuno et al., 2019) and experiments in rodents (Garcia-Alloza et al., 2011; Martins et al., 2019; Sanchez-Bezanilla et al., 2019) indicate Aβ increase in the vicinity of ischemic injury. Experimental studies have also shown that cortical infarct can lead to Aβ accumulation in remote brain regions (Zhang et al, 2012a; Kluge et al., 2018; Sanchez-Bezanilla et al., 2019), such as ipsilateral thalamus (van Groen T et al., 2005; Zhang et al., 2012b; Ong et al., 2017).

## 1.2 Role of astrocytes in normal brain and response to injury.

Astrocytes are the most common type of macroglia cells that together with neurons have neuroectodermal embryonal origin. In contrast, microglia are represented by neural macrophages that have mesodermal embryonal origin (Janowska et al., 2019).

Physiological role of astrocytes includes structural and metabolic support of the brain neurons, regulation of ion concentration in extracellular space, participation in the maintenance of the blood-brain barrier, uptake and release of neurotransmitters, and modulation of synaptic transmission (Pekny and Pekna, 2014; Rossi, 2015; Butt and Verkhratsky, 2018).

Evidence suggests that astrocytes are less susceptible than neurons to ischemia, which promotes their protective role in stroke (reviewed by Sims and Yew, 2017). If a patient survives stroke, the infarcted brain tissue is demarcated by the glial scar that can gradually result in partial, sometimes almost complete, functional recovery due to neuronal plasticity. Early disruption of scar formation has been shown to prevent functional restoration (Li et al., 2008; reviewed by Sims and Yew, 2017). Glial cells surrounding infarction, namely astrocytes in crosstalk with microglia, have been shown to play a key role in scarring as well as in neuronal plasticity associated with recovery. Activation of astrocytes in response to brain injury is called reactive astrogliosis (reviewed by Sims and Yew, 2017; Pekny et al., 2019).

Important homeostatic mechanism of astrocytes is their ability to control cell volume by excreting osmotically active molecules, such as taurine, thus preventing deleterious effect of stroke-related oedema (Pasantes-Morales and Vázquez-Juárez, 2012). Astrocytes also prevent oedema by forming a paravascular pathway (called glymphatic system in analogy with lymphatic system) that facilitates flow of the cerebrospinal fluid through the brain (Iliff et al., 2012). This flow is aided by aquaporin-4 channels in the astrocyte membrane (Papadopoulos et al., 2004).

In brain injury, astrocytes are exposed to molecules released from dead and dying cells, passing through a leaky blood-brain barrier, or produced by leukocytes that enter the CNS. These molecules, such as transforming growth factor alpha, ciliary neurotrophic factor, interleukin 6, leukaemia inhibitory factor, or oncostatin M induce changes of astrocyte genomic expression leading to a wide range of molecular, structural, and functional alterations that help restoring homeostasis (Balasingam et al, 1994; Winter et al., 1995; Klein et al., 1997; Rabchevsky et al., 1998; Sriram et al, 2004; Hostenbach et al., 2014). JAK-STAT3 pathway plays a key role in mediating molecular signalling from cell membrane to the nucleus resulting in astrocyte activation (reviewed by Ceyzeriat et al., 2016). Several proteins become upregulated, including components of intermediate filaments (nanofilaments) like glial fibrillary acidic protein (GFAP), vimentin, nestin and synemin, being responsible for changes in astrocyte morphology and induction of proliferation (Bardehle et al., 2013; Pekny and Pekna, 2014; Pekny et al., 2019). Some proliferating astrocytes in the stroke penumbra migrate towards the centre of the lesion and form the glial scar by secreting extracellular matrix molecules. Scar formation prevents spreading of the lesion (Bush et al., 1999; Myer et al., 2006). More peripherally located astrocytes do not proliferate but become hypertrophic, with thickened processes, mainly remaining within their domains (reviewed by Pekny et al., 2019).

Reactive astrocytes also control neuronal plasticity, the basis of functional recovery. This is mainly dependent on astrocytes located more distantly from the lesion. These cells produce factors stimulating neuronal sprouting and synaptogenesis, such as thrombospondin 1 and 2, brain-derived neurotrophic factor (BDNF), glia-derived neurotrophic factor (GDNF), and ciliary neurotrophic factor (reviewed by Gleichman and Carmichael, 2016). Astrocytes can also inhibit neural repair after stroke. For example, ephrin-A5 that is upregulated in periinfarct reactive astrocytes blocks neuronal outgrowth and minimizes repair (Overman et al, 2012).

### 1.3 Role of microglia and circulation-derived cells in stroke.

Microglia provides for immune response of the nervous system by eliminating microbes, dead cells and protein aggregates as well as by secreting various soluble factors contributing to immune response and tissue repair (Colonna and Butovsky, 2017). As resident macrophages of the brain, microglia cells are the first immune cells to respond to ischemia (Lambertsen et al., 2019).

Within hours after stroke microglia in brain tissue around the lesion acquire amoeboid shape with few short processes (Morrison and Filosa, 2013). This microglial reaction is thought to be induced by loss of contacts due to degeneration of neighbouring cells and release of molecules from damaged cells (Kim et al., 2014). Over the next few days, reactive microglial cells increase in the perifocal region due to proliferation and migration from surrounding tissue (Schroeter et al., 2002).

Microglial activation is associated with several phenotypical changes that can be arbitrary classified as pro-inflammatory and anti-inflammatory type, called M1 and M2, respectively (Ransohoff, 2016). This process is called polarization of microglia (Hu et al., 2012). It is generally accepted that, in the acute stage of stroke (within 1 day), proliferation and activation of microglia cause a strong inflammatory reaction detrimental to the brain, whereas in the chronic stage (several days after onset), microglia can produce a variety of protective cytokines (e.g., insulin-like growth factor 1) contributing to neural repair (Xu et al., 2020).

Interestingly, a population of cells expressing both astrocyte and microglial markers have been recently identified in the injured brain (Wilhelmsson et al, 2017). These cells were not found in normal brain, and their role and genesis are unknown. There is a possibility that these cells form due to fusion of macroglia and microglia cells. Another possibility that some initially silent genes become upregulated in one of the cell types.

The periinfarct tissue is also influenced by cells invading from the circulation. Blood-derived monocytes migrated into the damaged brain tissue mature into macrophages. These cells are indistinguishable from microglia based on morphology or widely used marker proteins such as CD68 or IBA1 (reviewed by Sims and Yew, 2017). Other leukocytes, mainly neutrophils, also enter the infarct and periinfarct area (Jin et al., 2010).

### 1.4 Modulation of astrocyte activation in stroke.

The diverse role of astrocytes during stroke development opens the opportunities for its modulation that can decrease brain damage and improve recovery. Astrocyte activation has been shown to be not only beneficial, mainly in acute phase of stroke, but also unfavourable, due to inhibiting adaptive neural plasticity and other recovery mechanisms. Excessive scar formation is another possible outcome of reactive gliosis that limits recovery (Sims and Yew, 2016; Pekny et al, 2019).

