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Summary of the Dissertation

NG2-glia proliferation and differentiation following CNS injuries

NG2-glia proliferace a diferenciace po poškození CNS

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The dissertation is available for inspection at the Department for Ph.D. Study of the Dean´s Office, Second Faculty of Medicine, Charles University, V Úvalu 84, 150 06 Praha 5 (phone 224 435 836).

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NG2-glia proliferace a diferenciace po poškození CNS

Abstract in Czech

NG2 glie mají velký proliferační a diferenciační potenciál za fyziologických i patologických podmínek. Jsou velmi dobře známé jako prekurzory oligodendrocytů, avšak po poškození centrálního nervového systému (CNS) hrají důležitou roli v regeneraci. Z tohoto důvodu jsme zkoumali jejich vlastnosti po různých typech mozkových poškození jako je fokální cerebrální ischemie (FCI), kortikální bodná rána (SW) a demyelinizace (DEMY) u mladých (tříměsíčních) myší, u kterých jsou NG2 glie značené pomocí tdTomato pod promotorem Cspg4. V případě FCI jsme se také věnovali faktoru věku s využitím osmnáctiměsíčních myší. Abychom chování NG2 glií prozkoumali, provedli jsme mnoho technik na různých úrovních, jako je RT-qPCR na úrovni jedné buňky, RNA sekvenování a sekvenování na úrovni jedné buňky, imunohistochemie a technika patch-clamp. Tento přístup nám umožnil rozlišit dvě hlavní populace (NG2 glie, oligodendrocyty), z nichž každá obsahuje čtyři odlišné subpopulace. Profilování exprese dále odhalilo, že subpopulace NG2 glií exprimující GFAP (marker reaktivních astrocytů) je přítomna pouze přechodně po FCI. Po méně závažném poranění, konkrétně SW a DEMY však výrazně převažují subpopulace odrážející různá stádia zrání oligodendrocytů. Rozdílná genová exprese napříč ischemií a věkem odhalila sníženou expresi genů zodpovědných za údržbu/stabilitu axonů a synapsí a zvýšenou aktivaci interferonu typu I (IFN-I) u starých myší. Tyto výsledky vykreslují obraz komplexní heterogenity NG2 glií – jejich multipotentního fenotypu po poranění CNS a poukazují na ischemii jako na komplexní onemocnění související s věkem.

Klíčová slova

astrocyty, bodná rána, demyelinizace, fokální mozková ischemie, oligodendrocyty, stárnutí

NG2-glia proliferation and differentiation following CNS injuries

Abstract in English

NG2 glia display wide proliferation and differentiation potential under physiological and pathological conditions. They are very well known as precursors of oligodendrocytes, however, following central nervous system injury (CNS) they play an important role in regeneration. For this reason, we examined these features following different types of brain disorders such as focal cerebral ischemia (FCI), cortical stab wound (SW), and demyelination (DEMY) in young (3-months-old) mice, in which NG2 glia are labeled by tdTomato under the Cspg4 promoter. In the case of FCI, the factor of age was also studied using 18-months-old mice. To address these issues, we employed many techniques on tissue/cellular levels, such as single-cell RT-qPCR, single-cell/bulk RNA-sequencing, immunohistochemistry, and the patch-clamp technique *in situ*. First, such approach enabled us to distinguish two main populations (NG2 glia, oligodendrocytes), each of them comprising four distinct subpopulations. Next, the expression profiling revealed that a subpopulation of NG2 glia expressing GFAP, a marker of reactive astrocytes, appears transiently after FCI. However, following less severe injury, namely the cortical SW and DEMY, subpopulations mirroring different stages of oligodendrocyte maturation markedly prevail. Additionally, differential gene expression across ischemia and age uncovered downregulation of axonal and synaptic maintenance genetic program and increased activation of type I interferon (IFN-I) in aged mice. These results paint a picture of the complex heterogeneity of NG2 glia-their multipotent phenotype following CNS injuries and point to ischemia as a complex age-related disease.

Keywords

aging, astrocytes, demyelination, focal cerebral ischemia, oligodendrocytes, stab wound

1. Background

1.1. Central nervous system injuries

Injuries of central nervous system (CNS), including ischemic stroke, are major promoters of death and disability worldwide (Corps et al., 2015, Benjamin et al., 2018). The medical costs of strokes are forecasted to increase from $71.6 billion in 2012 to $184.1 billion by 2030 (USA). Despite the immeasurable burden on patients and families, there are no effective treatments to protect the CNS and promote functional recovery after acute injuries (Kim et al., 2019). A major roadblock to developing effective therapies is the lack of understanding of the cellular and molecular mechanisms that promote secondary neuronal damage and functional deficits after injury. To promote recovery, could be the utilization of naturally residing precursor brain cells - NG2 glia, which are capable differentiate to other cell types, regulating the metabolic environment, and directly modulating neuronal functions (Galichet et al., 2021). A better understanding of this enigmatic cell type will shed light on the pathogenesis and potential treatment strategies for numerous CNS disorders, such as ischemia or neurodegeneration.

1.2. NG2 glia under physiological and pathological conditions

NG2 glia represent a fourth resident glial cell population in the mammalian CNS that is distinct from astrocytes, mature oligodendrocytes, and microglia (Nishiyama et al., 2016, Nishiyama et al., 2009). NG2 glia are the most active cycling population with enormous proliferative capacity within the adult brain (Kirdajova and Anderova, 2020). NG2 glia represent a very flexible glial cell type, which react to the different pathologies in the brain and spinal cord. After activation, they change their morphology, proliferation rate, and differentiation. The type of NG2 glia response to injury is strongly dependent on the insult and the developmental stage (Song et al., 2017). It was shown that the accumulation of NG2 glia near the injury could have opposite effects. The physical barrier of NG2 proteoglycan participates in the growth-inhibitory environment (Tan et al., 2005) or NG2 glia provide an

adhesive substrate for axonal growth cones and promote their growth in the glial scar of ischemia (Yang et al., 2006). Besides the proliferation, it was shown that activated NG2 glia are able to differentiate into reactive astrocytes (Fig. 1) or even neurons after ischemia in the brain (Honsa et al., 2016, Komitova et al., 2011, Kirdajova et al., 2021) or in the spinal cord injury (Hackett et al., 2018, Huang et al., 2018). NG2 glia react also to neurodegeneration. but it seems that the response of NG2 glia observed in Amyotrophic lateral sclerosis and Alzheimer`s diseases is rather due to demyelination than due to neurodegeneration (Cruz et al., 2003, Sirko et al., 2013). Since NG2 glia are mainly precursors of oligodendrocytes, they are the perfect candidate to respond to demyelination. In fact, adult NG2 glia differentiate into oligodendrocytes capable of remyelinating axons (Zawadzka et al., 2010) and restoring nearly normal nerve conduction.

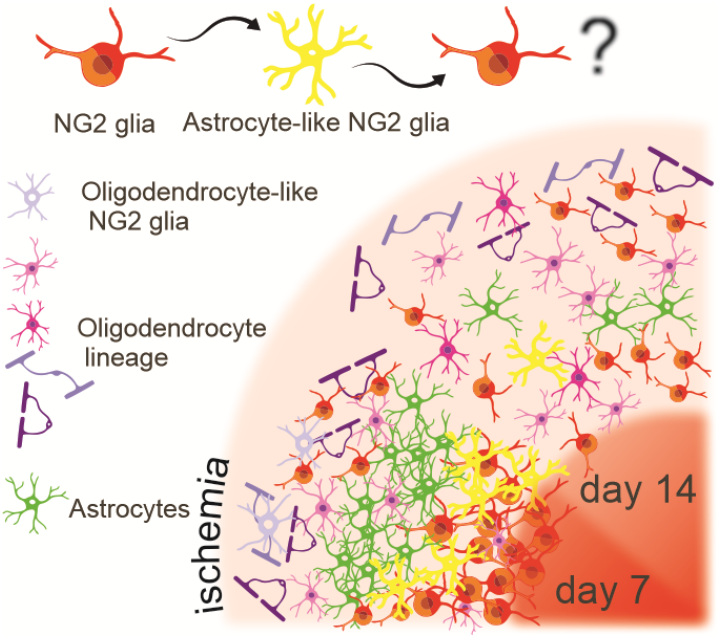


Figure 1: Scheme of cell types after focal cerebral ischemia seven and 14 days after injury. Formation of compact glial scar occurs along borders to infarct zone and includes astrocytes (green cells), NG2 glia (yellow cells) and cells derived therefrom such as astrocyte-like NG2 glia, oligodendrocyte-like NG2 glia, and oligodendrocytes (Kirdajova et al., 2021)

1.3.Ischemia

Brain ischemia stems from cardiac arrest or stroke, in which poor blood flow to the tissue causes glucose and oxygen deprivation in the brain parenchyma. Glucose and oxygen deficiency disrupts oxidative phosphorylation, which results in energy depletion and ionic disbalance, followed by cell membrane depolarization, calcium overload, and extracellular accumulation of excitatory amino acid glutamate (Belov Kirdajova et al., 2020). According to the location and extent of the injury, we

recognize two types of ischemia: focal and global cerebral ischemia (Yao et al., 2018). Global cerebral ischemia stems from an overall decrease in blood flow due to cardiac arrest or near-drowning. As the name suggests, focal cerebral ischemia (FCI) is due to the occlusion of specific arteries of the brain, caused by thrombosis or embolism (VanGilder et al., 2012a). A cortical stab wound (SW) can be considered also as a type of FCI since in this type of injury arteries are also damaged (Anderová et al., 2004, Komitova et al., 2011).

