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Glutamátové receptory NG2 gliových buněk: genové profilování a funkční změny po ischemickém poškození mozku

Glutamate receptors in NG2-glial cells: gene profiling and functional changes after ischemic brain injury

Diplomová práce

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 2.5.2017

Podpis

Poděkování:

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Abstrakt

Glutamát je hlavním excitačním neuropřenašečem v mozku savců a jeho přenos je zodpovědný za vyšší mozkové funkce, jako jsou učení, pamět či kognice. Účinek glutamátu je zprostředkován různými typy glutamátových receptorů, jejichž vlastnosti byly doposud studovány převážně v neuronech. Glutamátové receptory jsou ovšem exprimovány také v NG2 gliových buňkách, nicméně jejich role, jak ve zdravém tak i v poškozeném mozku, není zcela známá. Cílem této práce bylo objasnit složení a funkce těchto receptorů u NG2 gliových buněk za fyziologických podmínek a po fokální cerebrální ischemii. K tomuto účelu jsme použili transgenní myši, ve kterých jsou NG2 gliové buňky značeny pomocí fluorescenčního proteinu, což nám umožnilo jejich přesnou identifikaci. Analýza expresního profilu glutamátových receptorů v NG2 gliových buňkách byla provedena pomocí metody RT-qPCR na úrovni jedné buňky. Dále byla použita imunohistochemie a fluorescenční metoda zobrazování intracelulárních hladin vápenatých iontů (calcium imaging) pro detekci glutamátových receptorů na úrovni proteinu a charakterizaci jejich funkčních vlastností.

Klíčová slova: NG2 gliové buňky, glutamátové receptory, RT-qPCR, fluorescenční zobrazování intracelulárních hladin vápníku, fokální cerebrální ischemie

Abstract

Glutamate is the main excitatory neurotransmitter in the mammalian brain and its transmission is responsible for higher brain functions, such as learning, memory and cognition. Glutamate action is mediated by a variety of glutamate receptors, though their properties were until now studied predominantly in neurons. Glutamate receptors are expressed also in NG2-glia, however their role under physiological conditions as well as in pathological states of the central nervous system is not fully understood. The aim of this work is to elucidate the presence, composition and function of these receptors in NG2-glia under physiological conditions and following focal cerebral ischemia. For this purpose we used transgenic mice, in which NG2-glia are labeled by a fluorescent protein for their precise identification. To analyze the expression pattern of glutamate receptors in NG2-glia we employed single-cell RT-qPCR. Furthermore, we used calcium imaging to characterize their functional properties.

Key words: NG2-glia, glutamate receptors, RT-qPCR, calcium imaging, focal cerebral ischemia

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List of abbreviations

$[Ca^{2+}]_i$	intracellular calcium concentration
AC	adenylyl cyclase
aCSF	artificial cerebrospinal fluid
ADP	adenosine diphosphate
ALDH1L1	aldehyde dehydrogenase 1 family member L1
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATD	amino-terminal domain
ATP	adenosin triphosphate
BLAST	basic local alignment search tool
BrdU	bromodoexyuridine
CA1	Cornu Ammonis 1
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine monophosphate
cDNA	clone deoxyribonucleic acid
CNS	central nervous system
C_q	quantification cycle
Cre	Cre recombinase
Cre-ERt	tamoxifen-inducible Cre recombinase
CSPG4	chondroitin sulfate proteoglycan 4
Cspg4	gene encoding chondroitin sulfate proteoglycan 4
CTZ	cyclothiazide
D1-21	days of experiment
DNA	deoxyribonucleic acid
DCX	marker of newly derived neuronal cells
dNTP	deoxyribonucleotide triphosphate
EGFP	enhanced green fluorescent protein
F	fluorescence intensity
FCI	focal cerebral ischemia
GABA	ɣ-aminobutyric acid
GCI	global cerebral ischemia
GFAP	glial fibrillary acidic protein
GLT-1	glutamate transporter 1

Glu	glutamate
GluA	subunit of AMPA receptor
GluK	subunit of kainate receptor
GluN	subunit of NMDA receptor
GLUT	glucose transporter
Gly	glycine
Gria	genes encoding AMPA receptor subunits
Grik	genes encoding kainate receptor subunits
Grin	genes encoding NMDA receptor subunits
i.p.	intra-peritoneally
iGluRs	ionotropic glutamate receptors
IP3	inositol triphosphate
KAR	kainate receptors
KCl	potassium chloride
LBD	ligand-binding domain
LDHA	Lactate dehydrogenase isoenzyme A
LTD	long-term depression
LTP	long-term potentiation
MCA	middle cerebral artery
MCAo	middle cerebral artery occlusion
MCT	monocarboxylate transporter
$MgCl_2$	magnesium chloride
mGluRs	metabotropic glutamate receptors
MK-801	Dizocilpine
Na_2HPO_4	disodium hydrogen phosphate
NaCl	natrium chloride
$NaHCO_3$	sodium bicarbonate
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NG2	neural/glial antigen 2
NG2+	neural/glial antigen 2 positive
NMDA	N-methyl-D-aspartate
NMDG	N-methyl-D-glucamine
NO	nitric oxide
O2A	oligodendrocyte-type-2 astrocyte

O4	immature oligodendrocyte antigen
OLIG2	oligodendrocyte transcription factor
Olig2	gene encoding oligodendrocyte transcription factor
OPCs	oligodendrocyte precursor cells
PCA	principal component analysis
PDGFRα	platelet-derived growth factor receptor-α
Pdgfra	gene encoding platelet-derived growth factor receptor-α
PDGFRβ	platelet-derived growth factor receptor- β
Pdgfrb	gene encoding platelet-derived growth factor receptor- β
PLC	phospholipase C
PTB	pentobarbital
qPCR	quantitative polymerase chain reaction
Q/R	glutamine/arginine
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-qPCR	reverse transcription – quantitative polymerase chain reaction
S.E.M.	standard error of the mean
SERCA	sarco/endoplasmic reticulum Ca^{2+} ATPase
tdTomato	red fluorescent reporter protein
tdTomato+	red fluorescent reporter protein positive
TMD	transmembrane domain
TTX	tetrodotoxin

Introduction

Cerebral ischemia is a result of cardiac arrest or the occlusion of one of the major brain arteries, i.e. stroke, and is the third most common cause of death in developed countries. The pathological changes responsible for brain damage are caused by the reduction of blood supply resulting in the death of neurons and consequently impaired brain function (Who.int, 2016). Since the only effective strategy to fight the consequences of ischemia is the restoration of blood flow (Blakeley & Llinas, 2007), many studies are focusing on cellular mechanisms underlying ischemic brain damage. Clarifying the roles of individual brain cell types in the pathology of ischemia could lead to new therapeutic approaches.

Unsurprisingly, the primarily studied cell type regarding cerebral ischemia is neurons since the death of these cells is the main cause of brain function loss. Therefore, numerous clinical trials were conducted on the base of neuroprotection (Danton & Dietrich, 2004) or replenishment of neuronal cells using pluripotent stem cells, however most of them were not effective (Kalladka & Muir, 2014; Kwon et al., 2012). Nevertheless, since glial cells were acknowledged to be an important part of the brain tissue as well, the focus spanned also to non-neuronal mechanisms in the pathology of cerebral ischemia (Nedergaard & Dirnagl, 2005). The numerous essential roles of glial cells in the central nervous system (CNS) include K^+ buffering, glutamate clearance, metabolic support of neurons or modulation of synaptic transmission. Since these functions are impaired in the pathophysiology of cerebral ischemia, glial cells become an important target for innovative therapeutic approaches.

Importantly, ischemia-induced cellular damage is mediated predominantly by excessive accumulation of glutamate in the extracellular space leading to Ca^{2+} overload in cell types possessing functional glutamate receptors. This triggers a number of lethal processes ultimately causing cell death (Won, Kim, & Gwag, 2002). Recent studies have shown the presence of various glutamate receptor types in glial cells as well, however their role is not yet utterly clarified neither in the healthy brain nor in the pathological events of cerebral ischemia.

1. Glial cells

Since glial cells are unable to generate an action potential, they were considered to possess mainly a supportive structural function in the brain. However, nowadays glial cells are thought to have numerous important roles in proper neuronal functioning in the CNS as well as the peripheral nervous system (PNS; Verkhratsky and Butt 2007). Recently, a highly discussed topic regarding glial cells is their ability to proliferate and differentiate not only in the developing brain, but also in the adult and aged CNS. Therefore, elucidating mechanisms that retain proliferation/differentiation of glial cells following CNS injury might represent a key step towards the brain tissue regeneration (Leda Dimou & Götz, 2014).

Glial cells are basically divided in two groups. Glial cells of the PNS, which are mainly represented by Schwann cells and glial cells of the CNS, which comprise astrocytes, oligodendrocytes and NG2-glia (a subgroup of ectoderm originated macroglia) and a group of cells of mesodermal origin termed microglia (Verkhratsky & Butt, 2013) (Figure1).

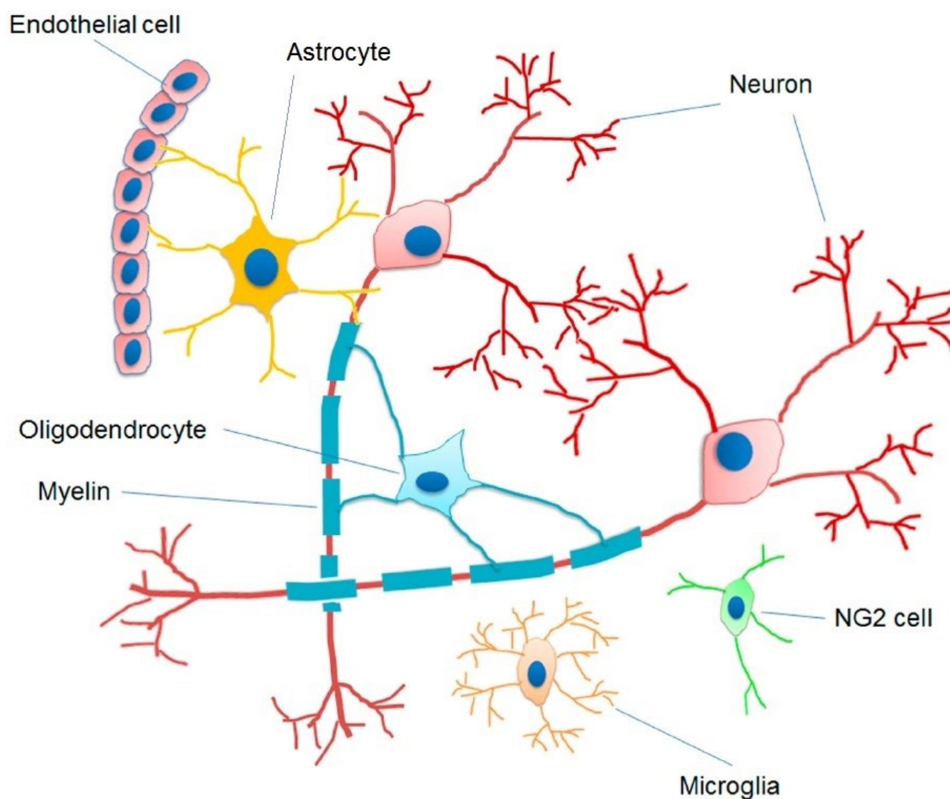

Figure 1: Illustration of CNS glial cell types and their connections (modified from Xiao et al. 2014). CNS, central nervous system

1.1 Astrocytes

It is well known that astrocytes more than other glial cells play a crucial role in preserving a sustainable environment for neurons. Aside from their structural function, the ability of astrocytes to maintain brain homeostasis represents a key feature, which is essential for proper neuronal functioning (Parpura & Verkhratsky, 2012). There are two types of astrocytes recognized in the brain. Protoplasmic astrocytes with short and branched processes, which are found mainly in grey matter and fibrous astrocytes with longer and more numerous processes located primarily in the white matter (Miller & Raff, 1984) (Figure2).

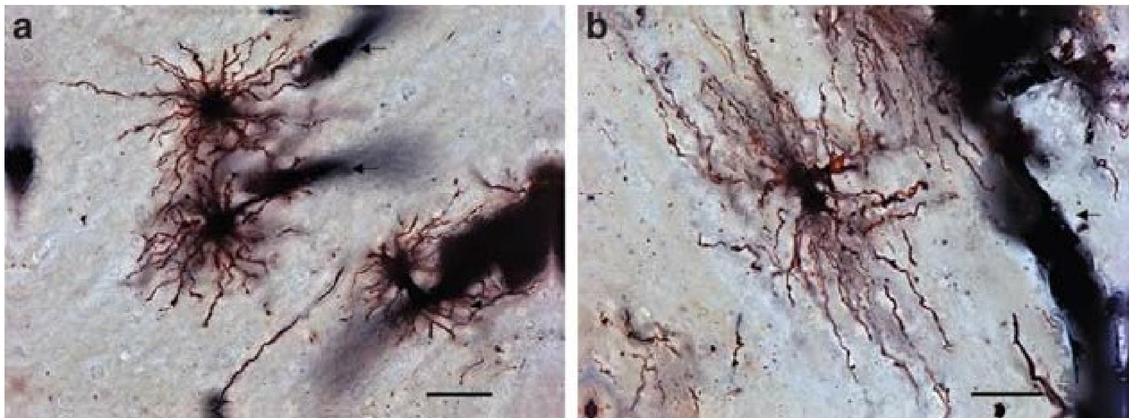

Figure 2: Golgi-stained protoplasmic and fibrous astrocytes in the human brain. Protoplasmic astrocytes from gray matter (a) showing shorter branched processes and a spherical cell body. Fibrous astrocytes in the white matter (b) presenting longer thorny processes extended in opposite directions. Both types of astrocytes form connections with blood vessels (arrows; Torres-Platas et al. 2011).