One possibility is to investigate the role of reactive astrocytes in stroke and other types of brain injury is genetic ablation of astrocyte intermediate filament proteins, such as GFAP and vimentin. For this purpose, genetically modified *GFAP*$^{-/-}$*Vim*$^{-/-}$ knockout mice have been engineered (Eliasson et al., 1999). These mice possess astrocytes that function normally under physiological conditions but do not develop compensatory hypertrophy of their processes after injury.

In acute stage of brain damage *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice showed delayed healing and more pronounced synaptic loss (Pekny et al., 1999; Wilhelmsson et al, 2004) while in neurodegeneration conditions (Alzheimer's or Batten disease) they were characterised by increased neuronal damage (Kraft et al, 2013; Macauley et al, 2011). Brain ischemia in *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice resulted in more extensive necrosis as well as in lower glutamine levels, decreased glutamate transport and increased oxidative stress observed in astrocytes (Pekny et al., 1999;

Li et al, 2008; De Pablo et al, 2013). These results suggest a protective role of astrocyte activation in early stage of brain injury.

On the other hand, several studies suggest a negative role of astrocyte activation at later stages of nervous system damage. For example, attenuated reactive gliosis in *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice results in a few positive effects, such as better synaptic regeneration (Wilhelmsson et al, 2004), better recovery after spinal cord injury (Menet et al., 2003), and better posttraumatic regeneration of the optic nerve in postnatal mice (Cho et al., 2005). Furthermore, the degradation of chondroitin sulphate proteoglycans produced by astrocytes can stimulate axonal regeneration (reviewed by Pekny et al, 2019). Also, *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice show better survival of neural grafts (Kinouchi et al., 2003) and better neuronal differentiation of transplanted adult neural stem cells (Widestrand et al, 2007).

# 2 Aims

Although Aβ deposition and other signs of post-stroke secondary neurodegeneration have been reported by several research groups (see above), little is known about the role of astrocyte activation in this process. In this study we investigate the influence of the attenuation of reactive gliosis in genetically ablated $GFAP^{-/-}Vim^{-/-}$ mice on secondary neurodegeneration and inflammation in the ipsilateral thalamus after photothrombotic stroke.

The specific aims of the study were to determine the effect of attenuation of reactive gliosis on:

(A) Aβ deposition in the thalamus after experimentally induced photothrombotic stroke.

(B) Post-stroke changes in the number of microglia cells as an indication of inflammation.

(C) Post-stroke changes in neuronal density, as an additional sign of neurodegeneration.

# 3 Method

## 3.1 Animals

*GFAP*$^{-/-}$*Vim*$^{-/-}$ mice, bred carrying null mutations in the GFAP and vimentin genes, (Eliasson et al., 1999) and C57BL/6-Sv129-129Ola wild type (WT) controls of a mixed genetic background were used for this study. Adult (10–12 weeks old, 22–27g body weight) male *GFAP*$^{-/-}$*Vim*$^{-/-}$ and age-matched WT mice were generated for this study by an in-house breeding facility. A total of 19 animals were used in the study. One animal was lost from failure to retrieve usable material from the relevant structures. For the Aβ study only 16 animals were used as this was an earlier timepoint with access to fewer specimen.

## 3.2 Photothrombotic induction of focal ischemia

Consistent cerebral infarct volume in *GFAP*$^{-/-}$*Vim*$^{-/-}$ and WT mice through focal cortical ischemia was induced by photothrombosis of the cortical microvessels with the photosensitive Rose Bengal technique as described previously by (Watson et al., 1986; Lee et al., 2007). 100 μL Rose Bengal solution in saline (10 mg/mL; Sigma-Aldrich, St Louis, MO) was injected intraperitoneally 5 min before illumination. A midline incision of the scalp was performed to expose the skull covering the caudal forelimb area and the rostral forelimb area (Jang et al, 2013). The exposed brain was illuminated for 10 min with a fiber-optic bundle of a cold light source (KL 1600 LED; Schott, Mainz, Germany) using a green filter. The scalp was then sutured, and mice were allowed to awake. The approximate location of induced brain infarct is shown in Fig. 1.

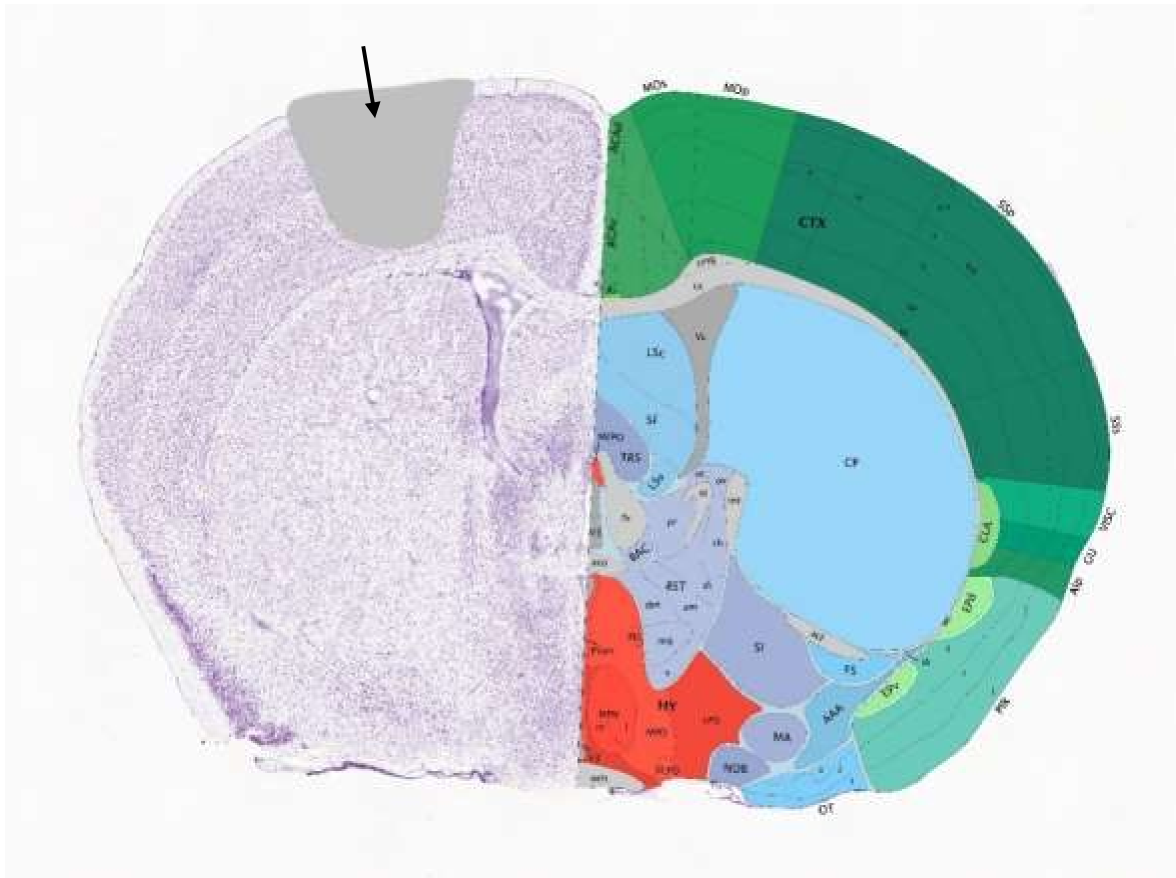


***Fig. 1*** *Approximate site of photothrombotic infarct in the primary motor cortex (MOp, arrow). Allen Institute for Brain Science. Allen Mouse Brain Atlas, 2004 ("http://atlas.brain-map.org/atlas?atlas=1&plate=100960240#atlas=1&plate=100960305&resolution=20.17&x=5409.156155873494&y=4022.1688511859943&zoom=-4&z=5").*

### 3.3 Tissue Preparation

7 weeks after photothrombosis, under deep Ketamine anesthesia, the mice were perfused transcardially with saline, and then with 4% paraformaldehyde. The entire brain was immersed in 4% paraformaldehyde overnight and infiltrated and the brain embedded in paraffin. The lesion volume was estimated using Haematoxylin and Eosin staining and measured as percentage of lesion area compared with the contralateral hemisphere, as previously described by Swanson et al (1990).