1.4. Demyelination (DEMY)

Demyelinating diseases of the CNS comprise a group of neurological disorders characterized by progressive loss of oligodendrocytes and myelin sheaths in the white matter tracts (Vega-Riquer et al., 2019). Myelin disorders were once thought to be confined to leukodystrophies (Waldman, 2018), inflammatory diseases such as Multiple sclerosis (Plemel et al., 2017), and injury such as periventricular leukomalacia (Back and Rivkees, 2005).

1.5. Age and ischemia

Central nervous system is sensitive to age with increasing deficits in neuronal functions including cognitive decline, motor, and sensory abnormalities during aging (Sousounis et al., 2014, Damoiseaux, 2017, Jeromin and Bowser, 2017). It is a complex and irreversible process accompanied by morphological, biochemical, and physiological changes and increased susceptibility to neurodegenerative diseases (Pan et al., 2020). Age was found to be one of the most important risk factors for brain infarction and its mortality (Hoyer, 1987). Older mice exhibit a differential response to stroke and have worse outcomes than adult mice (Chauhan et al., 2018). Abnormalities in glycolytic flux, lactate production, cessation of oxidation, and energy production were found to be more pronounced with advancing age, which indicates reduced plasticity of the brain to the pathological conditions (Hoyer, 1987).

2. Objectives

Hypothesis 1.: NG2 glia were shown to have multipotent capacity in the development and following injury, generating, beside oligodendrocytes, also astrocytes and even neural precursor cells (Kirdajova and Anderova, 2020). Since such observations were based on the expression of only few cell-type-specific markers, we hypothesize that multiple subpopulations exist within $NG2^+$ cells, especially after severe CNS injury, and they are characterized by distinct combination of genes. Therefore, the more precise analysis based on the expression of larger battery of genes is necessary to get better insight into multipotency of NG2 glia.

Aim 1.: To characterize the proliferation and differentiation potential of NG2 glia following FCI using genetic fate-mapping in combination with gene expression profiling.

Hypothesis 2.: Several studies suggested that astrocytes can originate from embryonic rather than postnatal or adult NG2 glia, in the intact CNS (Huang et al., 2019, Huang et al., 2014). In reaction to CNS pathology, some studies reported that a small fraction of NG2 glia can differentiate into astrocytes after spinal cord injury (Hackett et al., 2018) and after brain injury (Dimou et al., 2008, Honsa et al., 2016, Valny et al., 2018), while others showed that fewer NG2 glia, if any, become astrocytes (Kang et al., 2010, Zawadzka et al., 2010). We hypothesize that using the same transgenic mice, same method of astrocyte identification and protocol for tamoxifen administration in different CNS injuries confirm generation of astrocytes from NG2 glia in adult brain.

Aim 2.: To compare the fate of NG2 glia-derived astrocytes, between different types of CNS disorders in tamoxifen-inducible BAC transgenic mice.

Hypothesis 3.: Previous global gene expression studies of experimental stroke using microarrays (Mitsios et al., 2007, Roth et al., 2003) and more recently RNA-

Sequencing (Dergunova et al., 2018) have provided useful insights into the pathophysiology of ischemic stroke and uncovered many altered molecular pathways (VanGilder et al., 2012b). However, only few studies included aged animals. We assume that aging alterations on its own could be risk factor and reason for worse outcome after ischemia. Therefore, the profound comparation of transcriptional profile between young and aged brain after ischemia is needed.

Aim 3.: To dissect the interaction between stroke and aging at the genome-wide level using FCI on young adult (3-month-old) and aged (18-month-old) female mice.

3. Material and methodology

3.1. Animals

For purpose of characterization of the proliferation and differentiation potential of NG2 glia following CNS injuries we used 3-month-old transgenic mice Cspg4/tdTomato, in which tdTomato red fluorescent protein is expressed in NG2 glia and cells derived therein. In addition, to follow NG2 glia-derived astrocytes, the Cspg4/tdTomato mice were cross-bred with constitutive Gfap/EGFP mice, in which the visualization of astrocytes is feasible because of the enhanced green fluorescent protein under the control of the human promoter for GFAP, (Nolte et al., 2001). For determination interaction between stroke and aging at the genome-wide level young adult (3-month-old) and aged (over 15-month-old) C57BL/6J and FVB/NJ mice were used.

3.2. Induction of focal cerebral ischemia (FCI)

Adult mice underwent permanent middle cerebral artery occlusion, a procedure which has become a conventional model for inducing FCI. The sham-operated animals were considered controls (CTRL).

3.3. Induction of cortical stab wound (SW)

Sterile sharp knife was inserted vertically into the right cerebral hemisphere of adult mice, 1 mm deep to the dura surface. The sham-operated animals were considered CTRLs.

3.4. Induction of demyelination (DEMY)

Demyelination was induced by administration of 0.3 % cuprizone to mice *ad libitum* in chow for 5 weeks. After this period, they were returned to a regular diet and allowed to recover for 7 days. CTRL mice received regular mice chow, without cuprizone, for the entire period.

3.5. Tissue isolation and single cell suspension preparation

After transcardial perfusion and decapitation, brains were quickly removed from the skull. To prepare tissue slices or single cell suspension for our analyses, we isolated specific brain regions of the adult mouse brain (Fig. 2) and cut out for further processing. The tissue was cut into smaller pieces using a razor, incubated in papain solution for 45 minutes, mechanically dissociated with a pipette, and subsequently filtered through a cell strainer. Then single cells were collected using fluorescent activated cell sorting (FACS).

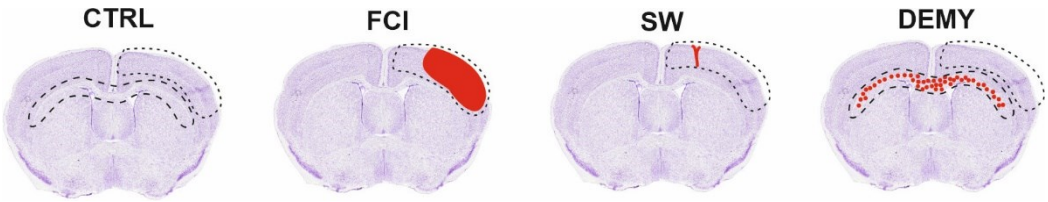


Figure 2: Scheme depicting brain regions (dashed lines), which were used for tdTomato^{+} cell isolation (Kirdajova 2021).

3.6. Bulk/single cell RNA-Sequencing and Single cell RT-qPCR

To analyze the transcriptome of the individual cells or bulk different techniques were used such as single cell RNA-Sequencing and single cell RT-qPCR (Rusnakova et al., 2013).

3.7. Single cell RT-qPCR data analysis

Kohonen self-organizing maps (SOM), dividing the cells into groups, were trained using GenEx 6 software. This classification of cells into groups was substantiated with the principal component analysis (PCA).

3.8. RNA-Sequencing data analysis

To analyze RNA-Sequencing data of NG2 glia differentiation, differential expression (DE) analysis was used. In case of comparison young/aged ischemic mice beside the DE analysis were also used gene set enrichment analysis (GSEA),

cell type proportion estimation, protein-protein interaction,custom gene set enrichment analysis.

3.9.Immunohistochemistry and confocal microscopy

Immunohistochemical analyses were used to evaluate proliferation/differentiation potential of NG2 glia after CNS disorders using specific protein markers (Table 1) and the incorporation of 5-ethynyl-2′-deoxyuridine (EdU). To compare the changes in young/aged ischemic mice interneuron/neuron markers were used (Table 1). Confocal fluorescence microscopes together with the ImageJ software were used for the analysis of fluorescence signals.

Table 1: Primary antibody used for immunohistochemistry

Cell type marker	Antibody	Species	Dilution	Company
Astrocytes	**GFAP coupled Alexa 488**	mouse	1:300	Ebioscience
	ALDH1l1	rabbit	1:500	Abcam
	VIM	mouse	1:1000	Abcam
	AQP4	rabbit	1:500	Millipore
Oligodendrocytes	**APC**	mouse	1:200	Merck
Pericytes	**PDGFRbeta**	rabbit	1:200	Santa Cruz
Proliferation	**KI-67**	rabbit	1:1000	Abcam
	KI-67 coupled FITC	mouse	1:200	ThermoFisher Scientific
	PCNA	mouse	1:800	Abcam
Interneurons	**PARVALBUMIN**	rabbit	1:500	Synaptic system
Neurons	**NeuN**	rabbit	1:100	Chemicon
Newly formated neurons	**Doublecortin**	rabbit	1:1000	Abcam

3.10. Patch-clamp measurements

The electrophysiological properties of NG2 glia were recorded using the patch-clamp technique in the whole-cell configuration. The current patterns were obtained by hyperpolarizing and depolarizing the cell membrane from the holding potential of -70 mV to the values ranging from -160 mV to 40 mV. The amplitudes of voltage-dependent currents were calculated in the FitMaster software.