For immunohistochemical recognition of astrocytes the most commonly used marker is the glial fibrillary acidic protein (GFAP), although it is preferentially expressed in white matter astrocytes and not expressed in all astrocytic processes. Regarding these limitations of GFAP as a marker of astrocytes, a new marker has been discovered, the aldehyde dehydrogenase 1 family member L1 (ALDH1L1). It is presumably expressed in all astrocytic cells throughout the whole brain regardless of age or pathological condition (Cahoy et al., 2008).

Among other astrocytic functions, such as exchanging metabolites with neurons, forming the blood-brain barrier, regulating cerebral blood flow, glial scar formation or modulation of synaptic activity by releasing gliotransmitters, their ability to maintain brain homeostasis plays a major role in proper functioning of neurons and synaptic

signaling. Astrocytes express a variety of receptors and transporters enabling them to control ion-, neurotransmitter- and metabolite concentrations, pH as well as osmotic balance (Simard & Nedergaard, 2004). Possessing this wide variety of transport systems, astrocytes are able to control the environment of the synaptic cleft using K^+ channels for K^+ uptake (Kofuji & Newman, 2004), aquaporins for maintaining osmolarity levels or neurotransmitter transporters, importantly glutamate transporters, for removing the excessive amount of this neurotransmitter from the extracellular space. Within astrocytes glutamine synthase transforms glutamate to glutamine, which is then transported back into neurons and there converted back to glutamate (Shen, 2013).

Moreover, astrocytes are crucial for providing necessary metabolites to neurons. Glycolysis in astrocytes, stimulated aside from other factors by glutamate uptake, results in converting glucose, transported to the cell from the blood, to pyruvate, which is subsequently transformed to lactate. Lactate, as the major neuronal energetic substrate, is then transported to neurons where it is converted back to pyruvate, which is then used in the Krebs cycle to produce energy in the form of adenosine triphosphate (ATP; Magistretti 2006) (Figure3).

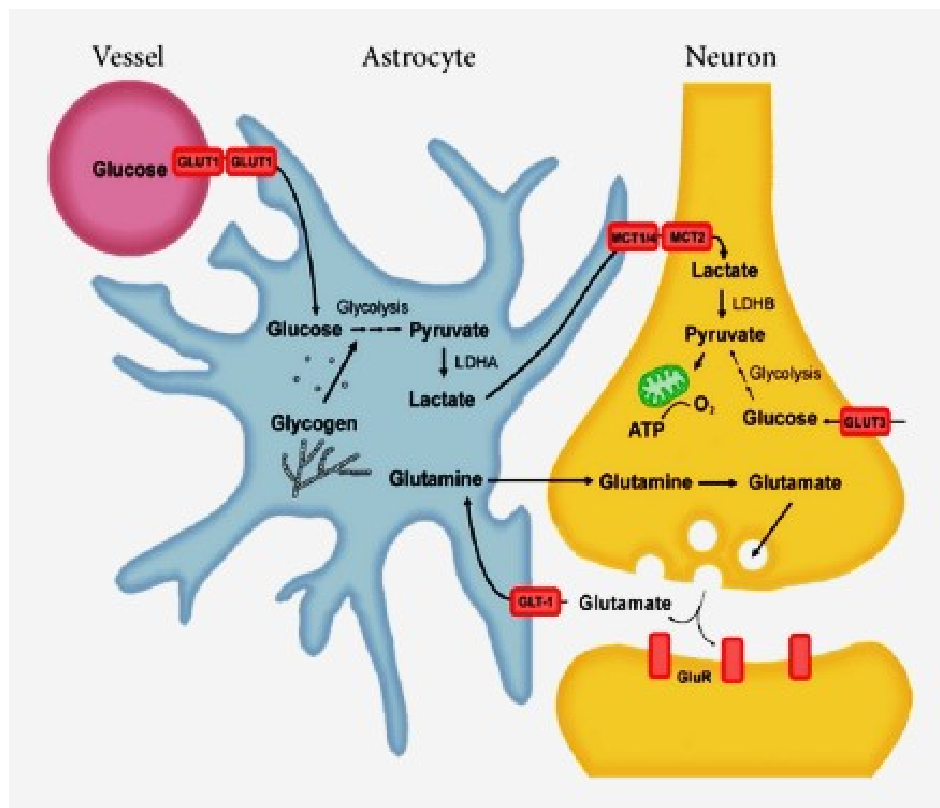

Figure 3: The lactate shuttle hypothesis. The glutamate released from neurons upon activation is taken up into astrocytes by glutamate transporters and there converted to glutamine. The glutamate uptake stimulates increased glucose uptake from blood vessels via glucose

transporters and elevates aerobic glycolysis (which can be also stimulated by breaking down intracellular stores of glycogen). LDHA then converts pyruvate to lactate, which is afterwards exported out of the cell via the MCT1/4 and transported into neurons by MCT2. Lactate is then converted by LDHB to pyruvate, which is utilized within oxidative phosphorylation in mitochondria. Glucose can alternatively enter neurons via GLUT3 (Newington, Harris, & Cumming, 2013). GLT, glutamate transporters; GLUT, glucose transporters; LDH, lactate dehydrogenase isoenzyme; MCT, monocarboxylate transporters

1.2 Microglia

The immune system in the CNS is mainly represented by microglia. Microglia can be activated by numerous stimuli, such as CNS injury, viral or bacterial infections or neuronal degeneration, and ultimately, they turn into cells, which are able of phagocytosis, proliferation and migration towards infected or injured locations, where they act as local macrophages (Kettenmann, Hanisch, Noda, & Verkhratsky, 2011). In their active state microglia produce and release inflammatory cytokines, chemokines, proteolytic enzymes, complement proteins and various other molecules, and moreover, they are capable of presenting antigens to T cells, thus majorly contributing to the immune response as well as affecting other glial cells during inflammation, injury or neurodegeneration (Ransohoff & Perry, 2009).

In their "non-active" state each microglial cell continuously scans the extracellular space of a defined region using its thin branched processes, nonetheless, the scanned domain is strictly bound not to overlap with other microglial regions (Nimmerjahn, Kirchhoff, & Helmchen, 2005).

Microglia are moreover thought to have a major role in postnatal development processes, mainly including postnatal neurogenesis and neuronal development (Walton et al., 2006) as well as contributing to synaptic pruning. Therefore, certain synaptic abnormalities may be possibly caused by impaired microglial function (Paolicelli et al., 2011).

1.3 Oligodendrocytes

The most commonly known function of oligodendrocytes in the CNS is wrapping neuronal axons with myelin and therefore, mainly contributing to action potential propagation (McTigue & Tripathi, 2008). Importantly, the production of

myelin and generation of myelin sheets is conditioned by communication between neurons and oligodendrocytes, since neuronal signaling, including electrical activity or certain growth factors, affect oligodendrocyte proliferation as well as differentiation and even their survival. This takes part mainly during the developmental stages after their differentiation from oligodendrocyte precursor cells (OPCs) in early postnatal life (Simons & Trajkovic, 2006). Interestingly, oligodendrocytes seemingly possess also a neuroprotective function producing some neurotrophic and growth factors as well (McTigue & Tripathi, 2008).

1.4 NG2-glia

NG2-glia represent the most recently distinguished glial cell population in the CNS. They have also been called OPCs, since the first cells described to develop from NG2-glia were oligodendrocytes. The term NG2 (neural/glial antigen 2) was entitled to these cells later when they were first found to express NG2 proteoglycan as the integral membrane chondroitin sulfate proteoglycan 4 (CSPG4; Stallcup & Beasley, 1987). Afterwards, NG2-glia slowly started to be recognized as the fourth major population of glial cells.

The morphology of NG2-glia differs in the CNS depending on the region. In general, they are described as cells with irregular shaped nuclei and numerous branched processes emerging from the cell soma. In comparison, the processes of NG2-glia in white matter extend from their elongated somas parallel to neuronal axons, while among the neuronal cell bodies in gray matter NG2-glial processes branch rather unevenly. As well as the morphology, the distribution of NG2-glia varies from region to region, their density ratio being approximately 1.5 times higher in white matter than in gray matter. In white matter NG2-glia represent 8-9% of all cells, while in gray matter they comprise only 2-3% (Dawson et al., 2003).

Identification of NG2-glia has been mainly enabled by their expression of NG2 proteoglycan (Figure 4), although this marker is also expressed in other brain cell types, such as pericytes (Ozerdem et al., 2001). To distinguish these two cell types, it has been proven to use the platelet-derived growth factor receptor-α (PDGFRα)

as a specific marker of NG2-glia (Hart, Richardson, Heldin, Westermark, & Raff, 1989), since pericytes express a different version of this receptor, the platelet-derived growth factor receptor- β (PDGFRβ; Lindahl et al., 1997).

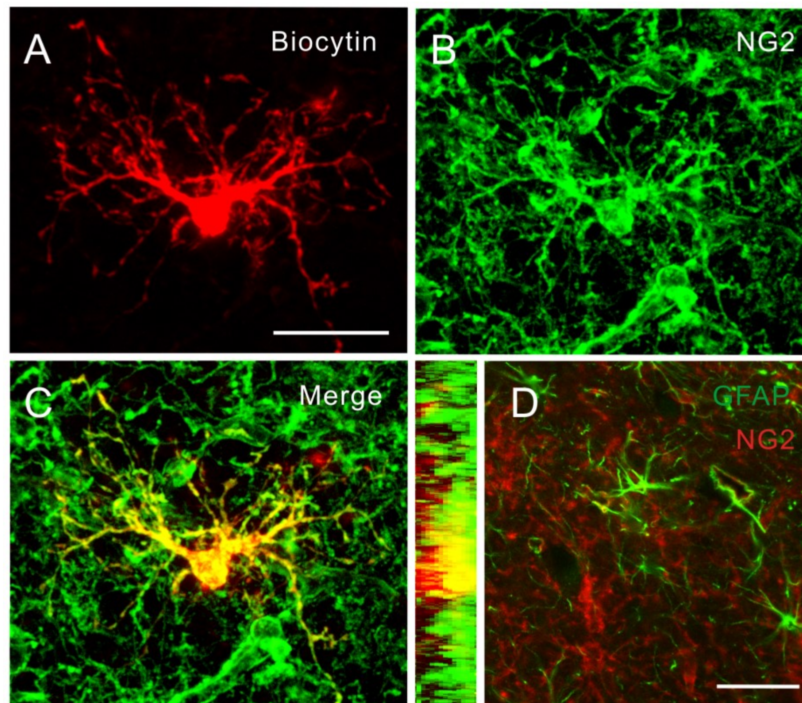

Figure 4: A two-photon image stack of a single NG2-glial cell filled with biocytin (A) merged with immunofluorescent staining against NG2 of the same slice (B) in (C). (D) Double immunofluorescent labeling of astrocytes (labeled by GFAP; green) and NG2^{+} cells (red; Lin et al. 2010). NG2, neural/glial antigen 2; GFAP, glial fibrillary acidic protein

1.4.1 Origin

NG2-glia arise at different developmental stages in different regions of the brain. During embryonal development, they are generated from the ventral germinal zones of the medial and lateral ganglionic eminences. The second, perinatal wave of NG2-glia generation originates from the dorsal and ventral ventricular zone (Kessaris et al., 2006). Importantly, in regions of adult neurogenesis and gliogenesis NG2-glia are generated even postnatally, e.g. in the subventricular zone of the lateral ventricles (Menn et al., 2006). The diverse provenance of NG2-glia might suggests distinct functional properties within NG2-glia population. However, NG2-glia arising from different zones display no significant functional differences with respect to their

membrane properties, responses to glutamate or γ-aminobutyric acid (GABA), basal proliferation rates or cell cycle duration (Tripathi et al., 2011).

1.4.2 Proliferation and differentiation

One of the major roles of NG2-glia is their capability of proliferation and differentiation under physiological and pathological conditions. Their proliferating capacity seemingly decreases with age, since in the healthy young adult brain up to 70-75% of all proliferating cells are NG2-glia while in the aged brain tissue NG2-glia represent only 38% of proliferating cells (Dawson et al., 2003). Similarly, the cell cycle of NG2-glia has been described to slow down with age with subtle variations across different brain regions (Young et al., 2013). Nowadays, the fate of NG2-glia is widely discussed, since it has been described that these cells have the potential to differentiate into various other cell types (Figure 5).