### 3.4 Immunohistochemistry

8 µm thick paraffin serial coronal brain slices from WT and *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice were cut with a microtome and grouped on glass slides. After deparaffinization and antigen retrieval using 10 mM hot citrate buffer with 2 mM EDTA (pH 6.2), sections were treated with hydrogen peroxide to block endogenous peroxidase and processed for single label immunostaining.

For immunostaining of Aβ, sections were first incubated with primary rabbit-anti-mouse polyclonal anti-Aβ IgG antibodies (BioLegend, San Diego, CA, USA, dilution 1:500) at 4°C overnight and then with secondary donkey-anti-rabbit biotinylated IgG antibodies (Jackson ImmuoResearch Laboratories, West Grove, PA, USA, dilution 1:250) at room temperature for 1 h. One negative control mouse, which underwent the whole protocol sans antibodies, was used and showed no signal.

For labelling of microglia, sections were incubated with primary rabbit anti-Iba1 antibodies (Wako Chemicals, Richmond, VA, USA, dilution 1:200) at 4°C overnight and then with biotinylated donkey anti-rabbit secondary antibodies (Jackson ImmuoResearch Laboratories, dilution 1:500) at room temperature for 1 h.

For visualisation of neurons, sections were incubated with biotinylated mouse anti-NeuN antibodies (Millipore, Billerica, MA, USA, dilution 1:100) at 4°C

After incubation with biotinylated primary or secondary antibodies, sections were treated with avidin-biotin complex (ABC) containing streptavidin-horseradish peroxidase (HRP) and then developed using diaminobenzidine (DAB) as a substrate according to the manufacturer instructions (Vector Laboratories, Burlingame, CA, USA). As a result, immunoreactive sites were labelled with dark brown DAB precipitates.  One section per slide was used as negative control and received the whole protocol sans antibodies were used in the Iba1 and NeuN stainings and showed no signals.

Under the microscope, structures with more intensive DAB staining were identified. Three slides per mouse were selected at a similar depth to show the same structures according to allen mouse brain map (fig. 2 and 3). These slides, with an average of six sections per slide, were then examined microscopically and the best of every two sequential sections were selected and photographed.

Specimens were observed and photographed in bright field using a Nikon Eclipse 80i microscope and digital camera at 20x magnification, acquiring one ipsilateral and one contralateral image per brain section.

Adjacent slides were then immunostained using primary antibodies against NeuN (Millipore, Billerica, MA), and Iba1 (Wako, Richmond, VA).

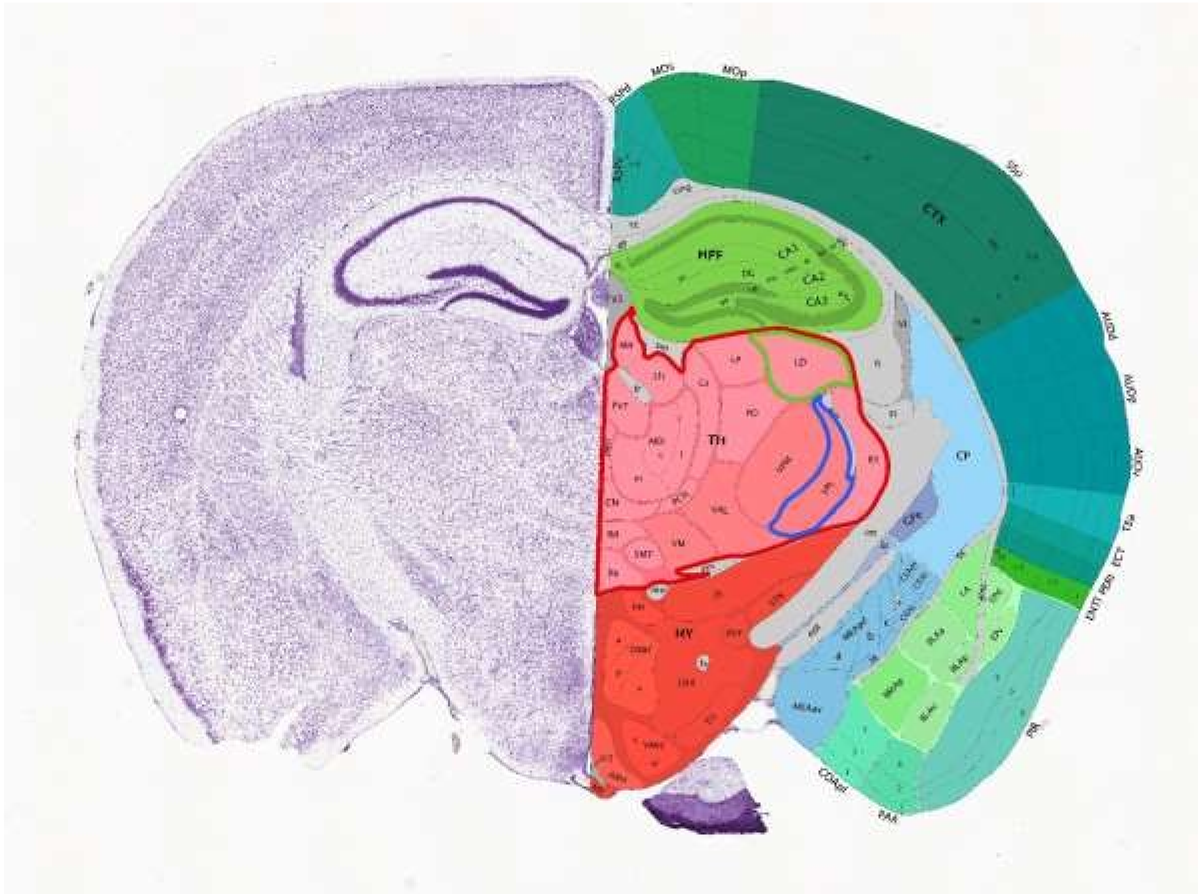


***Fig. 2*** *Thalamic nuclei at the approximate depth studied with immunohistochemistry in this study. Thalamus circled in red, LD circled in green, VPL circled in blue. Allen Institute for Brain Science. Allen Mouse Brain Atlas, 2004 ("http://atlas.brain-map.org/atlas?atlas=1&plate=100960240#atlas=1&plate=100960236&resolution=20.17&x=5409.156155873494&y=4022.8112645896085&zoom=-4").*