3.11. Western blot

The total protein content from the tissue homogenates was used for the analysis of several proteins (STAT and Interferon signaling). The primary antibodies were combined with horseradish peroxidase-conjugated secondary antibodies and peroxidase activity was detected.

3.12. Statistics

The results are expressed as the mean ± standard error of the mean (SEM). Values of $p < 0.05$ were considered significant, $p < 0.01$ very significant and $p < 0.001$ extremely significant.

4. Results

4.1. Proliferation and differentiation potential of NG2 glia following FCI

To disclose the changes in the expression profiles of NG2 glia and those derived from NG2 glia after FCI we analyzed their mRNA transcripts of 93 genes using single-cell RT-qPCR. We used Cspg4/tdTomato transgenic mice expressing tamoxifen-inducible cre recombinase under the control of the Cspg4 promoter.

Following ischemia, beside the NG2 glia from uninjured animals (Bona fide NG2 glia; BF-NG2 cells) we identified three other NG2 glia subpopulations (Fig. 3). The first subpopulation characterized by a high percentage of cells expressing oligodendrocyte marker genes, such as *Mbp* (42.3 ± 9.5 %), *Cldn11* (84.6 ± 6.3 %) and Tcf7l2 (94.2 ± 4.9 %) and the Wnt signaling effectors critical for oligodendrocyte maturation (Guo et al., 2015), was termed oligodendrocyte-like NG2 glia (OL-NG2 cells)(Fig. 3). Moreover, 38.5 ± 7.2 % of OL-NG2 cells started to express transient receptor potential cation channel subfamily V member 4 (*Trpv4*) (Fig. 3), which was shown to be expressed by committed oligodendrocyte precursors (Marques et al., 2016). The second subpopulation, astrocyte-like NG2 glia (A-NG2 cells), was characterized by the highest number of cells expressing astroglial markers, such as *Gfap* (43.2 ± 6.0 %) and *Aqp4* (51.6 ± 9.9 %) (Fig. 3). On the contrary, only 9.5 ± 5.7 % A-NG2 cell expressed *Mbp* (Fig. 3). The last subpopulation of ischemic NG2 glia was characterized by the highest percentage of cells expressing proliferation marker *Mki67* (84.1 ± 8.3 %) and marker of newly derived cells Nestin (*Nes*) (86.4 ± 8.7 %) (Fig. 3)(Anderova et al., 2011, Honsa et al., 2012). We named this subpopulation as proliferating NG2 glia (P-NG2 cells).

While NG2 glia isolated from uninjured mice were classified as BF-NG2 cells, all NG2 glia subpopulations were distributed unequally following ischemia (Fig. 4). The maximal changes were observed 3 and 7 days after FCI. The highest incidences of P- and A-NG2 cells were observed 3 days after FCI, unlike the OL-NG2, which culminated at day 7 after FCI. At day 14 after FCI the distribution of NG2 glial subpopulations became similar to that observed in CTRL (Fig. 4).

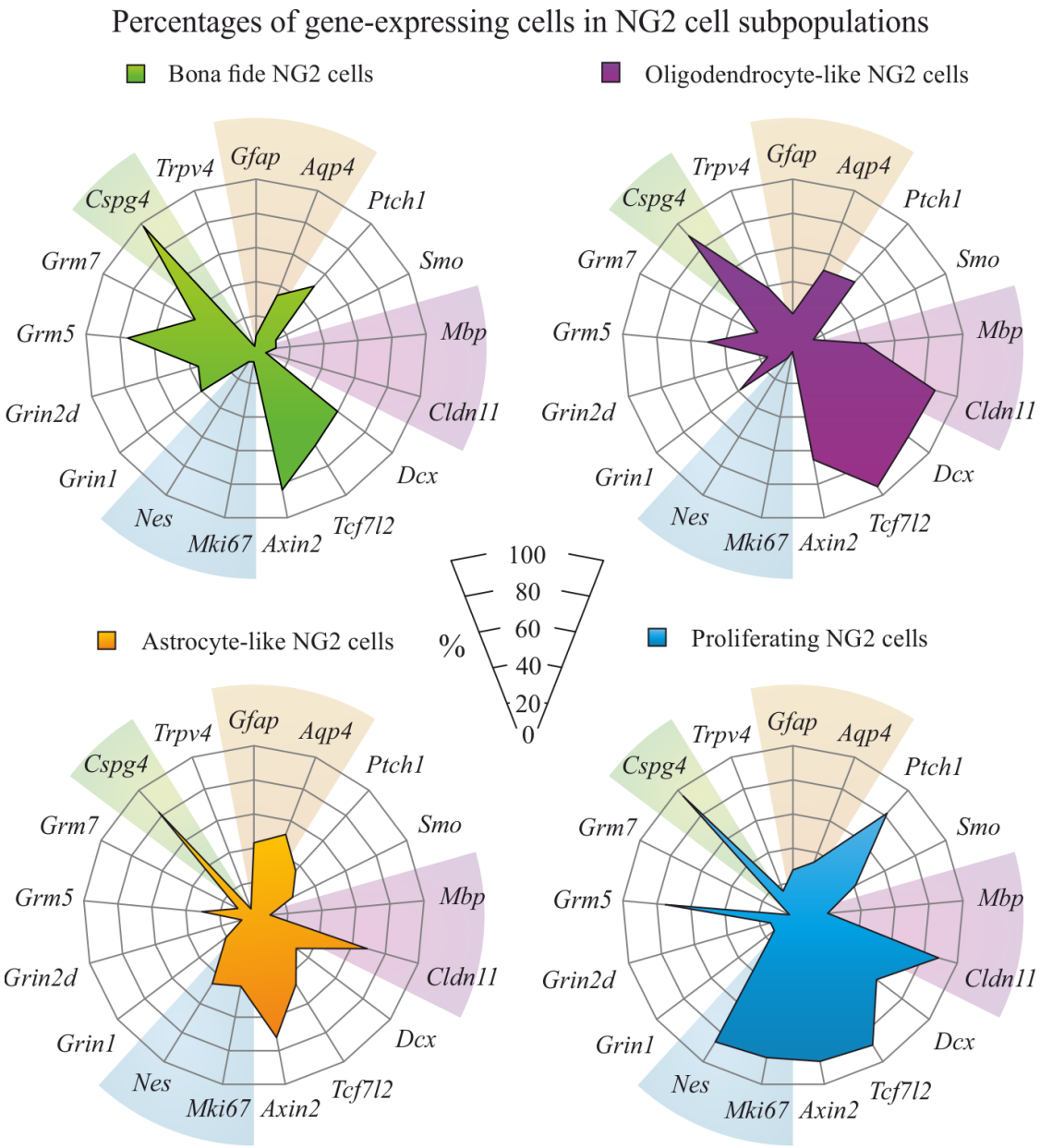


Figure 3: Four NG2 glia subpopulations were identified after focal cerebral ischemia (FCI) using SOM analysis. Subpopulations differ in percentage of cells expressing several genes; only genes, expression of which was changed significantly ($p < 0.05$) are depicted; the background colors indicate genes characteristic for particular subpopulation; statistics were calculated using two-way ANOVA test comparing each subpopulation with every other.

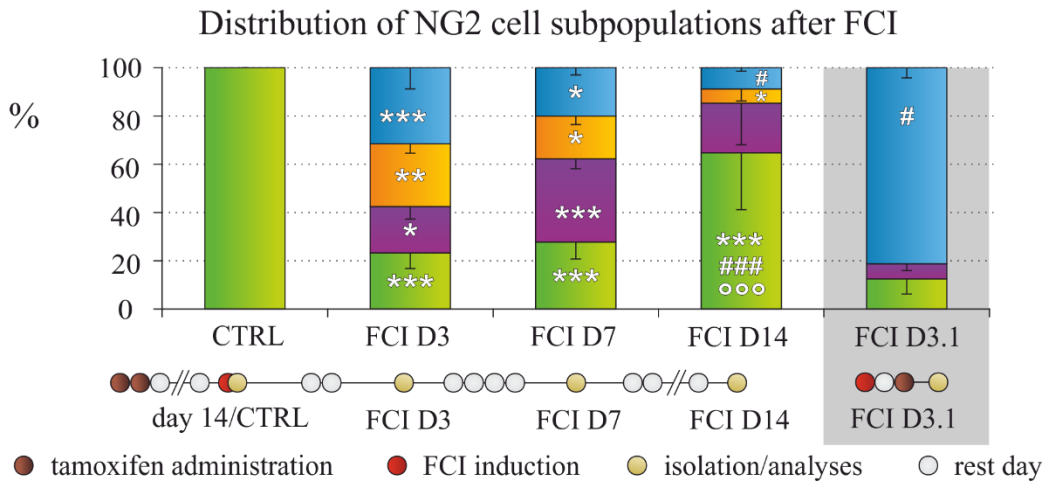


Figure 4: Four NG2 glia subpopulations were identified after focal cerebral ischemia (FCI) using SOM analysis. B) Oligodendrocyte-, astrocyte-like and proliferating NG2 glia subpopulations emerged after FCI and are distributed unequally within the period of 14 days after FCI. The scheme of tamoxifen administration is depicted under the graph. Statistics were calculated using two-way ANOVA with Bonferroni post-test comparing incidences of particular subpopulation among time-points and t-test to compare distribution of subpopulations between same time-points when different tamoxifen administration scheme was used; asterisks show significances compared to control (CTRL) group, hashtags show significances relative to FCI D3 group and circles show significances relative to FCI D7 group. *, #, $p < 0.05$; **,##, $p < 0.01$; ***, ###, °°° $p < 0.001$. FCI, focal cerebral ischemia; SOM, self-organizing Kohonen maps

4.2. Appearance of NG2 glia-derived astrocytes between different types of CNS disorders

Since we detected NG2 glia-derived astrocytes following FCI, the next question was whether this subpopulation also appears in other types of brain disorders such as SW or DEMY. In order to disclose changes in the gene expression of NG2 glia in glial scar formation (Wanner et al., 2013) and remyelination (Skripuletz et al., 2011), we isolated two different regions of the brain corpus callosum (CC) and cortex (CTX) from the CTRLs and mice seven days after the induction of FCI/SW or withdrawal of the cuprizone diet (DEMY) (Fig. 2).