The first cell type discovered to originate from NG2-glia (thereupon called OPCs) were oligodendrocytes. Since the expression of NG2 doesn't occur in mature oligodendrocytes, the *in vivo* proof of this theory had to be conducted by different markers, such as the immature oligodendrocyte antigen O4 or the oligodendrocyte transcription factor OLIG2, which both appear to be expressed in mature oligodendrocytes as well as in their precursors (Ligon et al., 2006; Reynolds & Hardy, 1997). Another approach that led to proving the NG2-glia-oligodendrocyte differentiation was bromodoexyuridine (BrdU) labeling, which is commonly used for identification of proliferating cells. Using this technique it has been shown that after BrdU injection the number of BrdU positive NG2-glia decreases in contrast with the rising number of BrdU positive oligodendrocytes (Bu, Banki, Wu, & Nishiyama, 2004). Nowadays, the usage of more advanced techniques allows studying the fate of NG2-glia using transgenic mouse models expressing Cre recombinase (Cre) under the control of several gene promoters active in NG2-glia, such as *Cspg4*, *Olig2* or *Pdgfra*. The expression of Cre under these specific promoters grants precise identification of NG2-glia and their progeny by using certain Cre reporters (e.g. various fluorescent proteins), which are expressed following Cre-mediated recombination. It is still highly discussed whether a certain population of NG2-glia remains undifferentiated, thus maintaining their own restoration (L Dimou & Gallo, 2015). Nevertheless, following the distribution of Cre reporter proteins, the differentiation of NG2-glia into oligodendrocytes has been

demonstrated in gray and white matter of the forebrain and the spinal cord (Zhu, Bergles, & Nishiyama, 2008).

NG2-glia have been described to give rise also to astrocytes when several studies demonstrated the differentiation of cultured oligodendrocyte-type-2 astrocyte (O2A) progenitor cells, which express NG2, into astrocytes under certain circumstances (e.g. the presence of fetal bovine serum or bone morphogenic proteins; Raff et al. 1983; Mabie et al. 1997). Similarly to the oligodendrocyte differentiation, NG2-glia-derived mature astrocytes lack the expression of NG2, which complicates identification of their origin in *in vivo* experiments. However, GFAP mRNA was detected in NG2 positive cells possibly suggesting some relationship between these two cell lineages (Matthias et al., 2003). Currently, it is generally accepted that NG2-glia are capable of differentiating into protoplasmic astrocytes in the gray matter of certain brain regions during embryonic development (Zhu et al., 2008), nonetheless, postnatally under physiological conditions their fate is most likely restricted to generating oligodendrocytes (Kang et al. 2010; Zhu et al. 2011). An interesting study described the effect of the presence of the *Olig2* transcription factor (a crucial regulator of oligodendrocyte specification and differentiation) on the fate of NG2-glia, demonstrating that deletion of *Olig2*, specifically in NG2-glia, results in converting their fate to astrocytes in the neocortex and corpus callosum. Such conversion occurs mainly during embryogenesis with some observations suggesting even postnatal conversion (Zhu et al., 2012).

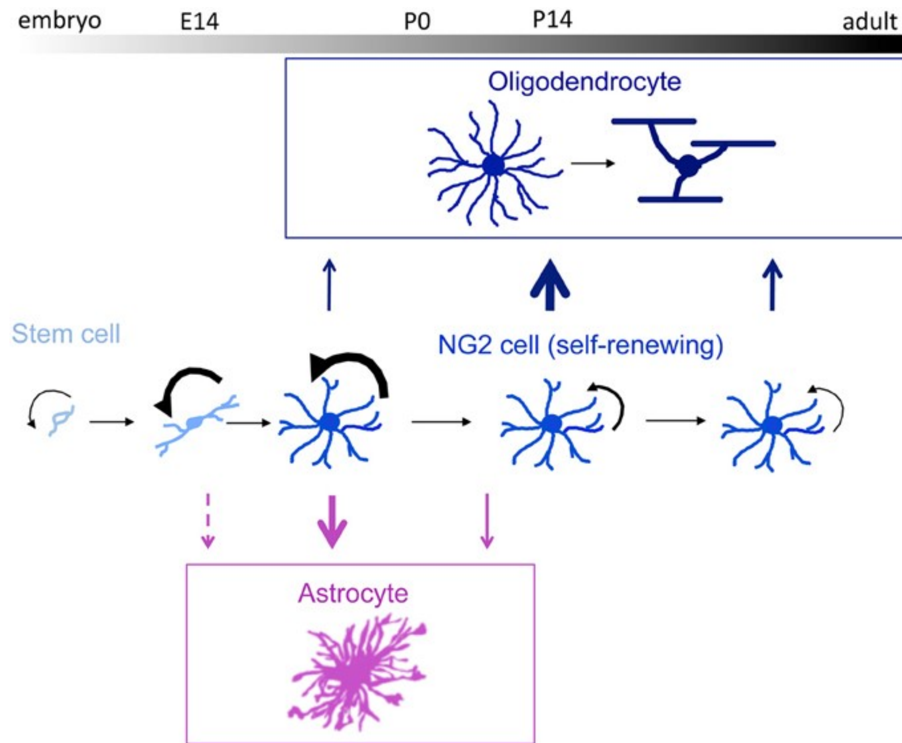

Figure 5: A scheme of NG2-glial proliferation and differentiation throughout development. Black arrows show the rate of proliferation, which is highest perinatally. Blue arrows show differentiation into oligodendrocytes, which is greatest during the third postnatal week. Purple arrows indicate differentiation into astrocytes, this being observed mainly prenatally. The thickness of the arrows projects the rate of proliferation or differentiation (Nishiyama, Suzuki, & Zhu, 2014).

In vitro studies of NG2-glia multipotency revealed their potential to generate also neurons (Belachew et al., 2003; Kondo & Raff, 2000). However, *in vivo* studies trying to prove this differentiation potential provided rather contradictory results. Two research groups detected the expression of certain neuronal or neural stem cell markers and transcription factors in NG2 positive cells (Rivers et al. 2008; Guo et al. 2010). Furthermore, a recent fate-mapping study using NG2/Cre transgenic mice showed cells possessing the electrophysiological properties of neurons, which were derived from NG2-glia in the hypothalamus (Robins et al., 2013).On the contrary, several recent studies using various types of Cre transgenic mouse strains denied the neuronal fate of NG2-glia as they did not find such evidence *in vivo* (Kang et al., 2010; Rivers et al., 2008; Zhu et al., 2011). Regarding these inconsistent findings additional studies are necessary to clarify NG2-glia neurogenic potential.

According to current findings the differentiation potential and proliferation rate of NG2-glia changes under pathological conditions, such as cerebral ischemia, neurodegeneration or traumatic brain injury. Therefore, NG2-glia represent an important glial subtype, which might take part in gliogenesis/neurogenesis following CNS disorders.

1.4.3 Neuron-glia synapses

Recently, an intensely studied function of NG2-glia under physiological conditions is their ability to form synapses with neurons. Thanks to the expression of various receptors or ion channels NG2-glia are able to respond to the action potential of neurons thus creating functional synaptic connections. Although they are able to depolarize their membrane in reaction to the synaptic activity of neurons, NG2-glia alone are not capable of creating action potential, thus being considered as non-excitable cells (Bergles et al., 2010). NG2-glia were described to possess glutamate receptors and GABA receptors as well, which enables them to react to excitatory and inhibitory signals from neurons. It is quite interesting that the neuronal inputs received by NG2-glia differ between white and gray matter. In gray matter NG2-glia receive both excitatory and inhibitory signals, whereas in white matter they receive only excitatory inputs (S. C. Lin & Bergles, 2004; Ziskin, Nishiyama, Rubio, Fukaya, & Bergles, 2007). Reactions to these neuronal signals are therefore thought to influence other important properties of NG2-glia, such as their proliferation and differentiation (Yuan et al. 1998; Kukley et al. 2007). However, as NG2-glia give rise to oligodendrocytes the occurrence of these neuron-glia synapses decreases and such connections are not found between neurons and oligodendrocytes in their mature form (De Biase et al. 2010).

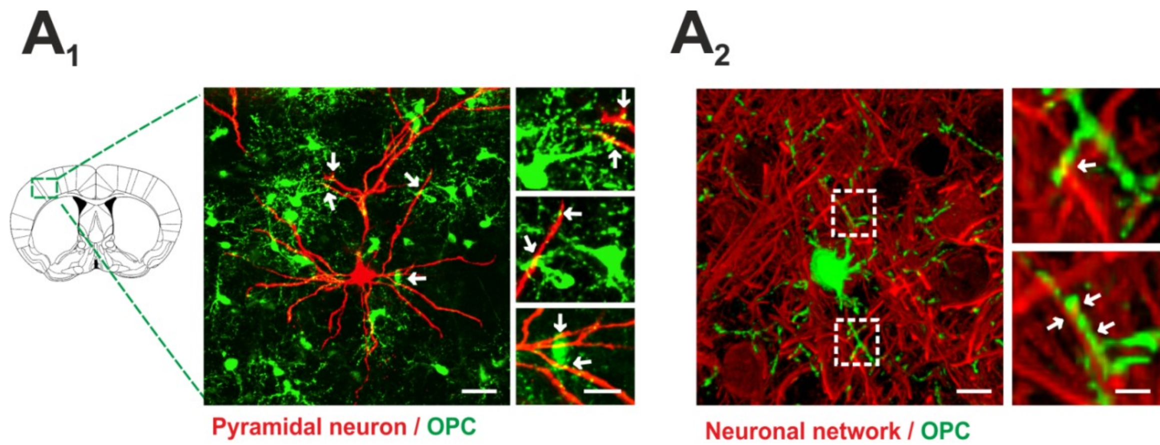

Figure 6: A_1 - Image of a pyramidal neuron form the somatosensory cortex filled with biocytin (red) in contact with NG2-glia (OPCs; expressing enhanced yellow fluorescent protein; green); connections marked by white arrows. Scale bars are 25 µm and 10 µm for higher magnifications. A_2 – NG2-glial cell (green) surrounded by neuronal network (red) in cortex in a 3D confocal stack, connections are highlighted. Scale bars are 10 µm and 2 µm for higher magnifications (modified from Sakry et al. 2014). OPCs, oligodendrocyte precursor cells

2. Glutamate receptors

Glutamate is the main excitatory neurotransmitter in the mammalian brain (Petroff, 2002) acting through two classes of receptors, which are categorized based on their pharmacology and mechanisms of action. Glutamate receptors are divided into two groups, ligand gated ion channels (ionotropic glutamate receptors; iGluRs) and G-protein coupled receptors (metabotropic glutamate receptors; mGluRs) (Figure 7). The distribution, structure and functions of glutamate receptors in the CNS are thoroughly described in neurons; however, they have been detected and studied in glial cells as well (Teichberg, 1991). In neurons glutamate receptors are, above all, responsible for postsynaptic excitation, thus enabling communication between neuronal cells. Furthermore, they take part in the higher cognitive functions, such as learning, decision making, language processing or memory by affecting and modulating synaptic plasticity (Debanne, Daoudal, Sourdet, & Russier, 2003). On the other hand their roles in glial cells have not yet been utterly clarified as glial cells are considered as non-excitable cells of the CNS (Nedergaard, Ransom, & Goldman, 2003). Therefore, glutamate receptors in glial cells are thought to have distinct structural and functional properties compared to those in neurons.

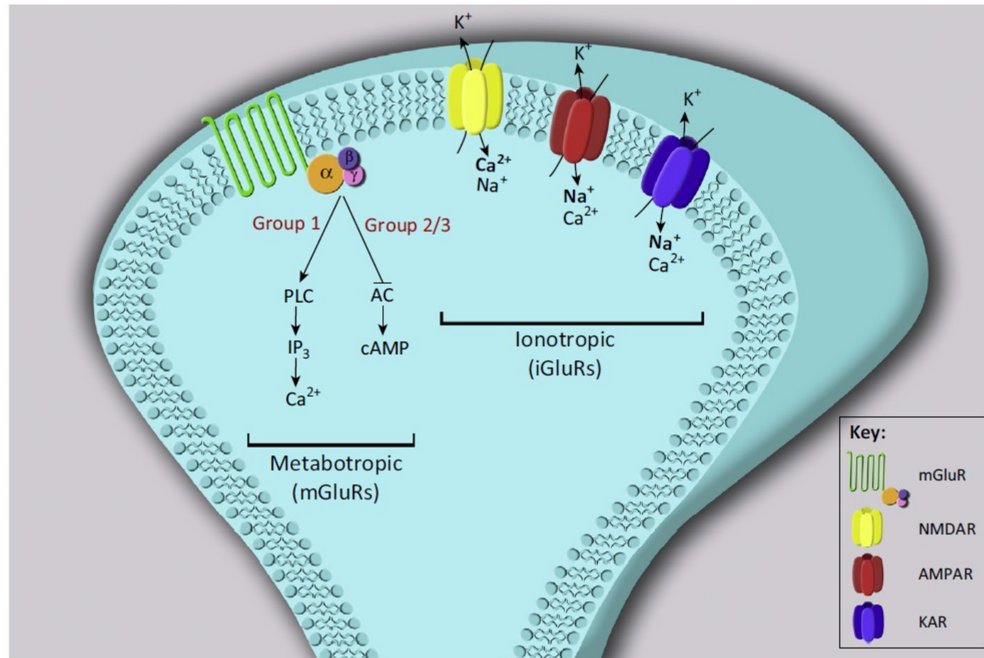

Figure 7: Glutamate receptor subtypes. mGluRs are coupled with G proteins and induce intracellular signaling cascades. iGluRs form ion channels and are selectively permeable to K^+, Na^+ or Ca^{2+} ions (dominant ion typed in bold) and are divided into NMDA receptors, kainate receptors and AMPA receptors (Hogan-Cann & Anderson, 2016). mGluRs, metabotropic glutamate receptors; iGluRs, ionotropic glutamate receptors; NMDAR, N-methyl-D-aspartate receptors; KAR, kainate receptors; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors; AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; IP3, inositol triphosphate; PLC, phospholipase C

2.1 Ionotropic glutamate receptors

iGluRs are essentially ligand-operated ion channels located on the cell membrane responding to glutamate binding by allowing the flow of certain ions (K^+, Na^+ or Ca^{2+}). They generally consist of four subunits enclosing a central pore. Importantly, the subunit composition affects the functionality of the receptor, e.g. permeability to certain ions (Traynelis et al., 2010) as shown in Figure 8. Therefore, based on structural homology and pharmacology (e.g. specific agonist binding), ionotropic glutamate receptors are categorized into four classes: N-methyl-D-aspartate (NMDA) receptors, kainate receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and delta receptors. Interestingly, the delta glutamate receptors are not capable of binding glutamate and serving as a ion channel, hence being categorized as members of this family merely because of their sequence homology (Lomeli et al., 1993). Glial

cells including NG2-glia were found to express iGluR subunits; the level of expression of specific subunits in NG2-glia is shown in Table 1.