*Fig. 3 Axonal projections from the right primary motor cortex labeled by rAAV tracers and visualized using serial two-photon tomography. Thalamus circled in green, ipsilateral LD circled in green, ipsilateral VPL circled in blue.LD and VPL Allen Institute for Brain Science. Allen Mouse Brain Connectivity Atlas, 2011 ("http://connectivity.brain-map.org/projection/experiment/siv/180720175?imageId=180720405&imageType=TWO_PHOTON,SEGMENTATION&initImage=TWO_PHOTON&x=20020&y=8020&z=3").*

### 3.5 Image analysis and statistics

Images were processed in ImageJ software (*https://imagej.net/*), removing artifacts and converting the image to inverted black and white and the correct image format for Metamorph Image Analysis software. Images were then tested in Metamorph and thresholds set to include DAB precipitates but exclude background noise. In this software the app Count nuclei was then

used and settings were set such that clearly visible puncta were counted, noise was not and each puncta was separate. The software then produced an excel file with total nuclei (DAB precipitates in this case), mean area and average intensity for each separate brain section. Similar procedures were repeated for Iba1- and NeuN-staining.

The mean number for each mouse were then calculated and mice divided into genotypes and negative controls. The results were analysed for normality using the Shapiro-Wilk and Kolmogorov-Smirnov tests in Graphpad Prism. Two-way ANOVA was used to compare independent variables (ischemic lesion and genotype) with Tukey's method for post hoc analysis. Multiple unpaired t-tests were performed to compare the laterality ratio between genotypes. A value of $P<0.05$ was taken as significant. Power was not assessed prior to the study as no earlier studies with similar variables exist and no expected means or variation was forthcoming.

### 3.6 Ethical reflection

The ethical considerations of this preclinical study most relate to the handling of animals, for which permission has been granted by the board. All tissue material has been acquired prior to study.

# 4 Results

## 4.1 Amyloid β deposition is increased in *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice after stroke.

Aβ deposition is used as a marker of neurodegeneration in several experimental models, though it has yet to be determined if it plays a pathogenic role in stroke or is just caused by the linked diseases, like Alzheimer disease.

Brain sections of *GFAP*$^{-/-}$*Vim*$^{-/-}$and WT mice were immunostained for Aβ 7 weeks after induction of photothrombotic stroke. The lateral dorsal (LD) nucleus of the thalamus was identified as a structure with marked and quantifiable Aβ deposits as assessed using computer assisted image analysis. Both *GFAP*$^{-/-}$*Vim*$^{-/-}$ and WT mice showed a dramatic increase of Aβ in the LD nucleus ipsilateral to the injury as compared to the contralateral LD nucleus (Fig. 4). This increase was significantly more pronounced in *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice than in WT mice. There was no significant difference in Aβ deposition in the contralateral LD nucleus between the two groups (Fig. 5).

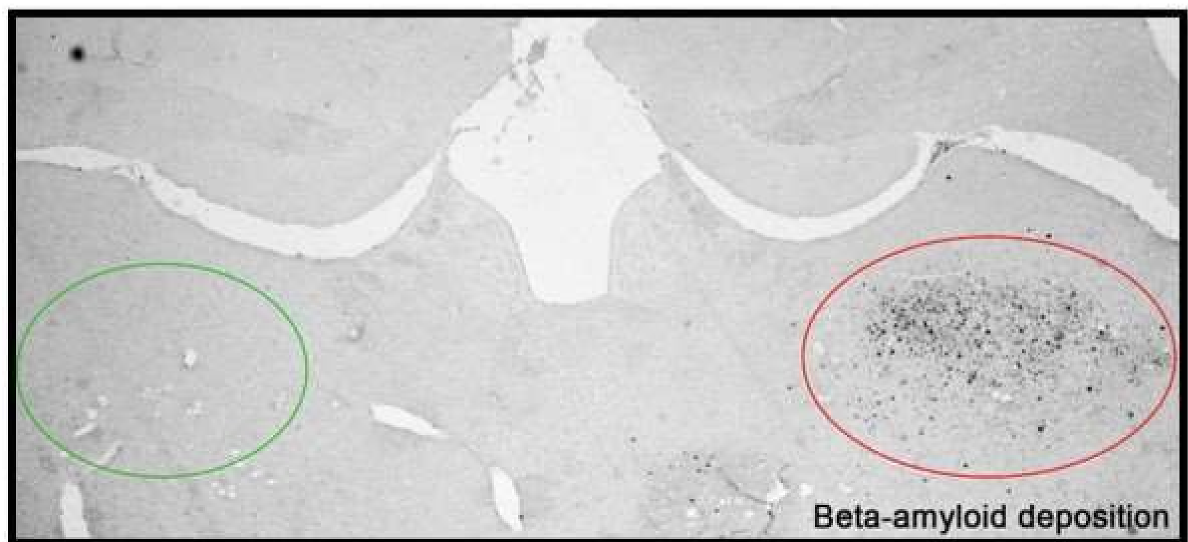


***Fig. 4.*** *Beta amyloid deposition in the ipsilateral and contralateral LD nucleus of GFAP*$^{-/-}$*Vim*$^{-/-}$ *mice.*