Similarly, to our previous data only from FCI, after the induction of three different types of brain disorders: FCI, SW, and DEMY, we found four subpopulations of NG2 glia and also four subpopulations of oligodendrocytes (Fig. 5).

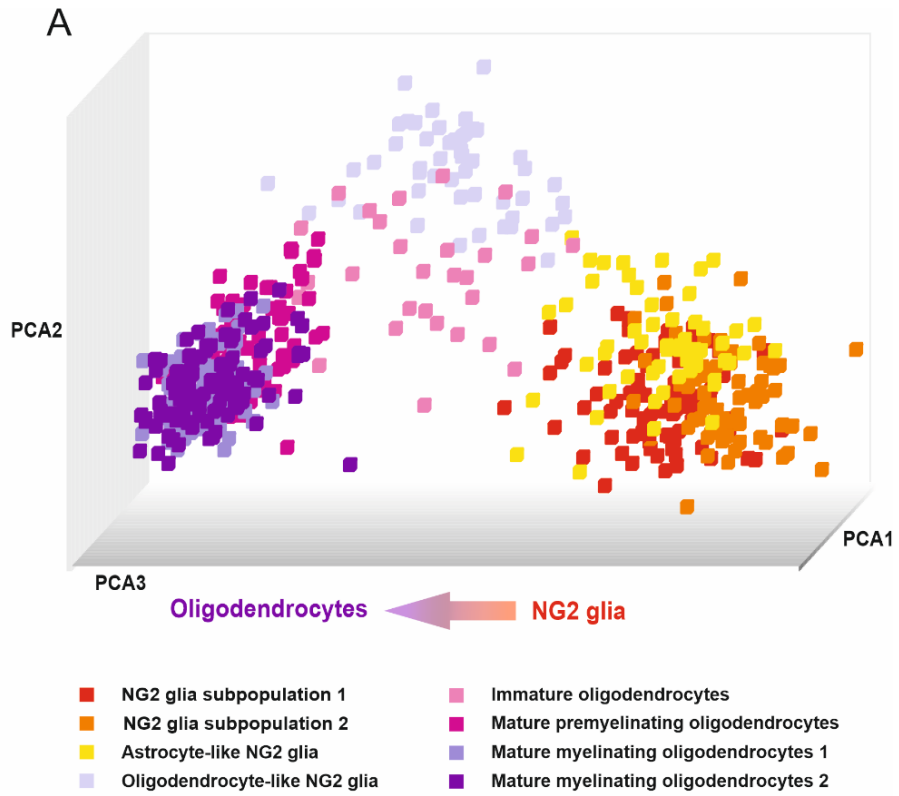


Figure 5: Principal component analysis (PCA) showing the distribution of four subpopulations of NG2 glia and four subpopulations of oligodendrocytes seven days after injury.

Two subpopulations of NG2 glia were termed NG2 glia (S1) and NG2 glia (S2), characterized by a high percentage of cells expressing typical markers of NG2 glia, such as *Cspg4* and *Pdgfrα* (Fig. 6). The expression pattern of these two groups was similar to NG2 glia from the uninjured brain, except for the fact that NG2 glia (S2) had a higher expression of several genes compared to the NG2 glia (S1) (Fig. 6). The third population of NG2 glia is a specific group of cells characterized by the highest number of cells expressing astroglial markers *Gfap* (37.5±7.1 %) and *Vim* (92.9±1.2 %) (Fig. 6) and was further termed astrocyte-like NG2 glia. These genes for intermediate filaments are typically upregulated in reactive astrocytes (Pablo et al., 2013), which could highlight the astrogliogenic potential of these cells. Both

NG2 glia (S2) and astrocyte-like NG2 glia subpopulations are characterized by a high expression of *Dcx* (55.2±7.0 % and 67.9±7.0 % respectively), as a marker of cell motility (Fig. 6). Moreover, astrocyte-like NG2 glia are characterized by their proliferation potential as this subpopulation has the highest percentage of cells expressing *MKi67* (39.3±7.0 %), a marker of proliferation (Fig. 6). This suggests that astrocyte-like NG2 glia retain the ability to proliferate thus differing to oligodendrogliogenesis, when this ability is lost (Hassannejad et al., 2019). The fourth subpopulation is oligodendrocyte-like NG2 glia, characterized by a high percentage of cells expressing oligodendrocyte-committed genes, such as *Mbp* (98.0±3.8 %), *Cldn11* (100.0±0.0 %), and *Tcf7l2* (100.0±0.0 %) and a lower percentage of NG2 glia-committed genes, such as *Cspg4* (28.6±5.4 %) and *Pdgfrα* (8.2±3.7 %) (Fig. 6). Another feature of this subpopulation is the expression of *Trpv4*, which was also described by Marques et al. in committed OPC (Marques et al., 2016) (Fig. 6).

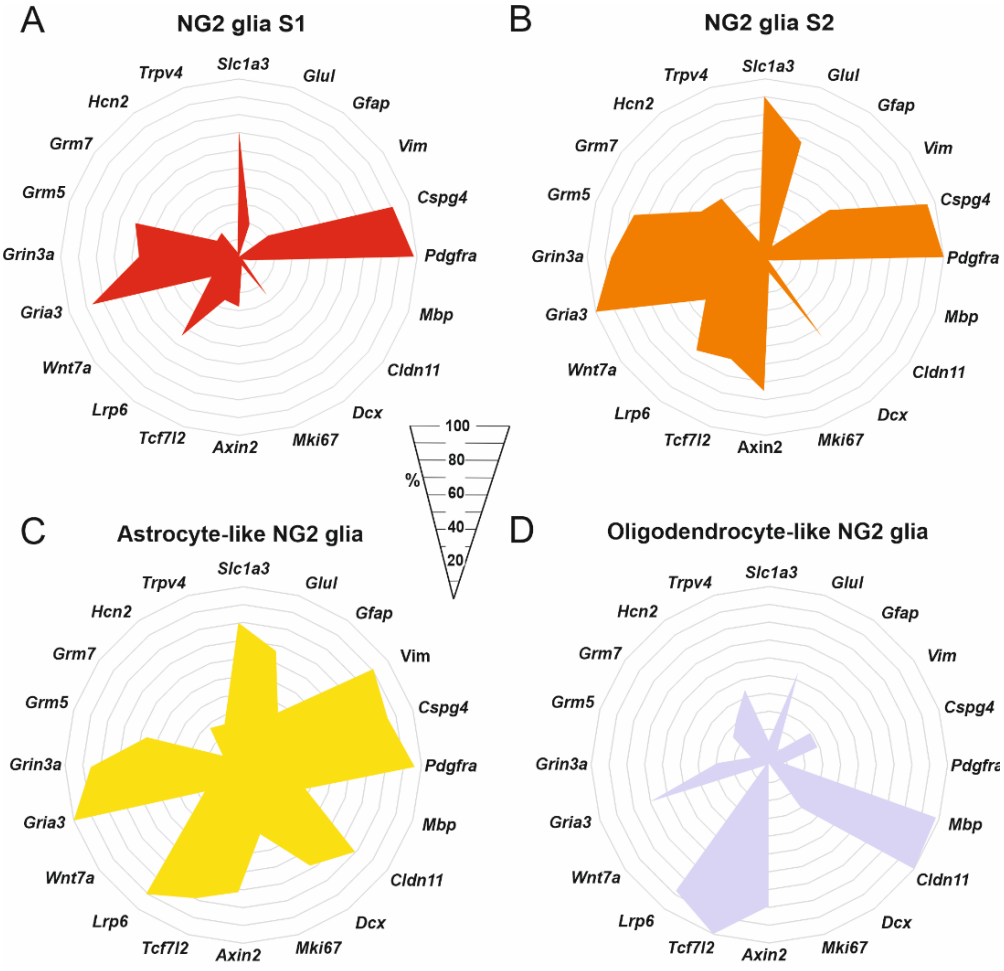


Figure 6: Four NG2 glia subpopulations identified using self-organizing map analysis. NG2 glia subpopulation 1 (A), NG2 glia subpopulation 2 (B), astrocyte-like NG2 glia (C), and oligodendrocyte-like NG2 glia (D). (A-D) Only genes, of which expression is changed significantly ($p < 0.05$), are depicted, with the exceptions of the marker genes. S, subpopulation.