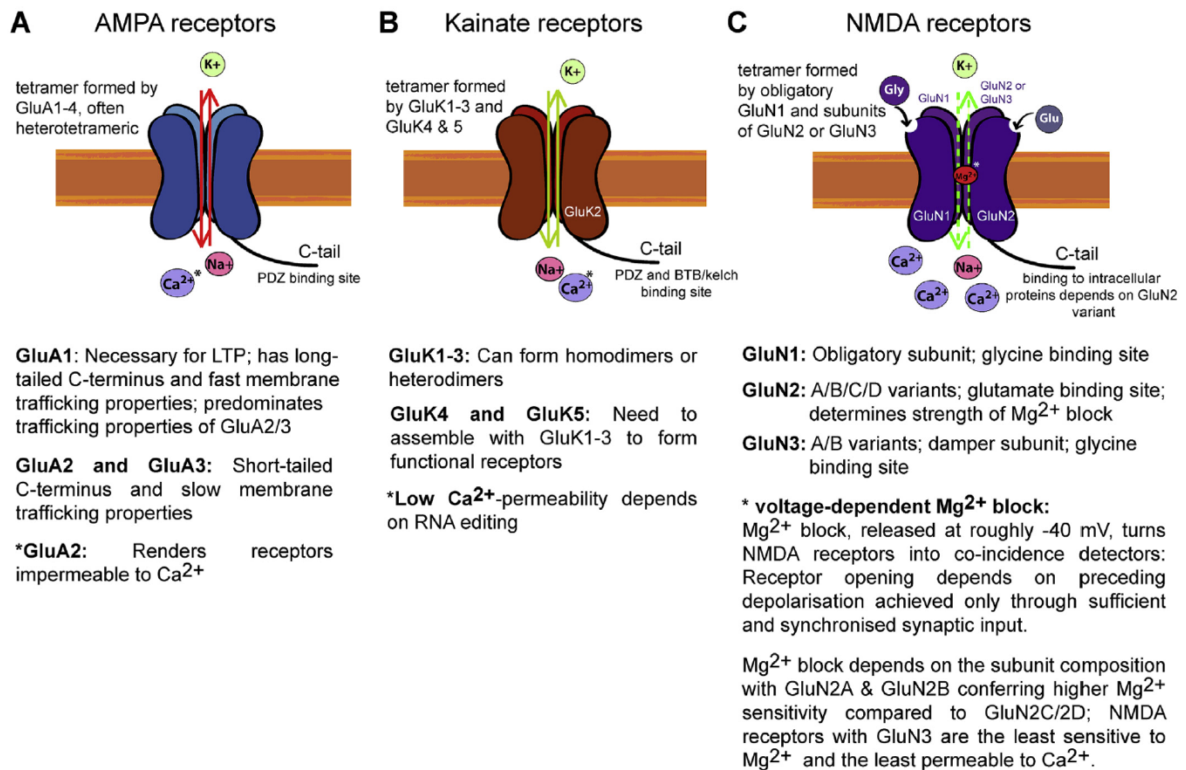

Figure 8: Types of ionotropic glutamate receptors and their possible subunit compositions including the differences in functional properties (Spitzer, Volbracht, Lundgaard, & Karadottir, 2016). AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA, N-methyl-D-aspartate; Gly, glycine; Glu, glutamate; PDZ, LTP, long-term potentiation; GluA, subunit of AMPA receptor; GluK, subunit of kainate receptor; GluN, subunit of NMDA receptor;

Gene Name	Also known as	Expression level in NG2^{+} cells (FPKM)	Additional information (Traynelis et al., 2010)
Gria2	GluA2, GluR2	108.4	AMPAR
Gria3	GluA3, GluR3	54.4	AMPAR
Grik5	GluK5, KA2	47.4	Kainate
Gria4	GluA4, GluR4	37.8	AMPAR
Grid1	GluD1, δ1	30.1	Orphan
Grin3a	GluN3A	23.5	NMDAR
Grik4	GluK4, KA1	19.3	Kainate
Grin1	GluN1, NR1	16.3	NMDAR
Grik3	GluK3, GluR7	14.9	Kainate
Grik2	GluK2, GluR6	14.0	Kainate
Grm5	mGluR5	13.3	Metabotropic (Group I)
Grik1	GluK1, GluR5	12.7	Kainate
Grid2	GluD2, δ2	11.9	Orphan
Gria1	GluA1, GluR1	10.4	AMPAR
Grm3	mGluR3	7.7	Metabotropic (Group II)
Grin2c	GluN2C, NR2C	5.5	NMDAR
Grin2d	GluN2D, NR2D	3.9	NMDAR
Grm4	mGluR4	2.1	Metabotropic (Group III)

Table 1: Expression levels of genes for different glutamate receptor subunits in NG2^{+} cells. The most expressed NMDA receptor subunit is GluN3A. AMPA receptor subunits show an overall higher expression with the most abundant subunit being GluA2. Genes for kainate receptor subunits are expressed as well, GluK5 being the most abundant. The expression of genes

encoding metabotropic glutamate receptors was detected as well. However, RNA-sequencing does not determine expression on protein level (Larson, Zhang, & Bergles, 2016). FPKM, fragments per kilobase of exon per million fragments mapped; NMDAR, N-methyl-D-aspartate receptors; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors GluA, subunit of AMPA receptor; GluK, subunit of kainate receptor; mGluR, metabotropic glutamate receptor; GluN, subunit of NMDA receptor *Gria*, genes encoding AMPA receptor subunits; *Grik*, genes encoding kainate receptor subunits; *Grin*, genes encoding NMDA receptor subunits; *Grm*, genes encoding mGluRs

2.1.1. NMDA receptors

NMDA receptors are heterotetramers, which can consist of GluN1, GluN2A-D and Glu3A-B subunits in various combinations (Hollmann & Heinemann, 1994). Their main agonists are glutamate or NMDA, however, the receptor activation requires binding of a co-agonist, such as glycine or D-serine. The glutamate binding site is located on the GluN2 subunits and the co-agonist binds to the GluN1 or GluN3 subunits (Johnson & Ascher, 1987). To form a properly functioning NMDA receptor it has to carry both the glutamate and glycine binding sites, therefore the most common combination is two GluN2 subunits and two GluN1 subunits or alternatively one GluN2 subunit, two GluN1 subunits and one GluN3 subunit. NMDA receptors containing only GluN1 and GluN3 subunits can form a functional ion channel but lack the glutamate binding site (Smothers & Woodward, 2007). The subunit combinations are specific to certain brain regions and such a large range of possible subunit combinations causes NMDA receptors to differ in various functional properties, such as ion permeability (Na^{+}, K^{+} and Ca^{2+}), desensitization time or specific antagonists (Sanz-Clemente, Nicoll, & Roche, 2013). Interestingly, the ion current flow through NMDA receptor channels is blocked by Mg^{2+} ions at resting membrane potential, thus being also voltage-dependent. Membrane depolarization causes release of Mg^{2+} ions from the channel thus enabling ion flow through the cell membrane (Paoletti & Neyton, 2007). NMDA receptors are permeable to Na^{+}, K^{+} and Ca^{2+} ions; this permeability is determined by subunit compositions. For instance, including the GluN3 subunit decreases the overall permeability of the receptor (especially for Ca^{2+} ions) besides causing highly reduced susceptibility to the Mg^{2+} channel inhibition (Cavara & Hollmann, 2008).

The function of NMDA receptors has been thoroughly studied in neurons, where they participate in neuronal network signaling and regulating synaptic plasticity, i.e. by

taking part in inducing long-term potentiation (LTP; essential for memory function) and long-term depression (LTD) (Fei Li & Tsien, 2009). The Ca^{2+} ions entering the cell through the NMDA receptor channels serve as second messengers in cellular signalization affecting various signaling pathways (Rasmussen, Jensen, Lake, & Goodman, 1976). Furthermore, NMDA receptors are thought to contribute to glutamate excitotoxicity by causing excessive calcium entry und thus inducing necrotic or apoptotic cell mechanisms during numerous CNS pathologies (e.g. Alzheimer's disease, epilepsy or stroke), hence therapies using various antagonists or allosteric modulators of these receptors have been tested in clinical trials. For example, an uncompetitive open-channel blocker called memantine is currently used to treat patients with moderate to severe form of Alzheimer's disease (Chen & Lipton, 2006; Muir, 2006).

2.1.1.1 NMDA receptors in glial cells

NMDA receptor subunits were detected in multiple types of glial cells (Conti, DeBiasi, Minelli, & Melone, 1996; Müller, Grosche, Ohlemeyer, & Kettenmann, 1993; Wang et al., 1996). The functional properties of glial NMDA receptors vary from those in neurons due to their different subunit composition. As several studies demonstrated, glial cells may include the GluN3 subunit in NMDA receptor configuration, thus making the NMDA receptors less sensitive to the Mg^{2+} block and decreasing their Ca^{2+} permeability (Burzomato, Frugier, Pérez-Otaño, Kittler, & Attwell, 2010; Káradóttir, Cavelier, Bergersen, & Attwell, 2005; Lalo, Pankratov, Kirchhoff, North, & Verkhratsky, 2006; Palygin, Lalo, & Pankratov, 2011; Verkhratsky & Kirchhoff, 2007).

The expression of functional NMDA receptors in astrocytes has been highly debated, but finally clarified by observing NMDA evoked inward currents and increased Ca^{2+} concentration in these cells (Dzamba, Honsa, & Anderova, 2013; Palygin, Lalo, Verkhratsky, & Pankratov, 2010). Despite that, the purpose of astrocytic NMDA receptors remains debatable. They could be possibly involved in gliotransmitter release (thus affecting synaptic function), nevertheless, such functions have not yet been shown. Astrocytic NMDA receptors also presumably take part in neuroinflammatory processes, e.g. by mediating the release of proinflammatory cytokines, in the meantime, this has been shown only in *in vitro* studies (Palygin et al., 2010). Several NMDA receptor subunits have been also detected in microglia with some functional features being observed in cultured cells, but whether microglia actually express functional

NMDA receptors has not been resolved (Murugan, Sivakumar, Lu, Ling, & Kaur, 2011). In oligodendrocytes NMDA receptors are predicted to contribute to myelination, since some receptor subunits have also been identified, although studies regarding functional properties of NMDA receptors in these cells present disparate results (Lindsay M. De Biase et al., 2010; Káradóttir et al., 2005).

2.1.1.2 NMDA receptors in NG2-glia

NG2-glia apparently express NMDA receptor subunits at least on mRNA level (see Table 1). The most highly expressed is the GluN3A subunit, therefore NG2-glial NMDA receptors are presumably less sensitive to extracellular Mg^{2+} but also less permeable for Ca^{2+} ions (Káradóttir et al., 2005). However, some studies discuss the purpose of NMDA receptors in NG2-glia, since they presented data showing that only ~60% of $NG2^{+}$ cells in the corpus callosum had a detectable NMDA receptor current, these currents being very small and blocked by Mg^{2+} at resting membrane potential (Ziskin et al., 2007). Moreover, NMDA receptor expression seemingly decreases as NG2-glia undergo differentiation into oligodendrocytes (Lindsay M. De Biase et al., 2010; Y. Zhang et al., 2014). It has also been shown that NMDA receptors are not required for proper NG2-glial functioning *in vivo*, such as cell survival, differentiation into oligodendrocytes or forming synapses with neurons but could possibly have an impact on regulating the expression of calcium permeable AMPA receptors (L. M. De Biase et al., 2011; Guo et al., 2012). Furthermore, several recent studies indicate that NMDA receptors could take part in remyelination after white matter injury, since their inhibition attenuated this process. Nevertheless, the participation of NG2-glia and oligodendrocytes is yet to be clarified (C. Li et al., 2013; Lundgaard et al., 2013).