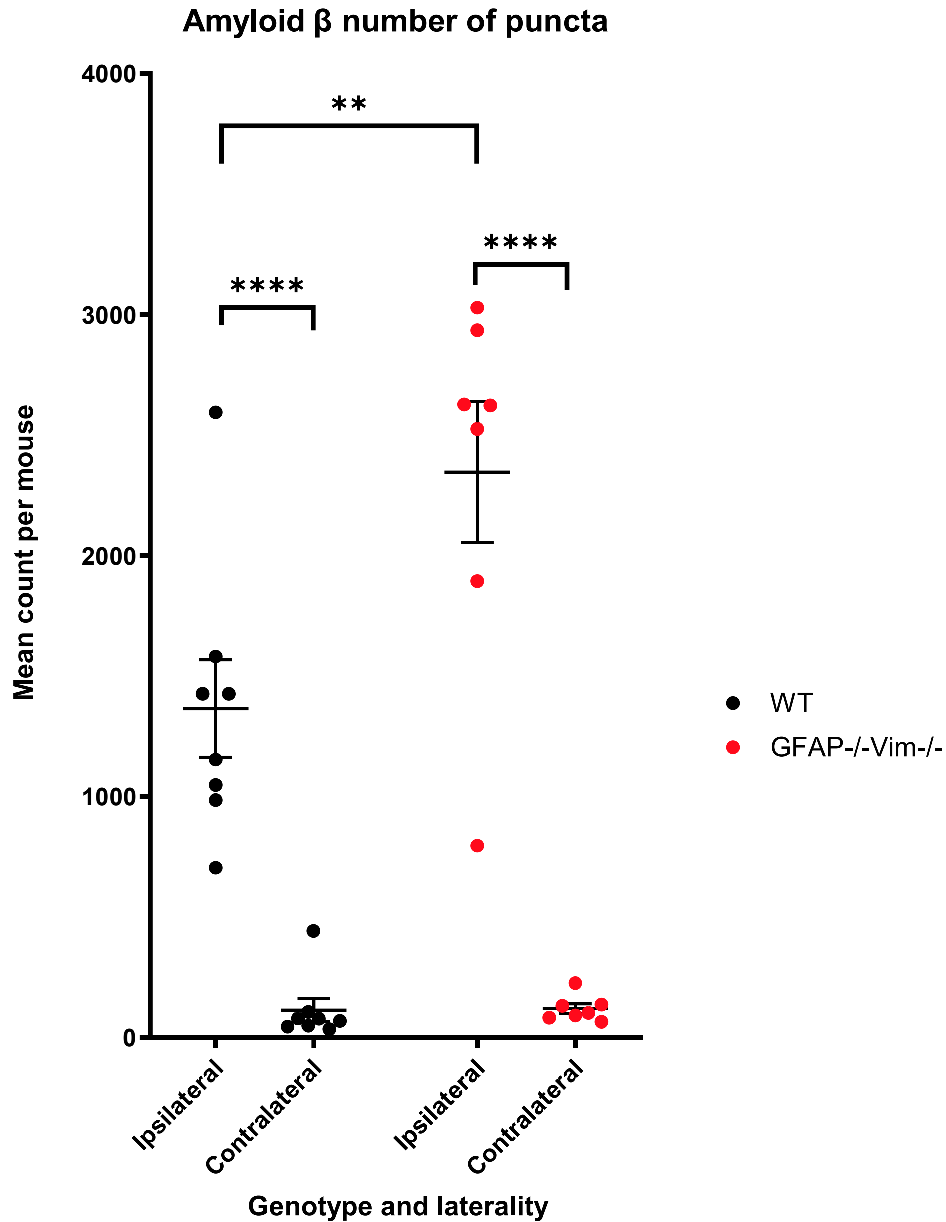


***Fig. 5.*** *Dot plots representing the number of Amyloid β (Aβ) deposits in the lateral dorsal (LD) nucleus of the thalamus 7 weeks after induction of the photothrombotic stroke as assessed using the Metamorph image analysis software. There were significantly more pronounced Aβ deposition in the LD nucleus of GFAP and vimentin negative (*$GFAP^{-/-}Vim^{-/-}$*)mice(n=7) compared to wild type (WT)(n=8) mice (p= 0.0044) (Two-Way ANOVA). Values are given as Mean ± standard error of the mean (SEM). *=p<.05, **= p<.01, ***= p<.001, ****=p<.0001*

### 4.2 Activation of microglia in *GFAP$^{-/-}$Vim$^{-/-}$* and WT mice after stroke

Iba1 is a cytoplasmic protein that is upregulated in activated macrophages and microglia. Here we used it as a marker for inflammation in sites distal to the induced injury. Computer aided quantification of Iba1 positive macrophages/microglia was performed in the thalamus, specifically the LD and VPL nuclei. The number of activated microglia cells in these nuclei after ischemia increased in both *GFAP$^{-/-}$Vim$^{-/-}$* and WT mice (Fig. 6). Although the numbers of activated microglia cells were not significantly different between ipsilateral regions of *GFAP$^{-/-}$Vim$^{-/-}$* and WT mice (Fig. 7), there was a significantly higher ratio between activated macrophages in ipsilateral to the injury LD nucleus and contralateral LD nucleus for *GFAP$^{-/-}$Vim$^{-/-}$* than for WT mice (Fig, 8). The same was not true for VPL nucleus.

***Fig. 6.*** *Iba1-positive cells in the LD nucleus (superior circle) and VPL nucleus (inferior circle) of GFAP$^{-/-}$Vim$^{-/-}$ mice.*

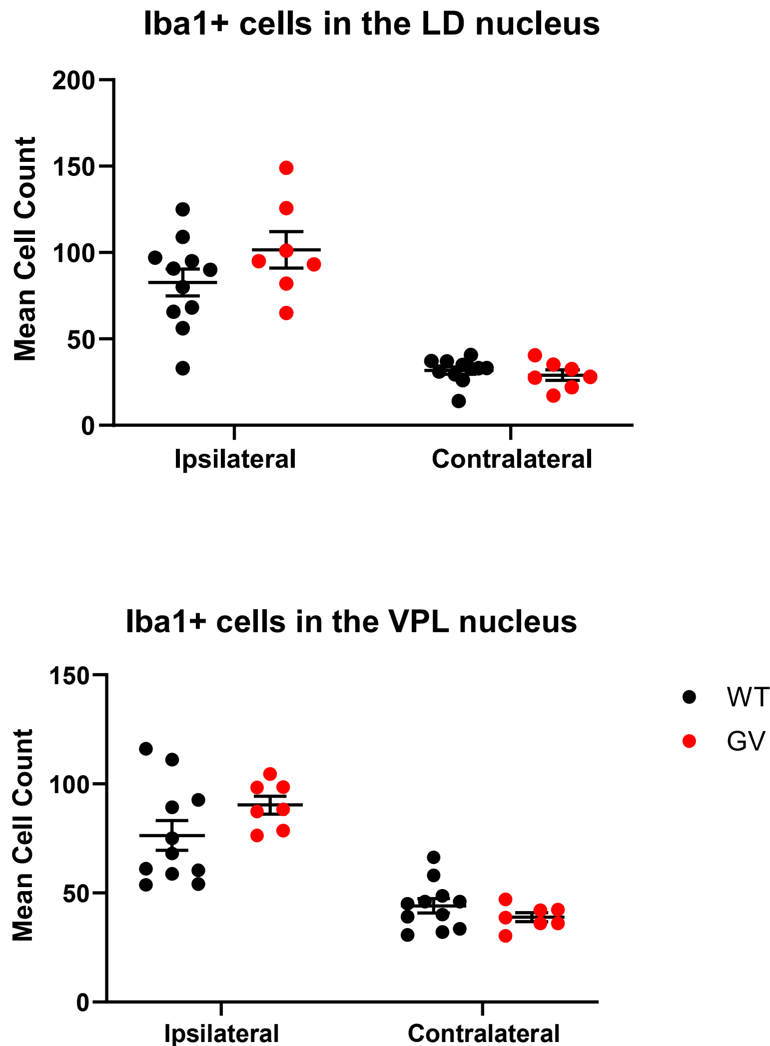


***Fig. 7.*** *Dot plots representing the number of activated (Iba1 positive) macrophages/microglia cells that were quantified with Metamorph image analysis software. The number of activated microglia cells increased after ischemia in the lateral dorsal (LD) and ventral posterolateral (VPL) nuclei ipsilateral to the lesion compared to contralateral for both GFAP and vimentin negative ($GFAP^{-/-}Vim^{-/-}$ )(n=7) and wild type (WT) (n=11)mice. No significant difference in the number of activated microglia cells were found between the two groups (Two-way ANOVA). Values are given as Mean ± standard error of the mean (SEM). *=p<.05, **= p<.01, ***= p<.001, ****=p<.0001*