The distribution of the four NG2 glia subpopulations varied among the regions of the uninjured brain, as well as in response to pathological stimuli. The proportion of the two subpopulations of NG2 glia between CTX and CC is already significantly different in the uninjured brain (Fig. 7A). Oligodendrocyte-like NG2 glia are equally distributed in the gray (CTX) and white (CC) matter and no astrocyte-like NG2 glia are present in the uninjured brain (Fig. 7A). Interestingly, astrocyte-like NG2 glia represent the prevailing subpopulation (48.5±8.5 %) after FCI, at the expense of NG2 glia (S1) and (S2).

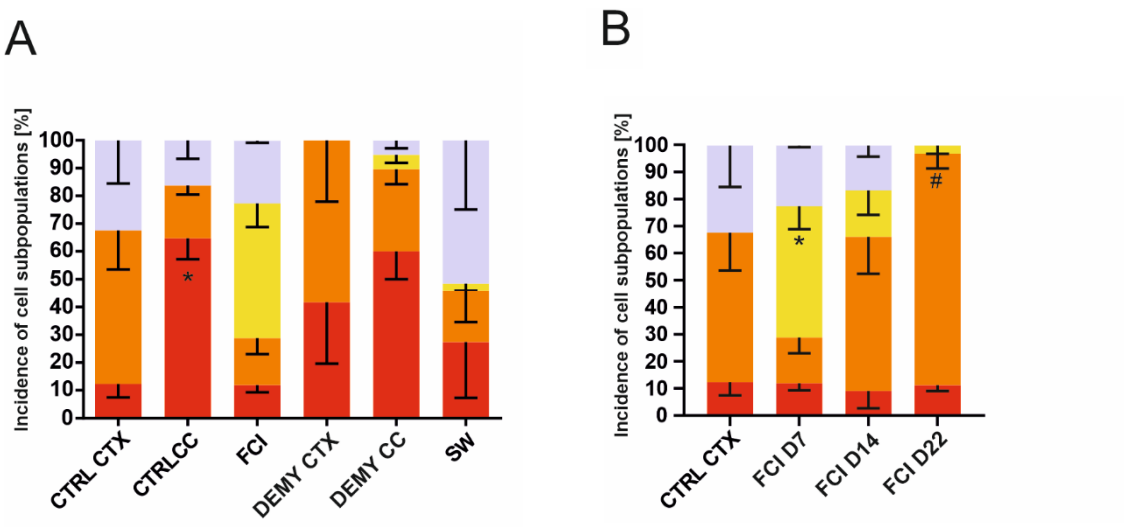


Figure 7: Distribution of four subpopulations of NG2 glia is unequal in controls (CTRLs) and following injuries (**A**) Changes in the distribution of four subpopulations of NG2 glia (**B**) at different time points after focal cerebral ischemia (FCI). Statistics were calculated using two-way ANOVA, comparing incidences of a particular subpopulation among groups. The percentage of subpopulations was calculated as an average of the percentage from different mice. Asterisks show significance compared to CTRL and hashtags show significances compared to FCI. * $p < 0.05$; ## $p < 0.01$; *** $p < 0.001$. CTX, cortex; CC corpus callosum; DEMY, demyelination; SW, stab wound.

In order to determine whether FCI-specific astrocyte-like NG2 glia is permanent or transient subpopulation within NG2 glia subpopulations, we performed single-cell RT-qPCR analysis 14 and 22 days after FCI (Fig. 7B). This subpopulation was still present 14 days after FCI, but with a decreasing tendency (Fig. 7B). In the late stages (22 days) astrocyte-like NG2 glia were not observed which was at the expense of NG2 glia (S2) similarly to oligodendrocyte-like NG2 glia (Fig. 7B).

To further verify if astrocyte-like NG2 glia only arise after FCI, we used immunohistochemistry. We clearly showed that tdTomato/GFAP$^+$ cells are only present after FCI in a higher number (31.4±1.9 %), compared to the CTRLs or the other types of injury (Fig. 8A). These cells were mainly located on the border of ischemia between NG2 glia and astrocytes (Fig. 8A) in the close vicinity of the lesion as already found by Valny et al. (Valny et al., 2018). We observed a GFAP immunoreactivity decay already at 14 days (Fig. 8B). This suggests that astrocyte-like NG2 glia represent a transient subpopulation, but unique to FCI. In summary, NG2 glia only form astrocyte-like NG2 glia following severe ischemic damage, while cortical SW or DEMY are not able to evoke such NG2 glia multipotency.

Based on the data, we found that astrocyte-like NG2 glia is a specific and distinct subpopulation of NG2 glia, only arising after FCI. Therefore, it was of interest to determine the electrophysiological properties of astrocyte-like NG2 glia 7 days after FCI. Astrocyte-like NG2 glia membrane currents resulted in a shift between the current pattern of NG2 glia and astrocytes (Fig. 9). Their passive membrane properties did not significantly differ from NG2 glia/astrocytes after FCI. However, they had considerably reduced K_{IR} (3.5±0.8 pA/pF), K_{DR} (4.2±1.1 pA/pF) and K_A (0.2±0.2 pA/pF) current densities, when compared to NG2 glia, while they did not differ from those obtained in cortical astrocytes (Fig. 9).

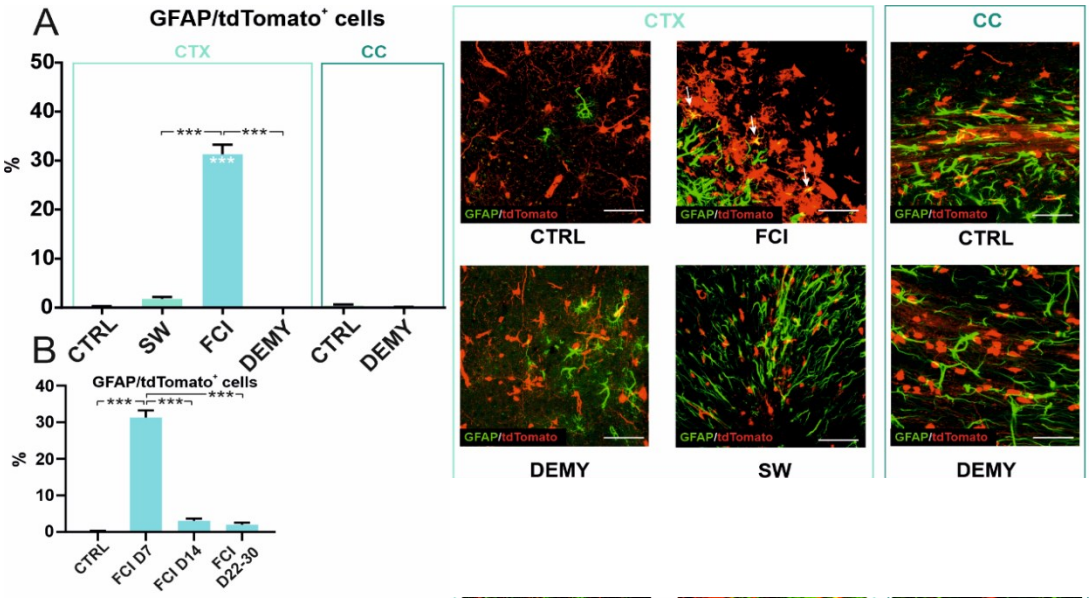


Figure 8: Graphs showing the percentage of tdTomato$^+$ cells expressing astrocyte marker glial fibrillary acidic protein (GFAP) and their representative images depicting the co-localization of astrocyte markers under physiological and pathological conditions (A) or after different time point after ischemia (B). Arrows indicate the co-localization of green and red signals. Statistics are calculated using one-way ANOVA. Asterisks show significances compared to the corresponding CTRL unless otherwise indicated. * $p < 0.05$; *** $p < 0.001$. Scale bars, 50 µm. CTRL, control; CTX, cortex; CC corpus callosum; FCI, focal cerebral ischemia; DEMY, demyelination; SW, stab wound.