2.1.2 AMPA receptors

Structurally AMPA receptors are assembled as heterotetramers composed of four subunits (GluA1-GluA4) in most cases formed as a symmetric dimer of dimers (Traynelis et al., 2010), however, the subunit composition depends on tissue localization as well as age or various diseases (Gorter et al., 1997; Kawahara et al., 2004). As the structure of most AMPA receptors comprises the GluA2 subunit, the properties of the GluA2 subunit are rather significant. AMPA receptors are generally

permeable only to Na^+ and K^+ ions and it is particularly the difference in the GluA2 subunit structure that allows Ca^{2+} ion influx through these receptors. The calcium permeable form of the GluA2 subunit is determined by post-transcriptional editing where the difference is caused solely by changes in a single amino-acid in a so called Q/R (glutamine/arginine) editing site of the GluA2 mRNA, the GluA2(Q) form enabling calcium permeability of the final AMPA receptor (Mishina et al., 1991). Notably, 99% of the GluA2 pre-mRNA is edited into the GluA2(R) form. It is not surprising that AMPA receptors lacking the GluA2 subunit are Ca^{2+} permeable as well, as the GluA1, GluA3 and GluA4 do not undergo the Q/R editing (Burnashev et al., 1992) (Figure 9). Another characteristic altering the function of AMPA receptors is the different splice variants of the subunits; these are called flip and flop and affect mostly the desensitization kinetics of the receptor. The final heterotetramer contains four glutamate binding sites, one for each subunit, which can bind the main AMPA receptor agonists: glutamate, AMPA and also kainate.

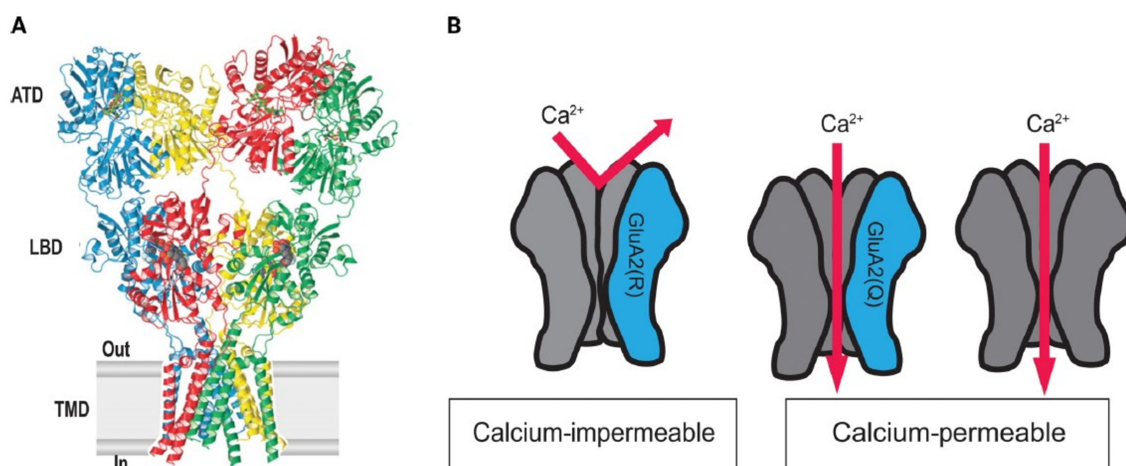

Figure 9: (A) The tetrameric structure of AMPA receptors composed of four subunits (GluA1–GluA4) in various combinations enclosing a ion channel pore. (B) The AMPA receptor tetramer usually contains the GluA2 subunit, which determines the calcium permeability of the channel. AMPA receptors containing the edited form GluA2(R) are impermeable to Ca^{2+} (left), whereas AMPA receptors containing the unedited GluA2(Q) form or completely lacking the GluA2 subunit are Ca^{2+} permeable (right, Wright and Vissel 2012). ATD, amino-terminal domain; LBD, ligand-binding domain; TMD, transmembrane domain; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; GluA, subunit of AMPA receptor

As expected, the roles of AMPA receptors in the CNS have been first described regarding neurons and neuronal synapses where they are responsible for fast excitatory

synaptic transmission. Moreover, AMPA receptors contribute to the LTP mechanisms of synaptic plasticity elevating the post-synaptic response to a certain stimulus either by increasing the number of AMPA receptors expressed on the cell membrane or by modulating their kinetic properties and channel conductance (Collingridge, Benke, Lüthi, & Isaac, 1998). Recently, it has been suggested that an increase in the synapse conductance is probably dependent preferably on the insertion of the Ca^{2+} permeable AMPA receptors into the post-synaptic cell membrane (Plant et al., 2006; Terashima et al., 2004). However, excessive AMPA receptor activation has been linked to numerous neurological diseases, such as ischemia, epilepsy or amyotrophic lateral sclerosis. Therefore, several AMPA receptor antagonists have been described to have a neuroprotective function, such as the competitive antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) or the noncompetitive inhibitor 2,3-benzodiazepine which is more selective and thus more promising in possible therapies (Ritz, Micale, Grasso, & Niu, 2008; Sólyom & Tarnawa, 2002).

2.1.2.1 AMPA receptors in glial cells

Determining AMPA receptors in glial cells was a rather complicated process, since for a long time AMPA and kainate receptors were classified together as non-NMDA glutamate receptors and were studied as one group. Later studies however accomplished to differentiate between these two receptor types and proved the presence of AMPA receptors in most glial cell types. The key approach in distinguishing AMPA and kainate receptors was employing proper pharmacological tools, such as cyclothiazide (CTZ), an allosteric AMPA receptor modulator preventing their desensitization (Partin, Patneau, Winters, Mayer, & Buonanno, 1993; Yamada & Tang, 1993) or various specific AMPA receptor blockers.

Most glial cells are thought to express Ca^{2+} permeable subtypes of AMPA receptors, not containing the GluA2 subunit. Calcium currents evoked by AMPA application were shown in astrocytes (Seifert & Steinhauser, 1995) and gene expression analyses confirmed the presence of AMPA receptor subunits mRNA (Seifert, Rehn, Weber, & Steinhäuser, 1997). The functions of AMPA receptors in astrocytes are still not utterly clarified, however, they presumably have a role in astrocyte-neuronal signaling as well as signaling between different types of glia (Seifert & Steinhäuser,

2001). Moreover, astrocytic AMPA receptors permeable to calcium can contribute in various CNS pathologies, e.g. inducing calcification (Petegnief et al., 1999). AMPA receptors were detected also in cultured microglia with the function of regulating the release of certain cytokines, thus possibly mediating neuron to microglia communication (Hagino et al., 2004; Noda, Nakanishi, Nabekura, & Akaike, 2000). In oligodendrocytes AMPA receptors are mostly described to participate in excitotoxic damage and death of these cells, hence affecting axonal myelination (Leuchtmann, Ratner, Vijitruth, Qu, & McDonald, 2003).

2.1.2.2 AMPA receptors in NG2-glia

The roles of AMPA receptors in NG2-glia were thoroughly described regarding their differentiation into oligodendrocytes (Borges, Ohlemeyer, Trotter, & Kettenmann, 1994; Holtzclaw, Gallo, & Russell, 1995). Moreover, the function of AMPA receptors in NG2-glia has been studied concerning neuron-glia synapses as described above. All AMPA receptor subunits are expressed in NG2-glia, the GluA2 subunit being the most abundant, thus suggesting NG2-glial AMPA receptors to be Ca^{2+} impermeable. However, numerous functional studies demonstrated the presence of Ca^{2+} permeable AMPA receptors as well (Bergles, Roberts, Somogyi, & Jahr, 2000; Ge et al., 2006). Various functions of AMPA receptors in NG2-glia have been described *in vitro*. For instance, AMPA receptors proposedly affect NG2-glial migration (Gudz, Komuro, & Macklin, 2006), proliferation and differentiation into oligodendrocytes. Some studies demonstrated inhibition of NG2-glial proliferation and differentiation by AMPA receptor agonist application further suggesting their role in K^+ channel modulation (Gallo et al., 1996; Yuan et al., 1998). While a recent study showed increased proliferation and differentiation of NG2-glia as a result of blocking AMPA receptors (Fannon, Tarmier, & Fulton, 2015). However, the rate of myelination was not changed, suggesting possible compilation of more signaling pathways in this complex process, thus more studies including *in situ* and *in vivo* analyses are yet to be performed. Importantly, AMPA receptors participate in signaling within the neuron-glia synapses possibly having a role in the plasticity of these synapses. A process similar to neuronal LTP has been observed in neuron-NG2-glia synapses in the hippocampus. The mechanism of this so called glial LTP probably resides in adding more Ca^{2+} permeable AMPA receptors to the postsynaptic glial membrane caused by positive feedback regulation (Ge et al., 2006). Taken together, these *in vitro* findings suggest important

roles of AMPA receptors in NG2-glia including axon myelination and possible remyelination after CNS injuries as well; hence it yet has to be determined if and how synaptic signaling between neurons an NG2-glia affects their functions in an undisturbed environment *in vivo*.

2.1.3 Kainate receptors

Kainate receptors have a typical tetrameric structure similar to NMDA and AMPA receptors. They are formed by GluK1-5 subunits in various combinations, however the GluK4,5 subunits cannot form a functional receptor unless assembled with the GluK1-3 subunits (Gallyas, Ball, & Molnar, 2003). Furthermore, RNA editing and different splice variants of the subunits allow numerous combinations resulting in diverse pharmacological and functional properties of the final kainate receptors. For instance, similarly to the AMPA receptor GluA2 subunit, RNA editing in the Q/R site affects Ca^{2+} permeability (Egebjerg & Heinemann, 1993). The subunit composition varies in different brain regions and different types of neurons and glia, thus suggesting variable roles of kainate receptors throughout the CNS.

As previously mentioned, kainate and AMPA receptors were earlier studied as one category of non-NMDA receptors, as it was scarcely possible to distinguish them pharmacologically. Later, more selective pharmacological compounds, such as 2,3-benzodiazepines (relatively selective antagonists of AMPA receptors), allowed observation of isolated kainate receptor currents (Paternain, Morales, & Lerma, 1995). The roles of kainate receptors in the CNS have been widely discussed as kainic acid was first described as a neurotoxin mediating seizures and damaging neurons (Ben-Ari, 1985; Herndon & Coyle, 1977; Nadler, Perry, & Cotman, 1978). After the identification of kainate receptors as a separate iGluR category and successful subunit cloning, there have been several proposed functions of this iGluR subtype. In neurons kainate receptors were described to evoke excitatory postsynaptic currents at synapses in the CA3 region of the hippocampus, however the receptor kinetics being overall slower than those of AMPA receptors (Castillo, Malenka, & Nicoll, 1997). Generally, kainate receptors are considered as presynaptic regulators of neurotransmitter release, where they were shown to regulate excitatory as well as inhibitory synapses (Pinheiro &

Mulle, 2008). Nevertheless, the mechanisms of kainate receptor signaling are still not entirely clarified and more questions arise regarding their possible role in synaptic modulation and plasticity.

2.1.3.1 Kainate receptors in glial cells

Considering glial cells, some studies analyzing kainate receptors as a separate group proposed several functional properties in CNS glia. In astrocytes kainate receptors are thought to mediate the release of gliotransmitters as a result of triggering Ca^{2+} waves within the astrocytic network. Therefore, their suggested role is acting as sensors of extracellular glutamate and possibly preventing excitotoxicity (Cassé et al., 2012; Liu, Xu, Arcuino, Kang, & Nedergaard, 2004). Activation of kainate receptors in microglia supposedly affects the release of certain cytokines, however the roles of AMPA and kainate receptors in this process are yet to be separated (Noda et al., 2000; Vezzani et al., 1999). Regarding oligodendrocytes kainate receptors are primarily studied as mediators of their excitotoxic death proposedly participating in demyelinating processes during CNS pathologies (Alberdi, Sánchez-Gómez, Marino, & Matute, 2002; Sanchez-Gomez & Matute, 1999).

2.1.3.2 Kainate receptors in NG2-glia

NG2-glia apparently express all kainate receptor subunits on protein level except the GluK1 subunit (Kukley & Dietrich, 2009; Patneau, Wright, Winters, Mayer, & Gallo, 1994). Conversely, the possible role of kainate receptors in NG2-glia has not yet been entirely determined, as most studies concentrated on AMPA receptors preferentially. However, a recent study by Kukley and Dietrich analyzed solely kainate receptors and suggested several functions *in situ*. Interestingly, in NG2-glia kainate receptors are located extrasynapticaly, hence their contribution in neuron-glia synapses is improbable. More likely the function of these receptors is to react to excessive glutamate outside the synaptic regions released by excitatory neurons and possibly even astrocytes. Whether and how this kainate receptor mediated reaction affects functional properties of NG2-glia remains unclear. Possibly they interact with other glutamate and GABA receptors in modulating NG2-glial proliferation and differentiation (Kukley & Dietrich, 2009).

2.2 Metabotropic glutamate receptors

Metabotropic glutamate receptors are receptors coupled with G-proteins and affect various signaling pathways. They are generally divided into three categories: group I, group II and group III. Group I mGluRs include mGluR1 and mGluR5 subtypes and are involved in intracellular calcium signaling via phospholipase C (PLC). Group II mGluRs include mGluR2 and mGluR3 subtypes and negatively affect adenylyl cyclase. Group III mGluRs work through the same signaling pathway as group II and consist of mGluR4, mGluR6, mGluR7 and mGluR8 subtypes. Nearly all types of mGluRs must undergo dimerization in order to form a functional receptor unit (Romano, Yang, & O'Malley, 1996).

All types of mGluRs are widely distributed throughout the CNS, their localization varying with different subtypes (Francesco Ferraguti & Shigemoto, 2006). They also possess diverse functions depending on the brain region and type of receptor. For instance, the group I receptors regulate gene expression, while the group II and III types presynapticaly modulate neurotransmitter release. Interestingly, mGluRs are studied as participants in synaptic plasticity, where they indirectly affect LTP (Bortolotto et al., 2005) and are directly involved in LTD (Jo et al., 2008; Moult, Corrêa, Collingridge, Fitzjohn, & Bashir, 2008).