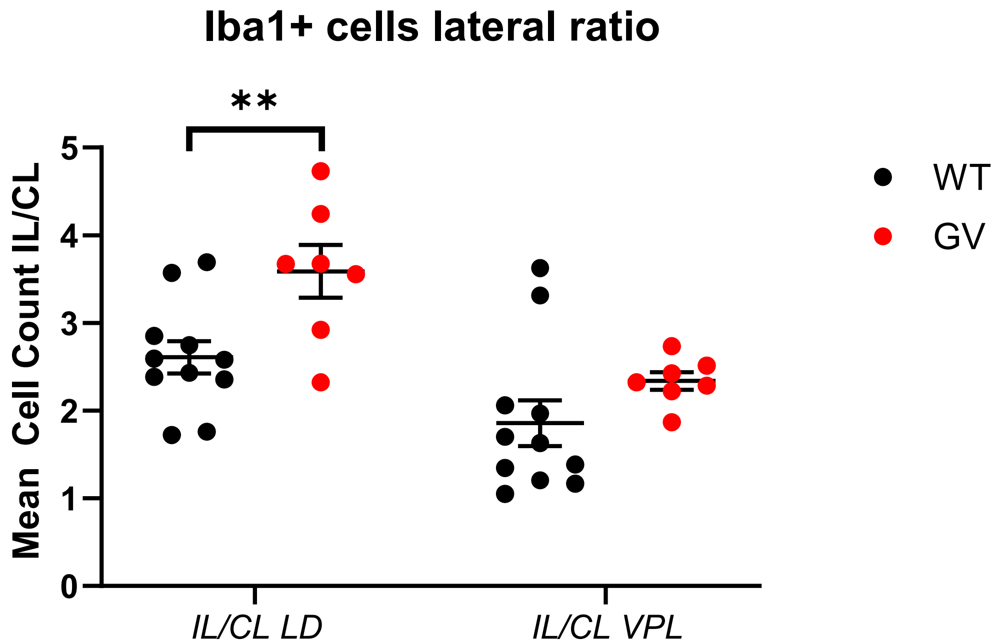


*Fig. 8. Dot plots representing the increase of activated microglia cells in GFAP and vimentin negative ($GFAP^{-/-}$ $Vim^{-/-}$)(n=7) and wild type (WT) mice (n=11) calculated as the ratio between the numbers of activated microglia cells in ipsilateral (ischemic) and contralateral thalamic nuclei of individual mice. The increase of activated microglia cells was significantly larger in $GFAP^{-/-}Vim^{-/-}$ mice for the lateral dorsal (LD) nucleus but not for the ventral posterolateral (VPL) nucleus (Multiple t-tests). Values are given as Mean ± standard error of the mean (SEM). IL, ipsilateral side; CL, contralateral side. *=p<.05, **= p<.01, ***= p<.001, ****=p<.0001*

### 4.3 *$GFAP^{-/-}Vim^{-/-}$* and WT mice show no decrease in thalamic neuronal density after stroke.

Immunohistochemical staining for the the neuronal marker NeuN was used to quantify neurons in the LD and VPL nuclei of the thalamus for comparison between *$GFAP^{-/-}Vim^{-/-}$* and WT mice after stroke induction (Fig. 9). There was no significant decrease in the density of neurons in the LD or VPL nucleus ipsilateral to the injury compared to the contralateral nucleus in either groups. There was no significant difference in neuronal density in the LD and VPL nuclei between *$GFAP^{-/-}Vim^{-/-}$* and WT mice (Figs. 10 and 11).

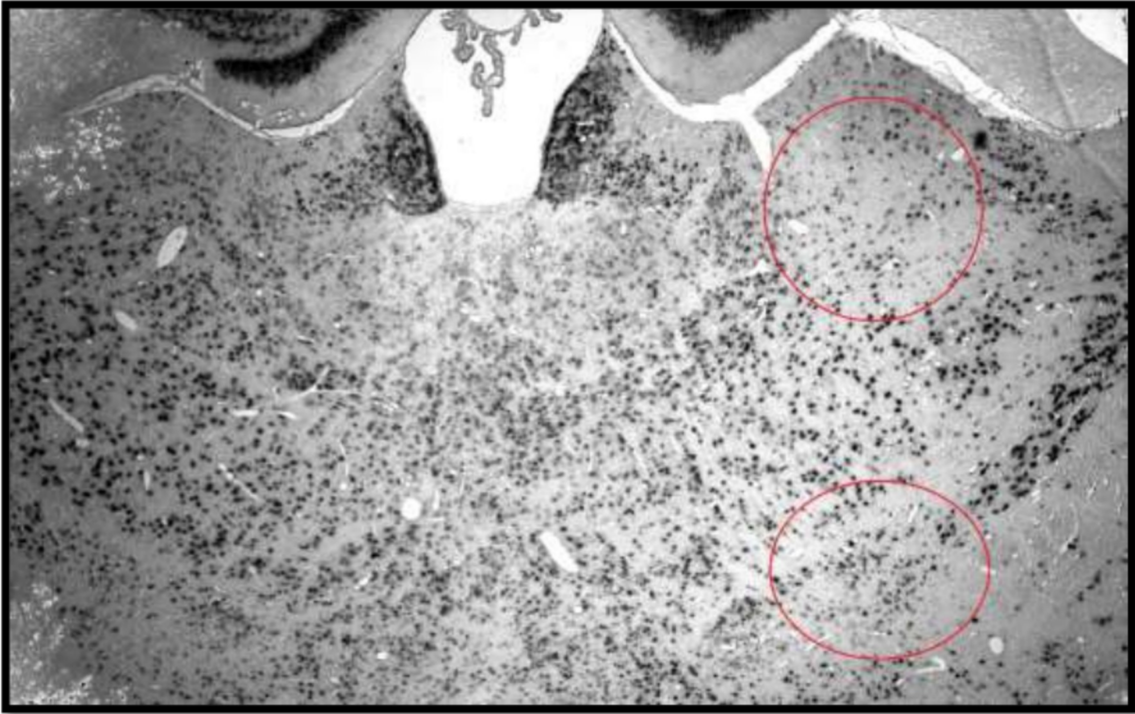

***Fig. 9***. *Neurons visualized with immunostaining for NeuN in GFAP*$^{-/-}$*Vim*$^{-/-}$ *mice. Ipsilateral LD and VPL nuclei are circled.*