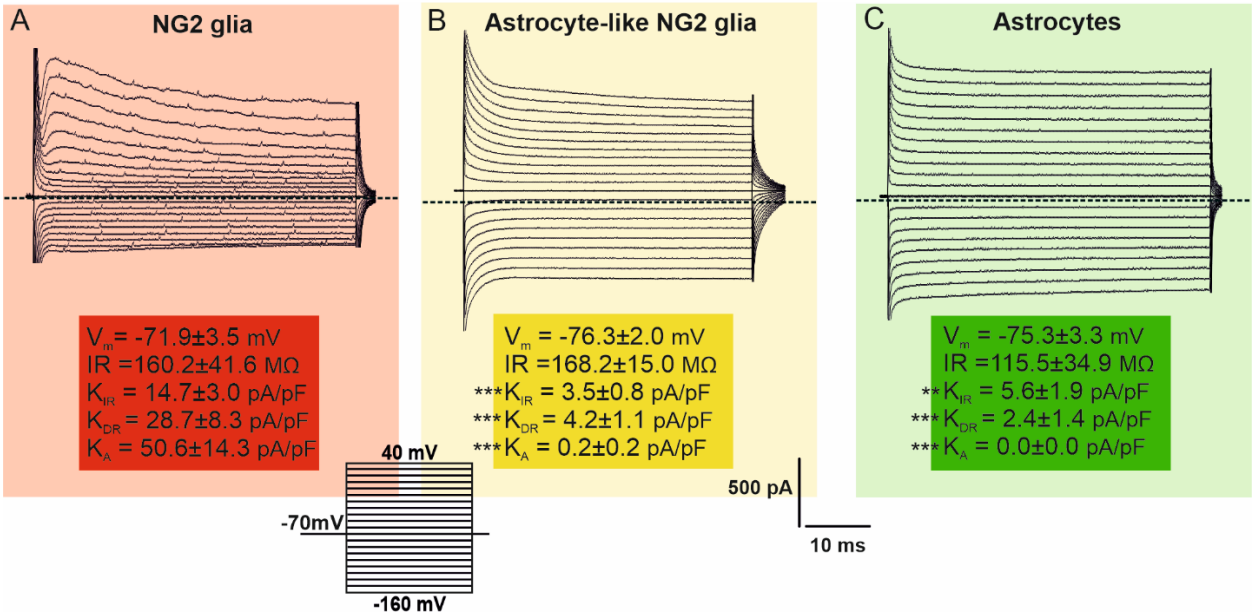


Figure 9: Current patterns of NG2 glia, astrocyte-like NG2 glia, and astrocytes seven days after focal cerebral ischemia (FCI). Typical current patterns of (A) NG2 glia, (B) astrocyte-like NG2 glia, (C) and astrocytes were obtained by hyper- and depolarizing the cell membrane from the holding potential of -70 mV to the values ranging from -160 mV to 40 mV, in 10 mV increments

(see the inset, bottom). Zero current is marked by the dashed line. Statistics were calculated using one-way ANOVA. Asterisks show significances compared to NG2 glia, ** $p < 0.01$; *** $p < 0.001$.

4.3. Interaction between stroke and aging at the genome-wide level using model of FCI

To explore the difference in the response to ischemia between young and aged brain leading to impaired regeneration, we performed RNA-Sequencing of CTX isolated from 3-month-old (young) and 18-month-old (aged) female mice at 3 days after FCI and from their age-matched CTRLs.

We found a substantial overlap between differentially expressed genes in young and aged mice, when aged mice differentially regulated more genes, often with a greater magnitude (Fig. 10A). Functional analysis with GSEA showed hundreds of significantly enriched gene ontology (GO) terms. The GO terms were related to inflammatory response (such as type I interferon (IFN-I) signaling, cytokine production, neutrophil degranulation), cell-cell interactions (such as integrin cell-surface interaction, extracellular matrix organization) and cell-cycle (such as regulation of DNA replication) and were upregulated to a greater extent in aged animals after injury (Fig. 10B). Similarly, gene sets associated with synaptic signaling and plasticity, neurotransmitter transport and potassium ion channels were downregulated exclusively, or to a greater extent in aged animals after ischemia (Fig. 10B).

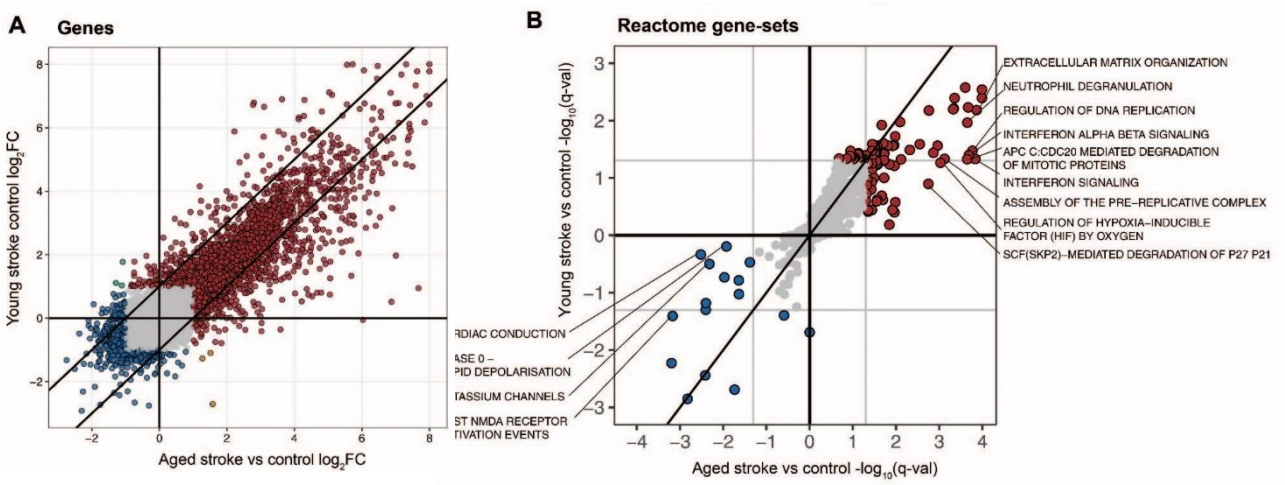


Figure 10: Comparison of differentially expressed genes and gene sets after ischemia between young and aged mice. A) Scatter plot comparing stroke-induced log_2 fold-change in young and aged mice. Genes with $|log_2FC| > 1$ are highlighted in color. B) Scatter plot comparing ischemia-induced alteration of Reactome pathways in young and aged mice. Pathways wih q-val < 0.05 are highlighted in color. Sign depicts UP (+) or DOWN (-) regulation.

In order to provide cell-specific context to the observed transcriptional profiles, we estimated relative changes of cell type proportions by computational deconvolution. We found a significant increase in all non-neuronal cell types following ischemia in both age groups ($p_{adj} < 2.2e\text{-}16$; Fig. 11). The largest increase was in microglial and endothelial marker genes (log_2FC 1.26-1.77) and the lowest in oligodendrocytic markers (log_2FC 0.47-0.60), which also significantly increased with normal aging ($p = 1.20e\text{-}19$; Fig. 11). The most prominent difference was in endothelial cell markers ($log_2FC_{young} = 1.26$, $log_2FC_{aged} = 1.74$). Furthermore, cell type proportion estimates revealed significant depletion of pyramidal/excitatory neurons during aging and following ischemia in both age groups ($p_{adj} < 1.00e\text{-}06$, $log_2FC_{young} = -0.56$, $log_2FC_{aged} = -0.63$, Fig. 11).

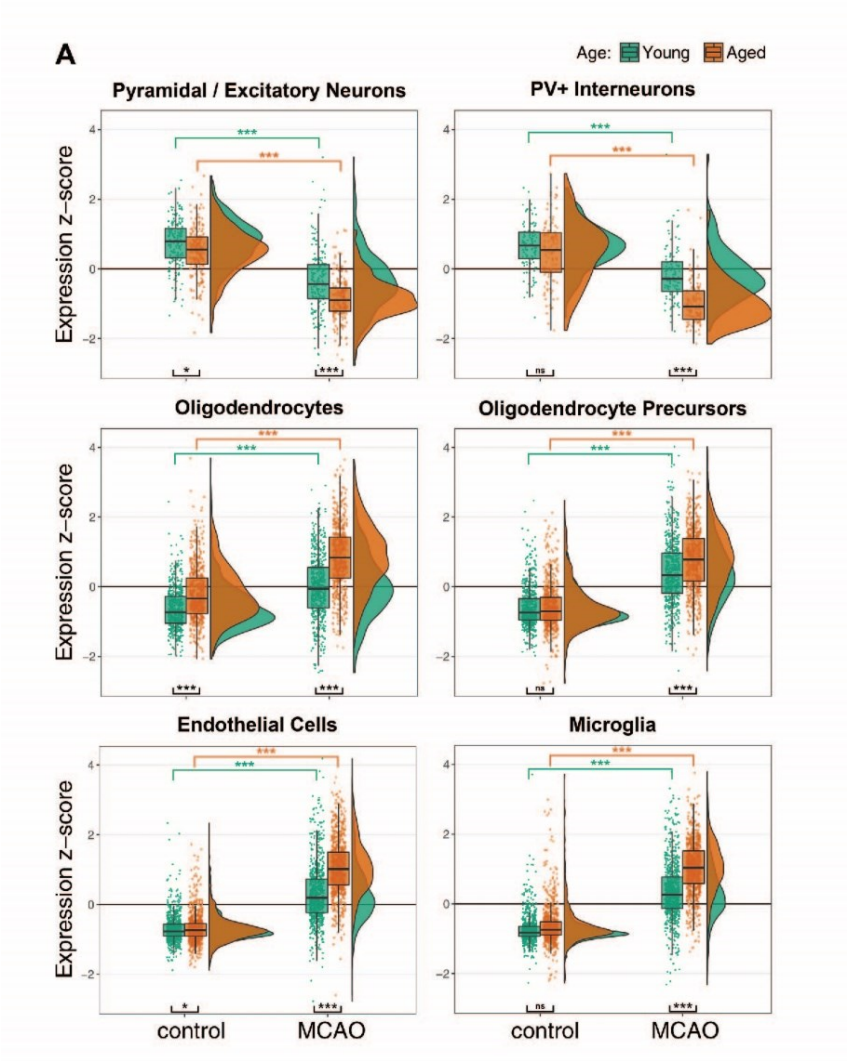
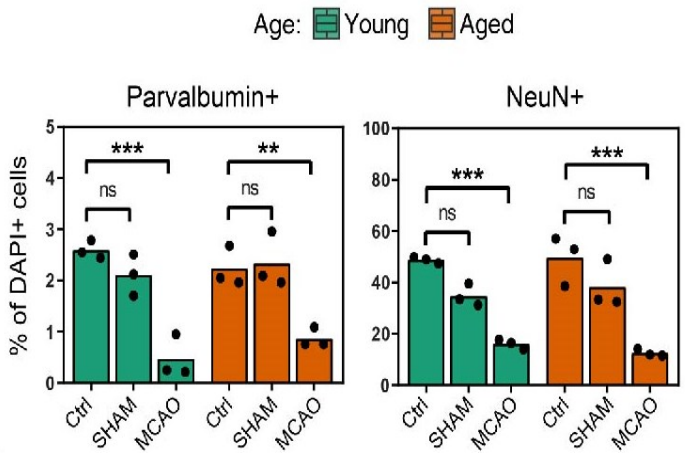


Figure 11: Cell type proportion estimation by transcriptome deconvolution. Raincloud plots showing z-scored expression of selected marker genes for major brain cell populations. Asterisks show Holm-corrected post-hoc t-test p-values.