Glial cells express mGluRs as well. In astrocytes the most abundant are mGlu3 and mGlu5 receptors (Cai, Schools, & Kimelberg, 2000), while other receptors subtypes have also been detected but their functional expression is debatable. The activation of astrocytic mGluRs results in regulating gliotransmitter release (Bezzi et al., 1998; Pasti, Volterra, Pozzan, & Carmignoto, 1997) or inducing gene transcription (Peavy & Conn, 1998), this including formation and release of certain neuroprotective growth factors (Bruno et al., 1998; Ciccarelli et al., 1999). They are also thought to take part in regulating astrocytic proliferation (Ikeda & Puro, 1995) and glutamate uptake (Vermeiren et al., 2005). In microglia mGluRs regulate the release of various cytokines, thus contributing to neurotoxicity during inflammation (Taylor, Jones, Kubota, & Pocock, 2005). The expression of mGluRs in astrocytes and microglia undergoes certain changes upon their activation, e.g. certain mGluR types show higher abundance in reactive astrocytes around lesions (Aronica et al., 2000; F Ferraguti, Corti, Valerio, Mion, & Xuereb, 2001). Therefore mGluRs in these types of glial cells supposedly

participate in regulating inflammatory processes during pathological conditions. Oligodendrocytes express mGlu3 receptors *in vivo* (Tamaru, Nomura, Mizuno, & Shigemoto, 2001), other mGluR types have been detected in cultured oligodendrocytes. The expression profile changes during oligodendrocyte development (Deng, Wang, Rosenberg, Volpe, & Jensen, 2004). OPCs ($NG2^+$ cells) express higher levels of mGluRs, specifically mGlu3 and mGlu5 types (Luyt, Varadi, Halfpenny, Scolding, & Molnar, 2004), while their expression decreases during their maturation. These observations suggest the role of mGluRs in the differentiation of NG2-glia (Figure 10), hence influencing remyelinating processes in CNS pathologies.

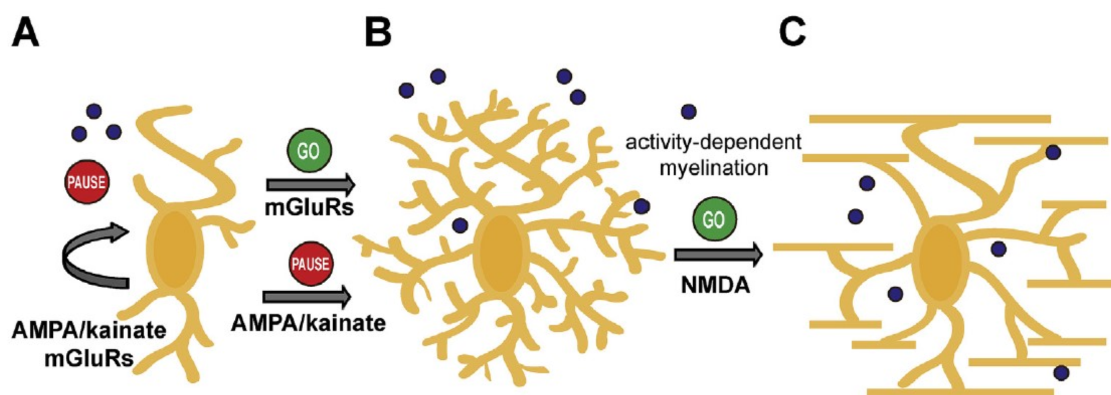

Figure 10: Summary of proposed effects of glutamate on NG2-glial proliferation and differentiation. Generally glutamate is thought to inhibit proliferation of NG2-glia (A), mGluRs seem to promote their differentiation, while AMPA/kainate receptor activation inhibits this process (B). NMDA receptors could contribute to activity-dependent myelination and remyelination after white matter injury (C; Spitzer et al. 2016). mGluRs, metabotropic glutamate receptors; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA, N-methyl-D-aspartate

3. Ischemic brain injury

Stroke is the third most common cause of death in developed countries, as approximately fifteen million people suffer from stroke each year. Moreover, it represents the most common type of CNS injury.

Ischemic brain injury is caused by either transient or permanent restriction of blood flow, mainly caused by an embolism or local thrombosis occluding a cerebral artery. This results in insufficient oxygen and glucose delivery needed for maintaining

cellular homeostasis. The processes of ischemia are very complex including various pathophysiological events, such as ionic imbalance, glutamate release and consequently cell death (Dirnagl, Iadecola, & Moskowitz, 1999).

The blood flow reduction and the following homeostatic imbalance lead to cellular energy failure and cell death, due to processes, such as neurotransmitter excitotoxicity, acidotoxicity, ionic imbalance, oxidative or nitritive stress, inflammation and peri-infarct depolarization (Doyle, Simon, & Stenzel-Poore, 2008). Due to the lack of oxygen mitochondria of the cells in the ischemic core produce less ATP, which leads to reduced activity of the Na^+/K^+ and Ca^{2+} ATPases and ultimately, to membrane depolarization and increased intracellular calcium concentration. The higher Ca^{2+} concentration causes the activation of Ca^{2+} dependent proteases, lipases and nucleases and overall induces cell death progression. Besides, due to their membrane depolarization, nerve cells from the ischemic core excessively release neurotransmitters resulting in excitotoxicity primarily caused by glutamate. Glutamate binds to AMPA and NMDA receptors, and their activation enhances the process of depolarization and contributes to increased Ca^{2+} influx and over-activation of Na^+ and Cl^- channels. This enables water to passively enter the cells, hence participating in edema formation. Moreover, the pH in ischemia is lowered due to higher concentrations of lactate, and thus pH-dependent ion channels contribute to the ionic imbalance as well. The overall distortion of cellular homeostasis and the increased intracellular concentration of Ca^{2+}, Na^+ and adenosine diphosphate (ADP) causes the production of reactive oxygen species (ROS) in affected cells (Dirnagl et al., 1999). Because of that, mitochondria release cytochrome C, which is one of the mechanisms of inducing apoptotic processes in cells surrounding the ischemic core. Within the ischemic core the described cascade together with rapid depletion of energy stores leads to necrotic cell death (Doyle et al., 2008; Siesjö, Bengtsson, Grampp, & Theander, 1989). Another component of ischemic damage is inflammation, i.e. the production of cytokines by activated microglia and astrocytes, as well as by cells of the immune system migrating to the site of injury from the bloodstream through the damaged hemato-encephalic barrier (Figure 11).

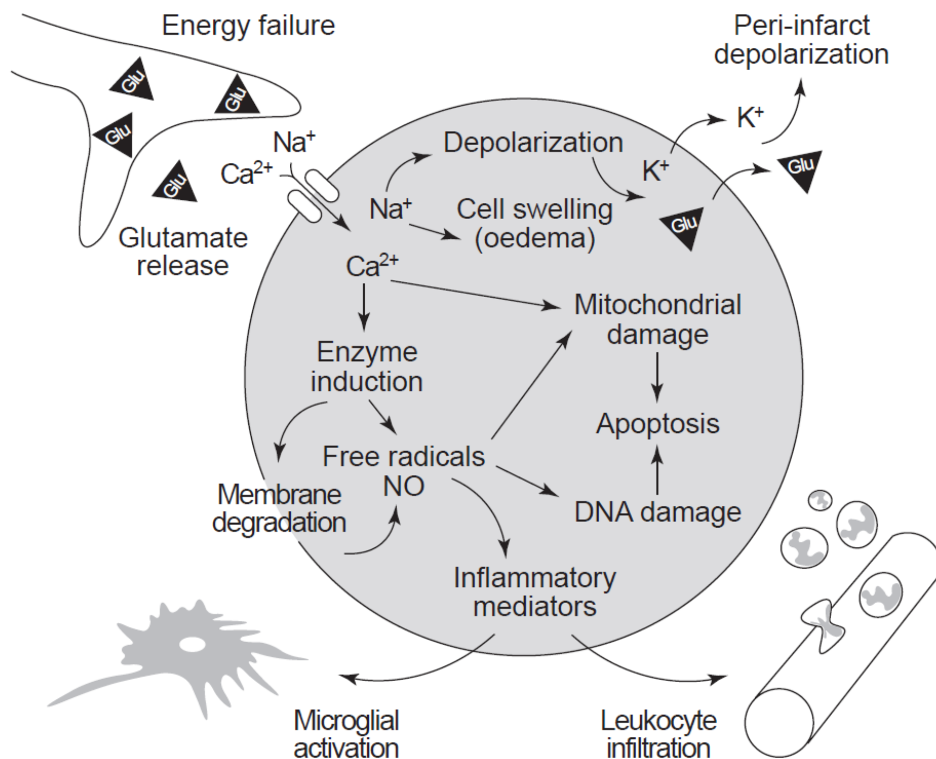

Figure 11: Pathophysiological mechanisms during focal cerebral ischemia. Depolarization of neurons is caused by energy failure. Excessive glutamate receptor activation dramatically increases intracellular Ca^{2+}, Na^{+}, Cl^{-} levels while K^{+} is released into the extracellular space. Higher concentrations of glutamate and K^{+} in the extracellular space can initiate a series of depolarization waves (peri-infarct depolarizations). Cell swelling is caused by water influx via osmotic gradients. Ca^{2+} as a second messenger over-activates numerous enzyme systems (proteases, lipases, endonucleases, etc.). Free radicals are produced damaging membranes (lipolysis), mitochondria and DNA and triggering apoptotic mechanisms. Moreover, free radicals induce the generation of inflammatory mediators, which activate microglia and lead to the migration of leukocytes from the bloodstream via upregulation of endothelial adhesion molecules (Dirnagl et al., 1999). Glu, glutamate; NO, nitric oxide; DNA, deoxyribonucleic acid

To study ischemic injury many animal models have been used, e.g. mice, rats, gerbils, rabbits, cats, dogs, pigs, sheep or even monkeys. Currently the most commonly used model are mice, since they are easily genetically manipulated and give rise to a great variability of transgenic strains (Traystman, 2003). On the other hand, their smaller size increases susceptibility to injury and leads to higher mortality rates, henceforth requiring more demanding surgical procedures (Taguchi et al., 2010).

3.1 Models of ischemia

The two basic types of ischemia include global cerebral ischemia (GCI) and focal cerebral ischemia (FCI). GCI is a result of cerebral blood flow reduction

throughout the whole brain or it's large parts, whereas FCI occurs after blood flow restriction in a clearly specific cerebral region.

3.1.1 Global cerebral ischemia

In the case of GCI, cerebral blood flow reduction in most brain parts causes neuronal damage in selectively susceptible regions, such as the CA1 region of the hippocampus. The easiest model of this type of ischemia is decapitation (Lowry et al. 1964) or formerly used neck tourniquet (Rybach & Buntebarth, 1984). Ventricular fibrillation with cardiopulmonary resuscitation is commonly used to simulate the clinical situation of cardiac arrest (Berkowitz et al., 1991). A very effective model in adult mice generating reproducible hippocampal damage is bilateral occlusion of both common carotid arteries in the combination of isoflurane induces hypotension. The main advantage of this method is a fast and simple surgery, which significantly reduces stress from the procedure (Onken, Berger, & Kristian, 2012).

3.1.2 Focal cerebral ischemia

FCI models can be divided into two main groups: transient and permanent ischemia. Transient ischemia forms variable degrees of damage depending on the duration, whereas permanent ischemia results in severe brain damage with the lesion size not changing in time (Hunter et al. 1995; Li et al. 1999; Fisher 2003). Regarding humans, the most common cause of stroke is occlusion of the middle cerebral artery (MCAo), thus this principle is generally involved in most FCI models. As a result of MCAo cerebral blood flow reduction occurs concurrently in the striatum and cortex, at the same time, however, the degree and expansion of the damage depend on the duration and the site of occlusion and also the rate of the surrounding blood supply. There are permanent as well as transient models of MCAo either in proximal or distal part of the artery (Figure 12). One of the simpler noninvasive methods is intraluminal MCAo, where the MCA is blocked permanently or transiently by a filament (nylon suture) advanced cranially through the internal carotid artery (Ansari, Azari, McConnell, Afzal, & Mocco, 2011). Another FCI model includes the formation of a local blood clot by injecting purified murine thrombin. This approach closely resembles human ischemic stroke and thus represents a promising method in studying thrombolytic therapies (Desk, Williams, & Health, 2007). A different variation is a

photochemical model of MCAo, which includes irradiating several branches of distal MCA by an argon laser beam after previous intravenous injection of a photosensitive dye leading to ischemic lesion formation after exposure to green light (560 nm; Watson 1997). A pharmacological model of MCAo employs endothelin-1, a vasoconstrictor which is applied either topically directly to the surface of the exposed MCA or by stereotaxic injection into the cortex area neighboring the proximal MCA (Sharkey, Ritchie, & Kelly, 1993). However due to the higher difficulty of surgical procedures performed on mice and due to their increased vulnerability, the majorly used method is the transcranial MCAo model, where the MCA is occluded directly on the brain surface. Permanent occlusion is mediated by electro-cauterization of the MCA, while temporal methods include clipping or ligatures of the artery. This method has a high rate of survival and relatively low variability thanks to localizing the lesion in a defined region of the cortex (Taguchi et al., 2010).