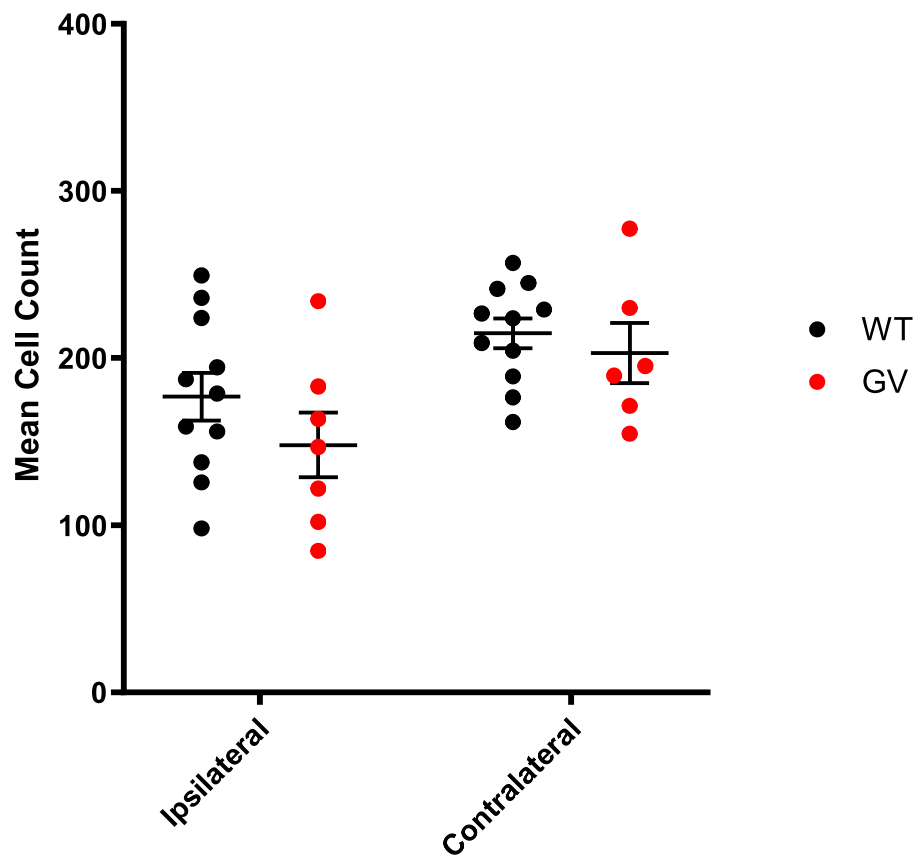


***Fig. 10.*** *Dot plots representing the number of NeuN-positive neurons quantified after stroke in the lateral dorsal (LD) nucleus of the thalamus of wild type (WT) (n=11) and GFAP and vimentin negative (GFAP$^{-/-}$Vim$^{-/-}$) mice (n=7), respectively. There was no significant decrease in neuronal density of the LD nucleus ipsilateral to the injury for either WT or GFAP$^{-/-}$Vim$^{-/-}$ groups. No difference between the two groups were found (Two-way ANOVA, multiple t-tests.). Values are given as Mean ± standard error of the mean (SEM). *=p<.05, **= p<.01, ***= p<.001, ****=p<.0001*

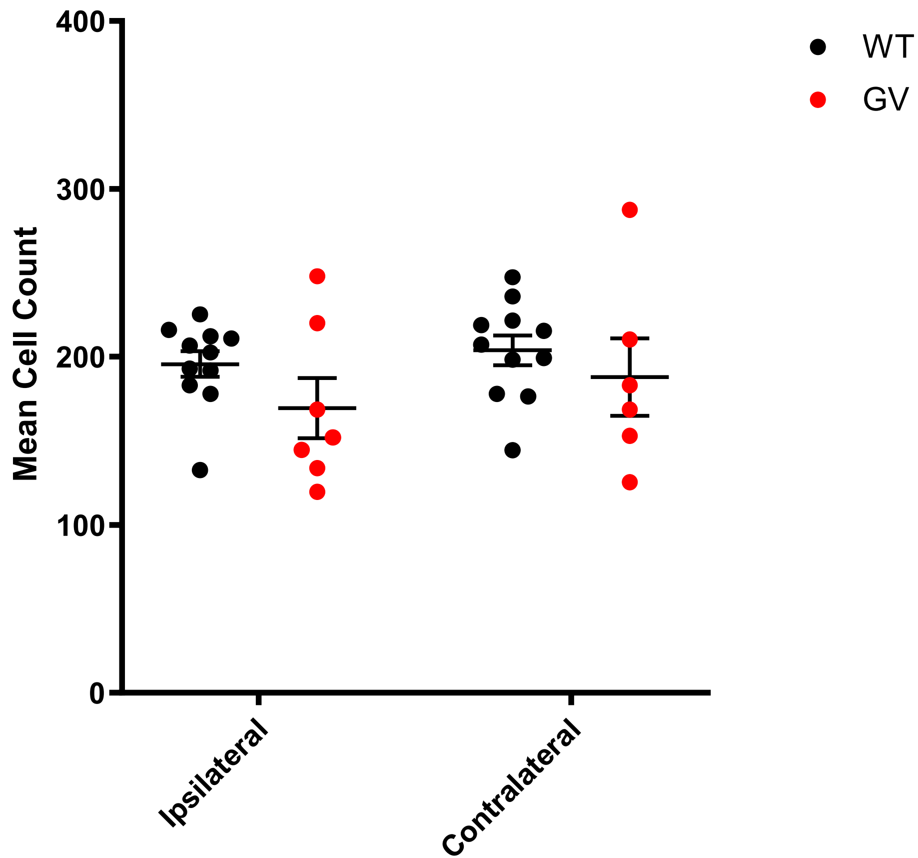


***Fig. 11.*** *Dot plots representing the number of NeuN-positive neurons quantified after stroke in the VPL nucleus of the thalamus of WT (n=11) and GFAP$^{-/-}$Vim$^{-/-}$ mice (n=7), respectively. Neuronal density in the VPL nucleus did not change significantly after stroke in either WT or GFAP$^{-/-}$Vim$^{-/-}$ mice (Two-way ANOVA, multiple t-tests.). Values are given as Mean ± SEM. *=p<.05, **= p<.01, ***= p<.001, ****=p<.0001*

# 5 Discussion

We have shown that the genetically ablated *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice, with affected astrocyte activation, are characterised by increased Aβ accumulation in the thalamus at a later stage (7 weeks) after photochemically induced stroke. These results indicate that reactive gliosis plays a protective role by preventing secondary neurodegeneration in following experimental stroke.

We have also demonstrated that 7 weeks after stroke induction the GFAP-/-Vim-/- mice show a higher relative increase in the number of activated microglia/macrophages in the LD nucleus of the ischemic side (as compared to the contralateral side) than do the WT mice. The increase of activated macrophages suggests a more pronounced inflammation in the LD-nucleus of the *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice following stroke that may be a response to a more pronounced secondary neurodegeneration, as assessed by Aβ accumulation. It is also not excluded that the attenuation of astrocyte activation may have a direct effect on the inflammation. No similar increase of activated macrophages was found in the VPL nucleus of the *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice, at least in this series of experiments, but it is also unknown whether this nucleus shows more Aβ accumulation.

That the VPL nucleus may be less affected in this cortical infarct model than the LD nucleus follows from counts of neuronal density. The latter decreased in the LD nucleus but remained essentially unchanged in the VPL nucleus. However, no statistically significant difference in the degree of neuronal loss have been found in the *GFAP*$^{-/-}$*Vim*$^{-/-}$ and WT mice. The fact that 7 weeks after stroke induction the VPL nucleus shows less damage than the LD nucleus could hypothetically be due to different neuronal connections of these thalamic nucleus with the infarcted cortical area. There is some discrepancy in the results regarding the effects of attenuation of gliosis on Aβ accumulation (that increased) and neuronal counts that were unchanged. Although both parameters are indicative of neurodegeneration it should be noted

that the counted neurons may include both degenerating and normal neurons, as well as regenerating and migrating nervous cells (Lindvall and Kokaia, 2015).