For this reason, we counted the numbers of $NeuN^+$ and parvalbumin $(PV)^+$ cells by immunohistochemistry (Fig. 12). We found that reduction of both $Neun^+$ and PV^+ cells after FCI was similar in both age groups, suggesting that the greater decrease of PV^+ associated expression in aged mice is a result of transcriptional downregulation, rather than greater PV^+ cell loss.

Figure 12: Immunohistochemical quantification of Parvalbumin (PV) and NeuN-positive cells. Asterisks show significance of Sidak-corrected post-hoc pairwise comparisons. Results of two-way ANOVA are shown on the right. Significance codes (***) <0.001; (**) <0.01; (*) <0.05; (ns) >0.05.



A hallmark of the differential response of aged animals to ischemia was activation of IFN-I signaling pathway (Fig. 10B). IFN-Is are key antiviral cytokines that elicit prototypical Interferon-stimulated genes (ISGs) encoding antiviral and inflammatory mediators (Owens et al., 2014). It has been showed that blocking the IFN-I signaling improves ischemia outcome in young mice (Zhang et al., 2017). We have therefore focused on the key players in the brain inflammatory responses – glia and endothelial cells (Lecuyer et al., 2016) – and aimed to identify how they contribute to IFN-I signaling following stroke. Microglia and oligodendrocytes heavily upregulated ISG expression following FCI in both young and aged animals. Endothelial cells displayed opposite behavior and significantly downregulated vast majority of measured ISGs in young, and to a much lesser extent in the aged mice. The expression of astrocyte ISGs did not change in a synchronized fashion, altogether showing a limited response of astrocytes to IFN after stroke in both age groups.

5. Debate

5.1. Proliferation and differentiation potential of NG2 glia following FCI

The was majority of studies about proliferation, differentiation and maturation of oligodendrocytes and their precursors under physiological and pathological conditions focus on oligodendroglial lineage in context of myelination/remyelination (Spitzer et al., 2016, Hughes and Appel, 2016, Chamberlain et al., 2016, Wheeler and Fuss, 2016). Therefore, we analyzed the gene expression profile of NG2 glia from the ischemic mouse brain.

Following CNS injuries NG2 glia start to proliferate massively and migrate to the site of injury (Anderova et al., 2011, Sirko et al., 2013). Besides that, NG2 glia are able to differentiate into reactive astrocytes; however, the rate of differentiation is injury-dependent as reviewed by (Dimou and Gallo, 2015). Here we identified three subpopulations of NG2 glia emerging following FCI, which are phenotypically distinct from uninjured NG2 glia. Whereas the most P-NG2 cells and A-NG2 cells were detected three days after FCI, OL-NG2 cells peaked at day seven after FCI. These results suggest that FCI induces preferentially rapid proliferation of NG2 glia and their participation in glial scar formation rather than generation of new oligodendrocytes.

This analysis revealed the heterogeneity of NG2 glia, which becomes much greater after FCI. We identified four phenotypically distinct subpopulations of NG2 glia emerging after FCI and concluded that NG2 acquired FCI-induced multipotent character.

5.2. Appearance of NG2 glia-derived astrocytes between different types of CNS disorders

Numerous reports describe NG2 glia differentiation into astrocytes (Huang et al., 2019, Huang et al., 2014, Honsa et al., 2012, Honsa et al., 2016, Dimou et al., 2008), however, the majority of such studies only focuses on one type of injury, such as ischemia (Honsa et al., 2012), cortical SW (Komitova et al., 2011), or a

demyelinating injury (Zawadzka et al., 2010). It is difficult to compare these studies which employed various types of cre transgenic mice, different modes of TX administration, and different identification of cell types. Therefore, we analyzed NG2 glia heterogeneity after different types of CNS disorders (FCI, SW, DEMY) without any inter-lab variability, using the same criteria for all the disorders.

Since a specific subpopulation of astrocyte-like NG2 glia, which emerges following FCI, was already reported in our previous publications (Honsa et al., 2016, Valny et al., 2018) here we clearly state that astrocyte-like NG2 glia represent a unique, FCI-specific subpopulation. It is neither present in the other two types of injuries (DEMY, SW), nor in neurodegenerative diseases such as AD (Valny et al., 2018). The subpopulation of astrocyte-like NG2 glia is not permanent and it disappears during the progression of glial scar, most likely at the expense of NG2 glia subpopulation 2. Nevertheless, it suggests that for a certain period following ischemia, NG2 glia are multipotential and represent, together with astrocytes, crucial players in post-ischemic regeneration. It has still not been clarified what factors contribute to the differential switch of NG2 glia towards the astrocytic phenotype. Our previous studies suggested Sonic hedgehog as a potential modulator (Honsa et al., 2016), however, additional studies are needed to understand the reprogramming of NG2 glia from oligodendrogenesis to astrogliogenesis. Since massive astrogliosis and inflammation occur following ischemia and have a great impact on the repair processes (Magaki et al., 2018), we hypothesize that the immune response, together with astrogliosis, may only initiate NG2 glia transient increase of astrocytic markers in severe types of CNS injuries. Recent findings show that bone morphogenic protein, which is secreted by astrocytes, inhibits the generation of myelinating oligodendrocytes, and promotes differentiation into the astrocyte phenotype (Magaki et al., 2018). It is not clear why cortical SW, which also leads to marked astrogliosis and microglia activation, does not result in a similarly increased number of astrocyte-like NG2 glia. A possible explanation for their unique generation following ischemia may dwell from the diverse concentrations of extracellular

glutamate/K^+, that occur during FCI and SW (Anderová et al., 2004, Hansen, 1978) and may result in a diverse severity of these CNS injuries.

Our findings suggest that astrocyte-like NG2 glia may represent important players in glial scar formation and the CNS repair process. For the reason that astrocytes do not migrate toward lesion and only a limited number of astrocytes divide (Sofroniew and Vinters, 2010), astrocyte-like NG2 glia could temporarily perform the functions of astrocytes in the vicinity of the lesion.

5.3. Interaction between stroke and aging at the genome-wide level using model of FCI

We systematically analyzed the impact of aging, stroke, and their interaction on genome-wide expression profiles. Several studies documented that inflammation is increased in the aged brain (Benayoun et al., 2019, Cribbs et al., 2012, Ori et al., 2015), and it has been reported that the changes occur predominantly in glial cells (Davie et al., 2018, Soreq et al., 2017).

We investigated how adult and aged brain responds to ischemia. The evaluation of cellular differences revealed that genes enriched in PV^+ GABAergic interneurons are more greatly downregulated in aged mice after ischemia. Dysfunction or loss of PV^+ interneurons is implicated in the pathology of numerous neuropsychiatric disorders, including schizophrenia (Del Pino et al., 2013, Lewis et al., 2005), AD (Verret et al., 2012), and depression (Sauer et al., 2015, Zhou et al., 2015). Our immunohistochemical analysis showed no change in PV^+ cell numbers between age groups, suggesting that decreased PV+ signal may occur through transcriptional downregulation. A similar result was reported in mouse model of autism, where decrease of PV^+ signal, previously interpreted as loss of PV^+ interneurons, occurs through transcriptional downregulation (Filice et al., 2016).

An outstanding feature of differential response of aged animals to ischemic stroke was upregulation of IFN-I pathway, which persisted for at least 14 days. IFN-Is are

antiviral cytokines with pleiotropic roles (Prinz and Knobeloch, 2012) implicated in a number of CNS pathologies including MS (Goldmann et al., 2015, McDonough et al., 2017b), AD (Friedman et al., 2018, Frigerio et al., 2019, Taylor et al., 2014) and ischemia (Chen et al., 2014, McDonough et al., 2017a, Zhang et al., 2017). Therefore, we have analyzed INF-I signaling in more detail. As the cell-specific context to the IFN-I signaling after ischemia has not been well described in the literature, we profiled the responses of the glia and endothelial cells—known as key players in CNS neuroinflammation (Lecuyer et al., 2016). Our results support the perspective that not only microglia, but also oligodendrocytes are active players in the acute inflammatory response after ischemia. On the contrary, endothelial cells expressed the highest levels of ISGs under control conditions, they downregulated IFN-I pathway after ischemia. This discrepancy may be associated with different roles of IFN-I signaling in the endothelial cells, in line with the role IFNb plays in the maintenance of the blood brain barrier (BBB) integrity (Owens et al., 2014).