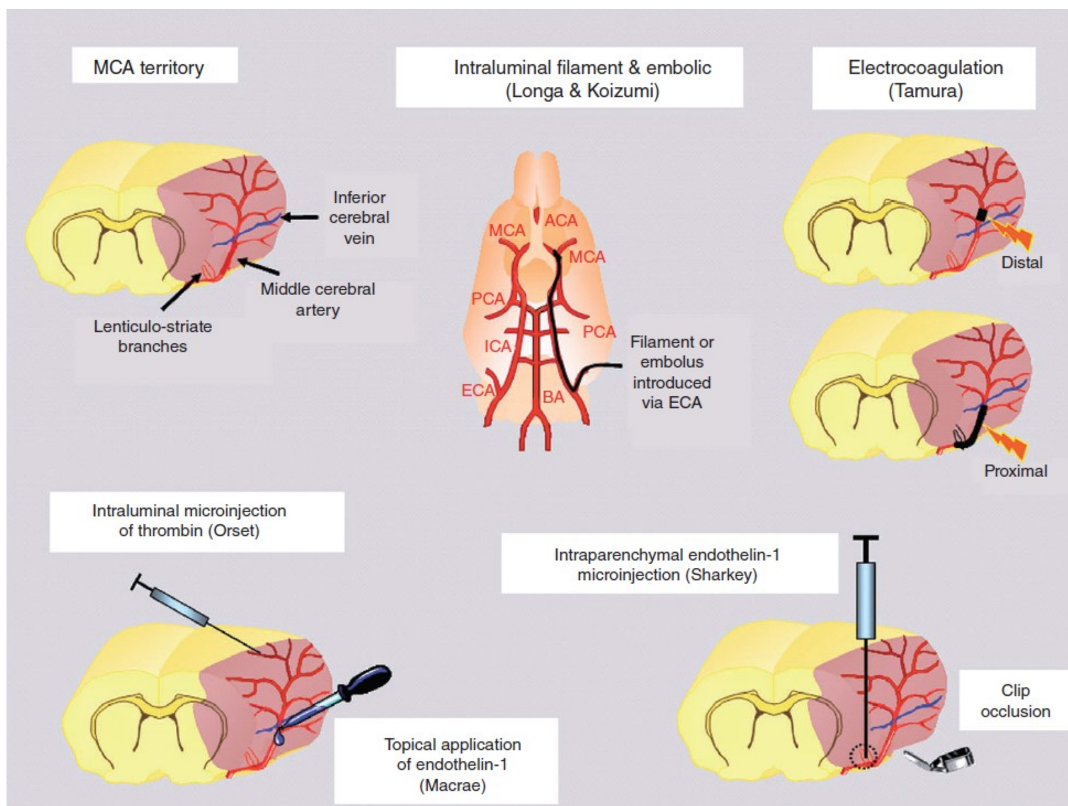

Figure 12: Different models of MCAo in rats and mice. Pink area in MCA territory diagram illustrates the regions supplied by MCA. Pink areas in electrocoagulation diagram illustrate regions affected by ischemia, hence demonstrating the possibility of inducing ischemia purely in the cortex by distal MCAo, while proximal MCAo induces cortical as well as subcortical ischemia (MacRae, 2011). ACA, anterior cerebral artery; BA, basilar artery; ECA, external carotid artery; ICA, internal carotid artery; PCA, posterior cerebral artery

4. NG2-glia in pathology

NG2-glia respond variably to different types of injury also depending on the developmental stage. They are able to change their morphology, proliferation rate and modify their differentiation, overall being a very flexible glial cell type. Acute CNS injury, such as ischemia, triggers a very fast reaction of NG2-glia (R. Zhang, Chopp, & Zhang, 2013). They undergo morphologic changes, which include enlargement of their cell somas, diverse thicker or shorter processes and increased expression of NG2 antigen (Levine, 1994). Furthermore, NG2-glia vastly increase their proliferation rate, starting one to two days post injury and further increasing up to seven days post injury. The number of proliferating cells remains relatively unchanged till the fourteenth day and then becomes to decrease (Simon, Götz, & Dimou, 2011). Another NG2-glial aspect during ischemia is their ability of migration towards the site of injury where they participate in forming the glial scar and are able to restore their density by triggering homeostatic proliferation of surrounding NG2-glial cells (Hughes, Kang, Fukaya, & Bergles, 2013). However, it was generally accepted that the glial scar is composed mainly of reactive astrocytes, thus the purpose of NG2-glia in this formation has not yet been fully understood. Despite that, due to the high proliferation rate and migration ability, the role of NG2-glia is supposedly more important than that of astrocytes, since this glial cell type showed a low proliferation rate and no migration after traumatic injury (Bardehle et al., 2013). The observed low proliferative capacity of astrocytes raises a question about the origin of reactive astrocytes within the glial scar, studies suggesting their generation from the original astrocytic population (Komitova, Serwanski, Richard Lu, & Nishiyama, 2011) are in contrast with those proposing their possible origin in NG2-glia (Honsa et al. 2012, 2016; Rusnakova et al. 2013). NG2-glia forming the glial scar also contributes to its function as a cellular barrier separating healthy and necrotic tissue. This formation has a positive role in reducing the entry of pathogens and immune cells and prevents spreading the ionic and neurotransmitter imbalance towards the healthy tissue. On the other hand the dense formation of the glial scar can have a negative role in regeneration processes by preventing the migration of cells and axon restoration (Pekny, Wilhelmsson, & Pekna, 2014).

NG2-glia were found to respond also to different types of neurodegenerative conditions, such as Alzheimer's disease or amyotrophic lateral sclerosis. NG2-glia

increase their proliferation and differentiate into new oligodendrocytes as a reaction to oligodendrocyte damage and myelin defects (Sirko et al., 2013), however, the proliferation rate after acute injury is much higher than in neurodegeneration processes (Behrendt et al., 2013). Furthermore, during inflammation NG2-glial proliferation has been observed only when accompanied with demyelination. Altogether these finding suggest, that the proliferative/differentiative/migratory reaction of NG2-glia is triggered by demyelination rather than neurodegeneration or inflammatory processes, however the mechanisms remain unclear.

As previously mentioned NG2-glia are able to differentiate not only to oligodendrocytes but also astrocytes. This characteristic is maintained even in pathological states, although the rate of differentiation into astrocytes has been observed either low (Komitova et al. 2011; Honsa et al. 2012, 2016) or completely absent (Kang et al., 2010; Simon et al., 2011). Interestingly, one study described the differentiation of NG2-glia into cells expressing a marker of newly derived neuronal cells (DCX) after FCI (Honsa et al., 2012), nevertheless, the ability of NG2-glia to generate neurons remains highly debatable.

4.1 Glutamate signaling

As previously described, NG2-glia possess a variety of glutamate receptors, suggesting that glutamate affects their characteristics. In injury or disease of CNS glutamate has both beneficial and harmful effects and since NG2-glia are capable of reacting to various pathologic states, understanding the role of glutamate in this process could possibly have wide implications.

In ischemia and inflammation glutamate can have a harmful effect due to the increase of its extracellular concentration, which can rise up to excitotoxic levels (Choi, 1994). Glutamate can be released by activated microglia, astrocytes and injured neurons (Mark et al., 2001). Moreover, the expression of glutamate transporters in neurons and glia can be downregulated in disease, hence causing them to fail in removing excessive glutamate from the extracellular space. Due to ionic disruptions in the CNS pathologies glutamate transporters can even be reversed resulting in more glutamate release (Kriegler & Chiu, 1993; Rossi, Oshima, & Attwell, 2000). Although oligodendrocytes

are sensitive to oxygen/glucose deprivation and glutamate excitotoxicity, fortunately, unlike neurons, myelin can be regenerated. The process of remyelination includes NG2-glia and depends on glutamate signaling from the demyelinated axons (Gautier et al., 2015; Lundgaard et al., 2013). Blocking AMPA/kainate or NMDA receptors and also inhibiting neuronal activity or vesicular glutamate release was shown to inhibit remyelination (Gautier et al., 2015; C. Li et al., 2013; Lundgaard et al., 2013). Taken together, in spite of glutamate having a toxic effect in CNS diseases, it is also needed for prompting myelin regeneration. It is suggested that this could depend on specifically localized and timed communication between axons and NG2-glia. Yet due to this dual role of glutamate, development of new therapies becomes rather complicated, and thus it is essential to deepen the understanding of glutamate signaling in these processes.

5. Aims of thesis

- To identify changes in the expression profile of NG2-glia following ischemic injury with a focus on glutamate receptor subunits

- To characterize functional properties of NG2-glial glutamate receptors before and after ischemic injury using calcium imaging

6. Materials and methods

6.1 Transgenic mice

All procedures involving the use of laboratory animals were performed in accordance with the European Communities Council Directive 24 November 1986 (86/609/EEC) and animal care guidelines approved by the Institute of Experimental Medicine AS CR Animal Care Committee on April 7, 2011.

Experiments were performed on two types of transgenic mice. Mice used for single-cell RT-qPCR were derived by crossing two mouse strains: B6.Cg-Tg(Cspg4-cre/Esr1*)BAkik/J (https://www.jax.org/strain/008538) and B6;129S6-*Gt(ROSA)26Sor*$^{tm14(CAG\text{-}tdTomato)Hze}$/J (https://www.jax.org/strain/007908) (further termed as Cspg4/tdTomato mice). For calcium imaging the Cspg4/tdTomato mice were additionally crossed with strain B6;129S-*Gt(ROSA)*$^{26Sortm95.1(CAG\text{-}GCaMP6f)Hze}$/J (https://www.jax.org/strain/024105) (further termed as Cspg4/GCaMP6f/tdTomato mice) (Jackson Laboratory, Bar Harbor, Maine, USA). In both strains tamoxifen administration triggers the expression of red fluorescent protein (tdTomato) specifically in NG2-glia and cells derived therefrom. The expression of GCaMP6f in Cspg4/GCaMP6f/tdTomato mice is restricted to NG2-glia as well, since it is expressed under the same promotor. GCaMP6f is a Ca^{2+} indicator generated by fusion of enhanced green fluorescent protein (EGFP), calmodulin and a peptide sequence from myosin light chain kinase M13. After Ca^{2+} binding, the molecule of the indicator changes its conformation and increases fluorescence.

Tamoxifen was administered intraperitoneally for two days following the scheme illustrated in Figure 13 (200 mg/kg, Sigma-Aldrich, St. Louis, MO, USA). For both ischemic and control mice 3-months old animals were used.

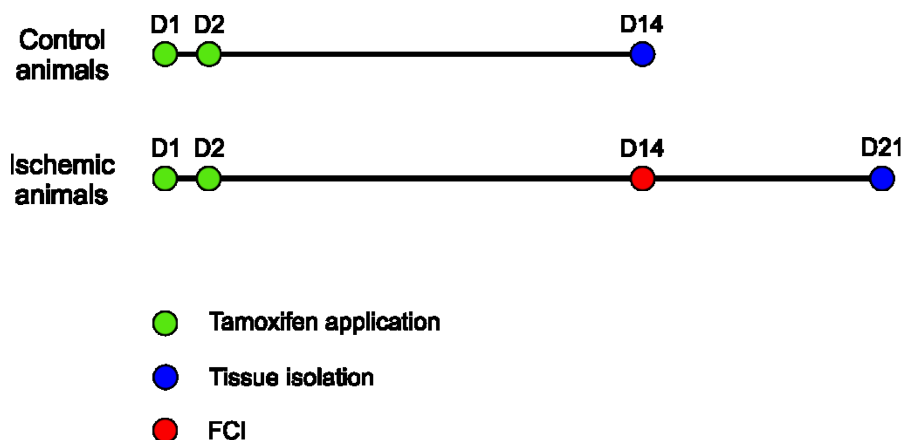

Figure 13: Scheme of tamoxifen application. D1-21, days of experiment; FCI, focal cerebral ischemia

The Cspg4/tdTomato mice are characterized by tamoxifen-inducible Cre recombinase (Cre-ERT) expression under the promotor for the NG2-glial marker Cspg4. The Cre recombinase is fused to a G525R mutant form of the mouse estrogen receptor, which does not bind its natural ligand (17β-estradiol) at physiological concentrations but binds synthetic estrogen receptor ligands 4-hydroxytamoxifen or tamoxifen. Cre-ERT is restricted to the cytoplasm and can access the nucleus after tamoxifen binding. In the nucleus the activated Cre recombinase cuts-out tdTomato stop sequence and allows transcription of this reporter protein.

6.2 Solutions

Composition of all used solutions is listed in Table 2, pH values were adjusted using pH-meter MiniLab IQ125 (IQ Scientific Instruments, Carlsbad, CA, USA). Osmolality was checked with a vapor-pressure osmometer (Vapro 5520, Wescor, South Logan, UT, USA), and when needed, adjusted with mannitol. All solutions were gassed with appropriate gas mixture. Isolation solution and bicarbonate-based artificial cerebrospinal fluid (aCSF) were both gassed with 95% O_2/5% CO_2. All chemicals were purchased from Sigma-Aldrich, St. Louis, MO, USA.

Table 21: Composition of extracellular solutions used for brain isolation and Ca^{2+} imaging.