Our results suggest that at a later time point (7 weeks) after stroke induction the reactive gliosis plays a protective role by diminishing secondary neurodegeneration. It is however not clear how this protective astrocyte function develops at earlier and later time points after brain injury. This information is important for understanding the role of astrocytes in the whole process of post-stroke neurodegeneration and regeneration.

Previous studies have shown a positive role of reactive gliosis at the acute/semi-acute stage after ischemic stroke, due to its importance for scar formation that prevents spreading of the infarct to surrounding brain tissue. This was supported by the fact of increased neuronal damage in mice with disturbed astrocyte activation (Li et al, 2008; reviewed by Sims and Yew, 2017; Pekny et al., 2019). However, at later stages of stroke development, reactive gliosis has been shown to interfere with neuronal plasticity and prevent functional recovery. This follows from the facts that inhibition of astrocyte activation in the $GFAP^{-/-}Vim^{-/-}$ mice improved neuronal differentiation, axonal growth, and synaptic regeneration (reviewed by Pekny et al., 2019). Our finding of increased Aβ accumulation in the thalamus of the $GFAP^{-/-}Vim^{-/-}$ mice at a later stage of stroke suggests that reactive gliosis may play not only negative but also a positive role at this time point, namely by preventing neurodegeneration.

The mechanism of Aβ accumulation in the thalamus after cortical infarct as well as its increase due to the inhibition of reactive gliosis remains unclear. Zhang et al. (2012b) have demonstrated that at 7 to 14 days after cortical infarction in hypertensive rats Aβ accumulated in the thalamic VPN neurons resulting in their death. Neuronal death was prevented by the inhibition of autophagy suggesting the role of lysosomal destabilisation by Aβ in neuronal damage according to previously described mechanisms (Zheng et al., 2011). Thus,

accumulation of Aβ in the thalamus 7 weeks after experimental stroke induction also can be due to the activation of autophagy (e.g., as a response to stress induced by cortical infarct), although the work of Zhang et al. (2012b) was performed using different experimental animals and Aβ accumulation was assessed at earlier time point after brain injury.

Hypothetically, reactive gliosis can promote clearance of Aβ accumulated in the brain. This possibility is supported by the ability of macro- and microglia to phagocytose Aβ deposits (Fujita et al., 2020).

# 6 Conclusion

The fact that reactive astrogliosis has both positive and negative effects on the brain tissue at different periods of stroke development raises a question regarding optimal modulation of astrocyte activity in therapeutic purposes. Should astrocyte activity be enhanced or suppressed and at what time points? What interventions can be used? Is it necessary to regulate astrocyte activity at all?

Modification of astrocyte activation with therapeutic purposes is a developing area. A few examples can be mentioned. Chondroitinase ABC has been shown to promote functional recovery after experimental stroke in rats, supposedly through activation of astrocytes in response to glucosaminoglycan degradation (Hill et al., 2012; Gherardini et al., 2015). Injecting of bone marrow stromal cells can accelerate recovery after induced stroke by mediating astrocytic responses in periinfarct tissue that can be related to the increased production of GDNF (Shen et al., 2010; Liu and Chopp, 2016). Minocycline, a widely used anti-inflammatory agent can improve recovery through increasing proliferation of periinfarct astrocytes (Chu et al., 2010; Liebigt et al., 2012). Some treatments were shown to promote recovery after stroke through reduced expression of GFAP, such as the N-methyl-D-aspartate receptor antagonist memantine (Lopez-Valdes et al., 2014) and the Rho kinase inhibitor, fasudil (Abeysinghe et al., 2016).

Further development of stroke management through astrocyte targeted therapies depends on the better knowledge of the role of astrocytes at different periods after the onset of the brain injury as well as on creation of new drugs modulating astrocyte activity.

**Populärvetenskaplig sammanfattning**

## Reaktiv glios och dess effekt på sekundär neurodegeneration hos möss

Stroke är idag en av de mest förekommande orsakerna till död och funktionsnedsättning. Den vanligaste formen av stroke är ischemisk stroke, så som av blodproppar i hjärnans kärl då hjärnvävnad skadas av syrebrist som följd. Effekterna av en stroke kan fortskrida efter att skadan är skedd och även sprida sig till andra delar av hjärnan i vad som kallas sekundär neurodegeneration. Idag kan vi behandla ischemin genom att återställa blodtillförseln genom blodförtunnande läkemedel eller att operativt ta bort proppar. Sedan kan vi rehabilitera med träning för att öka återhämtningen av funktion. Vi har inget direkt sätt att påverka hjärnans förmåga att ta över funktioner som förlorats. via läkemedel som annars skulle kunna underlätta funktionsåterkomst. Astrocyter är den vanligaste typen av gliaceller i hjärnan; hjälparceller som stöder, skyddar och håller den kemiska balansen i hjärnan. Dessa astrocyter reagerar på en skada genom att kraftigt ändra vilke gener i cellen som uttrycks i vad som kallas en reaktiv glios, vilket i akutskedet av ischemisk stroke skyddar hjärnan från ytterligare skada genom att bla kapsla in toxiska ämnen som bildas när celler dör. Studier har visat att även om denna reaktion skyddar hjärnan i akutskedet så kan den också hindra nybildandet av nervceller. Man kan alltså tänka sig att på rätt sätt och vid rätt tidpunkt med läkemedel dämpa denna reaktiva glios för att förbättra återhämtningen efter en stroke. Vilken betydelse reaktiv glios har för utvecklandet av sekundär neurodegeneration efter ischemisk stroke är inte känt. Som ett steg i denna forskning tittade vi här på möss som inte har genetiska förutsättningar att bilda protein nödvändiga för att gå i reaktiv glios, vid 7 veckor efter en skada som ska efterlikna en ischemisk stroke. Vi använde cellinfärgning för att analysera markörer som är kopplade till neurodegeneration: β-amyloid (Aβ) som ökar i de vanligaste formerna av demens; en annan markör som uttrycks i makrofager och mikroglia (immunceller som "äter upp" och bryter ner främmande eller skadliga ämnen och kommunicerar med resten av immunförsvaret) och kan

påvisa inflammation; och en markör förnervceller; för att se om mössen hade större förlust av dessa celler.  Vi visade på en ökad mängd Aβ efter stroke hos möss som inte kunde gå i reaktiv glios och mer inflammation (fler makrofager i skadade sidan jämfört med den oskadade sidan). Det här tolkar vi som att astrocyternas förmåga att gå i reaktiv glios påverkar sekundär neurodegeneration och inflammation i avlägsna delar av hjärnan även i det här senare skedet, efter 7 veckor. Den här informationen kan vara viktig att väga in när man ser till nyttan och risken med framtida potentiella behandlingar efter stroke. Fler studier behövs för att kartlägga den långtgående effekten av reaktiv glios på SND efter ischemisk stroke.

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