In conclusion, detailed insights into transcriptional response to ischemia described in this study may contribute to our understanding of the interplay between ischemic pathology and aging, and open avenues for the future search for effective therapeutic approaches.

6. Conclusions

In the present work, we studied the proliferation and differentiation potential of NG2 glia following different types of CNS pathologies and how the pathophysiology of the brain is altered during aging. To be able to examine those different features of NG2 glia we had to crossbreed many different transgenic mice and employed several laboratory methods that helped us to identify changes from mRNA, protein, and functional points of view.

First, analysis of the differentiation potential of NG2 glia confirmed that under physiological conditions, they serve mainly as precursor cells for oligodendrocytes. However, following permanent FCI, NG2 glia acquire a multipotent phenotype. As a result, they differentiated not only to oligodendrocytes but also to astrocytes. Besides the differentiation potential, NG2 glia massively proliferate in acute phases after ischemia and migrate toward the lesion suggesting their important role in glial scar formation (Hypothesis 1, Aim 1).

Furthermore, we examined if NG2 glia differentiation potential into astrocytes is only triggered by FCI compared with other types of CNS injuries. We disclosed that an astrocyte-like NG2 glia subpopulation is only present transiently after FCI and following less severe injury, namely the cortical SW and DEMY in corpus callosum and cortex, subpopulations mirroring different stages of oligodendrocyte maturation markedly prevail. Moreover, we proved that astrocyte-like NG2 glia are a specific transient subpopulation located in the ischemic glial scar, actively involved in the cell cycle displaying a current pattern, which is similar to that identified in cortical astrocytes (Hypothesis 2, Aim 2).

Finally, the transcriptional response to ischemia during adulthood and aging in female mice uncovered age-dependent alterations in processes predominantly associated with inflammation and interferon signaling as well as axonal and synaptic maintenance. Furthermore, our results showed that differential stroke outcome is associated with the overactivation of pro-inflammatory pathways and other

potentially detrimental factors in aged mice, rather than activation of the neuroprotective program in young mice (Hypothesis 3, Aim 3).

Taken together, NG2 glia perform multiple functions in both normal and pathological conditions in the brain. They have multipotent properties of self-renewal and repair in many kinds of brain injuries. In response to injury, NG2 glia are not only capable to proliferate and migrate to the lesions but also differentiate into oligodendrocytes or astrocytes. In conclusion, given that NG2 glia are actively involved in fast response to CNS injuries, we suggest that future studies of NG2 glia may unravel their potential therapies of CNS disorders.

Additionally, our results paint a picture of ischemia as a complex age-related disease and provide insights into the interaction of aging and stroke on a cellular and molecular level. Detailed insights into transcriptional response to ischemia described in this study may contribute to our understanding of the interplay between ischemic pathology and aging, and open avenues for the future search for effective therapeutic approaches.

7. Summary in Czech

7.1. Proliferační a diferenciační potenciál NG2 glií po fokální mozkové ischemii

V této práci jsme studovali genetické mapování osudu NG2 glií s využitím genového profilování na úrovni jedné buňky a techniky proteinové exprese. Identifikovali jsme tři subpopulace NG2 glií, které vznikají po FCI: což jsou proliferující NG2 buňky a buňky podobné astrocytům a oligodendrocytům; takové buněčné typy byly dále potvrzeny imunohistochemicky. Souhrnně jsme identifikovali několik dosud neznámých rozdílů mezi expresními profily NG2 glií a charakterizovali specifické geny přispívající k fenotypickým změnám NG2 glií po FCI.

7.2. Výskyt astrocytů odvozených z NG2 glií u různých typu poškození CNS

V této studii jsme porovnávali expresní profily NG2 glií po různých typech poranění CNS (FCI, SW, DEMY). Výsledky odhalily, že subpopulace NG2 glií exprimující GFAP, marker reaktivních astrocytů, byla přítomna pouze po FCI. Po méně závažných poraněních (SW, DEMY) však výrazně převažovala subpopulace odrážející různá stádia zrání oligodendrocytů. Celkově jsme dokázali, že astrocytům podobné NG2 glie jsou specifickou subpopulací NG2 glií objevujících se přechodně pouze po FCI. Tyto buňky, umístěné v post ischemické gliální jizvě, vykazují proudový profil podobný tomu, který byl identifikován v běžných kortikálních astrocytech.

7.3. Vztah mezi mrtvicí a stárnutím na úrovni celého genomu pomocí modelu fokální mozkové ischemie

V těchto experimentech jsme provedli komplexní analýzu RNA sekvenování během stárnutí, po ischemii a v jejich kombinaci u myší ve věku 3 a 18 měsíců. Odhalili jsme sníženou expresi genů zodpovědných za údržbu a stabilitu axonů a synapsí a zvýšenou aktivaci interferonu typu I (IFN-I) po mrtvici u starých myší. Tyto výsledky společně vykreslují obraz ischemické cévní mozkové příhody jako komplexního onemocnění souvisejícího s věkem a poskytují popis vztahu stárnutí a cévní mozkové příhody na buněčné a molekulární úrovni.

8. Summary in English

8.1. Proliferation and differentiation potential of NG2 glia following focal cerebral ischemia

In this work, we studied genetic fate-mapping of NG2 glia employing single-cell gene profiling and protein expression techniques. We identified three subpopulations of NG2 glia emerging after FCI: proliferative cells; astrocyte-like and oligodendrocyte-like NG2 cells; such phenotypes were further confirmed by immunohistochemistry. Collectively, here we identified several yet unknown differences between the expression profiles of NG2 glia and characterized specific genes contributing to phenotypical changes of NG2 glia after FCI.

8.2. Appearance of NG2 glia-derived astrocytes between different types of CNS disorders

In this study, we compared NG2 glia expression profiles following different CNS injuries (FCI, SW, DEMY). The results revealed that the subpopulation of NG2 glia expressing GFAP, a marker of reactive astrocytes, is only present after FCI. However, following less severe injuries (SW, DEMY), subpopulations mirroring different stages of oligodendrocyte maturation markedly prevail. Overall, we have proved that astrocyte-like NG2 glia are a specific subpopulation of NG2 glia emerging transiently only following FCI. These cells, located in the post-ischemic glial scar display a current pattern similar to that identified in cortical astrocytes.

8.3. Interaction between stroke and aging at the genome-wide level using a model of focal cerebral ischemia

In these experiments, we performed a comprehensive RNA-seq analysis of aging, ischemia, and their interaction in 3- and 18-month-old mice. We uncovered downregulation of axonal and synaptic maintenance genetic program and increased activation of type IFN-I signaling following stroke in aged mice. Together, these results paint a picture of ischemic stroke as a complex age-related disease and provide insights into the interaction of aging and stroke on the cellular and molecular level.

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10. List of publications

Publications related to the thesis:

1. **Kirdajova D**, Valihrach L, Valny M, Kriska J, Krocianova D, Benesova S, Abaffy P, Zucha D, Klassen R, Kolenicova D, Honsa P, Kubista M, Anderova M. Transient astrocyte-like NG2 glia subpopulation emerges solely following permanent brain ischemia. Glia. **2021** Nov;69(11):2658-2681. doi: 10.1002/glia.24064. Epub 2021 Jul 27. PMID: 34314531. (IF=7.4520, Q1)
2. Androvic P, **Kirdajova D**, Tureckova J, Zucha D, Rohlova E, Abaffy P, Kriska J,Valny M, Anderova M, Kubista M, Valihrach L. Decoding the Transcriptional Response to Ischemic Stroke in Young and Aged Mouse Brain. Cell Rep. **2020** Jun 16;31(11):107777. doi: 10.1016/j.celrep.2020.107777. PMID: 32553170. (IF=9.423, Q1)
3. Valny M, Honsa P, Waloschkova E, Matuskova H, Kriska J, **Kirdajova D**, Androvic P, Valihrach L, Kubista M, Anderova M. A single-cell analysis reveals multiple roles of oligodendroglial lineage cells during post-ischemic regeneration. Glia. **2018** May;66(5):1068-1081. doi: 10.1002/glia.23301. Epub 2018 Feb 2. PMID: 29393544. (IF=6.200, Q1)

Other publications:

4. Maleninska K, Janikova M, Radostova D, Vojtechova I, Petrasek T, **Kirdajova D**, Anderova M, Svoboda J, Stuchlik A. Selective deficits in attentional set-shifting in mice induced by maternal immune activation with poly(I:C). Behav Brain Res. 2022 Feb 15;419:113678. doi: 10.1016/j.bbr.2021.113678. Epub 2021 Nov 25. PMID: 34838932 (IF=3.332, Q3).
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7. **Kirdajova D**, Anderova M. NG2 cells and their neurogenic potential. Curr Opin Pharmacol. 2019 Dec 23;50:53-60. doi: 10.1016/j.coph.2019.11.005. [Epub ahead of print] Review. PubMed PMID: 31877531. (IF=5.547, Q1)
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11. Valny M, Honsa P, **Kirdajova D**, Kamenik Z, Anderova M. Tamoxifen in the Mouse Brain: Implications for Fate-Mapping Studies Using the Tamoxifen-Inducible Cre-loxP System. Front Cell Neurosci. 2016 Oct 20;10:243.

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