	Isolation solution	Bicarbonate-based aCSF
	Concentration [mM]	
NMDG	110	---
NaCl	---	122
KCl	2.5	3
$NaHCO_3$	24.5	28
Na_2HPO_4	1.25	1.25
Glucose	20	10
$MgCl_2$	7	1.3
$CaCl_2$	0.5	1.5
	pH	
pH	7.4	7.4
	mOsm/kg	
osmolarity	305±5	305±5

All chemicals were purchased from (Sigma–Aldrich St. Louis, MO, USA). NMDG, N-methyl-D-glucamine; aCSF, artificial cerebro-spinal fluid

6.3 Induction of ischemic injury

Ischemic injury was induced by a MCAo model of FCI (Figure 14). Prior to the induction of FCI, mice were anaesthetized with 3% isoflurane (Abbot, Illinois, USA) and maintained in 2% isoflurane using a vaporizer (Tec-3, Cyprane Ltd., Keighley, UK). A skin incision between the orbit and the external auditory meatus was made, and a 1-2 mm hole was drilled through the frontal bone 1 mm rostral to the fusion of the zygoma and the squamosal bone and about 3.5 mm ventrally to the dorsal surface of the brain. The MCA was exposed after the dura was opened and removed. The MCA was occluded by short coagulation with bipolar tweezers (SMT, Czech Republic) at a proximal location, followed by transection of the vessel to ensure permanent occlusion. During the surgery, body temperature was maintained at 37±1°C using a heating pad.

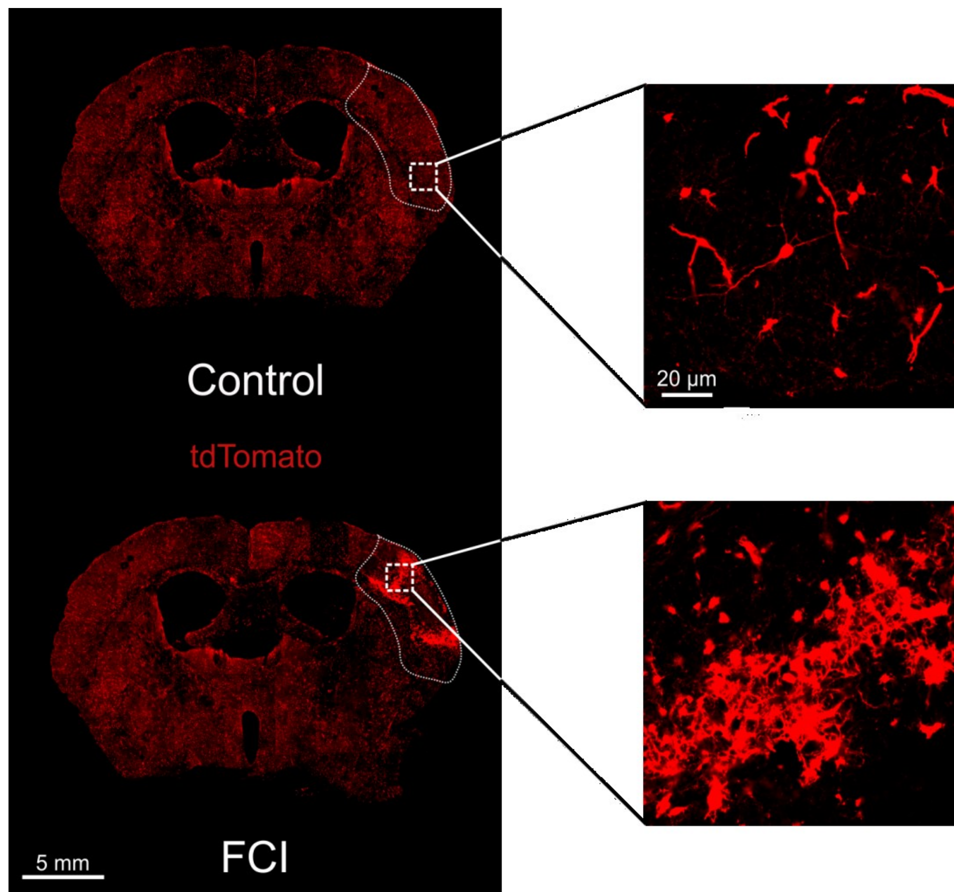

Figure 14: Scheme depicting tdTomato+ cells distribution and comparable brain regions used for tdTomato cell isolations for RT-qPCR and for calcium imaging measurements. Ischemic lesion is bordered by a high density of tdTomato+ cells. Detailed images picture tdTomato+ cells in healthy tissue and in ischemic border. FCI, focal cerebral ischemia

6.4 Preparation of acute brain slices

The brain slices were prepared from control mice as well as from those after MCAo. The animals were deeply anaesthetized with pentobarbital (PTB; 100 mg/kg, i.p.) and perfused transcardially with 20 ml of cold (4-8ºC) isolation solution. After decapitation the brains were dissected out and sliced into 200 µM thick slices using a vibration microtome (HM 650 V, Thermo Scientific Microm, Walldorf, GER) filled with constantly cooled isolation solution (max 4ºC). Afterwards, the slices were held at room temperature in gassed aCSF solution.

6.5 Single-cell gene expression profiling

Single-cell gene expression experiments were performed in collaboration with Laboratory of gene expression in Institute of Biotechnology AS CR, with intention to

characterize gene expression profile of tdTomato$^+$ cells. The experiments were performed with Cspg4/tdTomato mice. From the total of 95 characterized genes, only genes coding glutamate receptor subunits and genes coding NG2-glial and oligodendroglial markers were chosen for purpose of this work.

6.5.1 Preparation of cortical cell suspensions

Control mice or mice 7 days after FCI were deeply anaesthetized with PTB (100 mg/kg, i.p.), and perfused transcardially with cold (4–8°C) isolation buffer containing (in mM): NaCl 136.0, KCl 5.4, Hepes 10.0, glucose 5.5, osmolarity 290 ± 3 mOsmol/kg. To isolate the cerebral cortex, the brain (+2 to -2 mm from bregma) was sliced into 600 µm coronal sections using a vibrating microtome HM650V (MICROM International GmbH, Germany), and the uninjured or post ischemic parietal cortex was carefully dissected out from the ventral white matter tracks. The collected tissues were incubated with continuous shaking at 37°C for 45 min in 2 ml of papain solution (20 U/ml) and 0.2 ml DNase (both from Worthington, NJ, USA) and prepared in isolation buffer. After papain treatment the tissue was mechanically dissociated by gentle trituration using a 1 ml pipette. Dissociated cells were layered on top of 5 ml of Ovomucoid inhibitor solution (Worthington, Lakewood, NJ, USA) and harvested by centrifugation (140 x g for 6 min). This method routinely yielded ~2 x 10^6 cells per mouse brain. Cell aggregates were removed by filtering with 70 µm cell strainers (Becton Dickinson, NJ, USA), and the cells were kept on ice until sorting.

6.5.2 Collection of single tdTomato$^+$ cells by flow cytometry

The collection of single cells was performed using flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), which was manually calibrated to deposit single cells in the center of each well of the 96-well plates (Applied biosystems, Life technologies, Carlsbad, CA, USA), which were placed on a pre-cooled rack. Only viable and tdTomato$^+$ cells were collected. The viability of the cells was checked using Hoechst 33258 (Molecular Probes, Life Technologies, Carlsbad, CA, USA). Single cells were collected into 5 µl nuclease-free water with bovine serum albumin (1 mg/µl, Fermentas) and RNaseOut (20 U; Invitrogen, Life Technologies, Carlsbad, CA, USA). Collected cells in 96-well plates were immediately after sorting placed on a precooled rack (-80°C).

6.5.3 Synthesis of cDNA

cDNA was synthesized using SuperScript III RT (Invitrogen, Life Technologies, Carlsbad, CA, USA). Lysed single cells were incubated at 70°C for 5 min in 5 µl water containing 0.5 µM dNTP (Promega, Madison, WI, USA), 1 µM oligo (dT15) (Invitrogen, Life Technologies, Carlsbad, CA, USA) and 1 µM random hexamers (Eastport, Prague, CZ). 50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl_2$, 5 mM dithiothreitol, 20 U RNaseOut and 100 U SuperScript III (Invitrogen, Life Technologies, Carlsbad, CA, USA) were added to a final volume of 10 µl. Reverse transcription was performed at 25°C for 5 min, then at 50°C for 60 min, followed by 55°C for 15 min and finally terminated by heating at 70°C for 15 min. 4 µl from each sample was diluted 4 times and used for pre-testing and another 5 µl of sample was used for cDNA pre-amplification.

6.5.4 Primer design and optimization of assays

All primers used in this work were designed using Beacon Designer (version 7.91, Premier Biosoft International). All primers were designed to span an intron to avoid amplification of genomic DNA. BLAST (Basic Local Alignment Search Tool) searches revealed no pseudogenes. All assays of single cells were optimized so as to not generate primer dimers before cycle 45, to have a PCR efficiency of at least 90%, and to amplify all known splice forms documented by the National Center for Biotechnology Information (NCBI). Calibration curves with purified PCR products (QIA quick PCR Purification Kit; Qiagen, Germany) were used to establish the linearity of the assays. The formation of the correct PCR products was confirmed by electrophoresis on 20 g/L agarose gels for all assays and by melting-curve analysis of all samples. Five individual cells per assay were tested, and no genomic DNA amplification was observed.

6.5.5 qPCR

A CFX384 (BioRad, Hercules, CA, USA) was used for all qPCR measurements. To each reaction (10 µl) containing iQ SYBR Green Supermix (BioRad, Hercules, CA, USA) and 400 nM of each primer (Eastport, Prague, CZ), we added 3 µl of diluted cDNA. The temperature profile was 95°C for 3 min followed by 50 cycles of amplification (95°C for 15 s, 60°C for 15 s and 72°C for 20 s). All samples were

subjected to melting curve analysis. The same experimental setup was used to test pre-amplification.

6.5.6 Preamplification of cDNA and qPCR

To verify the pre-amplification protocol, the RNA from three animals was extracted and cDNA was prepared for the test of the protocol. Each reaction contained 25 μl of iQ Supermix (BioRad, Hercules, CA, USA), 5 μl of a mix of all primers (each in final concentration of 25 nM), 5 μl of non-diluted cDNA, and water added to a final volume of 50 μl. The temperature profile was 95°C for 3 min followed by 18 cycles of amplification (95°C for 20 s, 57°C for 4 min and 72°C for 20 s on a Biorad CFX96). The sample was diluted 5 times in water. The expression of all genes was measured in pre-amplified and non-pre-amplified samples. The average difference between pre-amplified and non-pre-amplified samples and the standard deviations of the differences were calculated and the same setup was used for the pre-amplification of single cells.

6.5.7 High throughput qPCR

The sample reaction mixture contained 2.4 μl of diluted pre-amplified cDNA, 0.25 μl of DNA Binding Dye Sample Loading Reagent (Fluidigm, San Francisco, CA, USA), 2.5 μl of SsoFast EvaGreen Supermix (BioRad, Hercules, CA, USA), and 0.01 μl of ROX (Invitrogen, Life Technologies, Carlsbad, CA, USA). The primer reaction mixture contained 2.5 μl Assay Loading Reagent (Fluidigm, San Francisco, CA, USA) and 2.5 μl of a mix of reverse and forward primers, corresponding to a final concentration of 4 μM. The chip was first primed with an oil solution in the NanoFlex™ 4-IFC Controller (Fluidigm, San Francisco, CA, USA) to fill the control wells of the dynamic array. The reaction mixture was loaded into each sample well, and primer reaction mixtures was loaded into each assay well of the 48x48 dynamic array. The dynamic array was then placed in the NanoFlex™ 4-IFC Controller for automatic loading and mixing. After 55 min the dynamic array was transformed to the BioMark qPCR platform (Fluidigm, San Francisco, CA, USA). The cycling program was 3 min at 95°C for activation, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 15 s, and elongation at 72°C for 20 s. After PCR, melting curves were collected between 60°C and 95°C with 0.5°C increments.

6.5.8 Data processing

Data from qPCR were pre-processed before expression analysis using GenEx (MultiD, version 5.3). Quantification cycle (Cq) values registered from amplifications that generated melting curves with aberrant melting temperature (Tm) were removed, Cq values larger than 26 were replaced with 26, and Cq values with products giving rise to a double peak in melting curves (corresponding to a mixture of expected and aberrant PCR products) were replaced with 26. All missing data, for each gene separately, were then replaced with the highest Cq value increased by 2, effectively assigning a concentration of 25% of the lowest measured concentration to the off-scale values. The Cq data were, for each gene separately, converted into relative quantities expressed relative to the sample with the lowest expression (maximum Cq) and finally converted to a logarithmic scale with base 2. The data were not normalized to any reference genes because of the large variation of all transcript levels among individual cells (Ståhlberg & Bengtsson 2010); with this pre-processing, the levels are expressed per cell.

6.6. Two-photon fluorescent calcium imaging

The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) measurements were performed using a two-photon microscope Olympus FV1200MPE (Olympus Life Science, Waltham, MA, USA) equipped with a 60x water objective. 200 μm thick acute brain slices previously kept in gassed aCSF in room temperature were transferred into the microscope perfusion chamber, which was continually supplied with gassed (5% CO_2 and 95% O_2) aCSF (20 ml/min). The receptor agonists and antagonists were prepared by their dilution in the aCSF and applied directly within the circular perfusion for 40 s; the used concentrations are listed in Table 3 and Table 4. Fluorescence intensity was measured in the somas of cells expressing the red fluorescent protein tdTomato, which was used to identify NG2-glia and cells derived therefrom. Pericytes were excluded from the analyses based on their morphology. The changes in $[Ca^{2+}]_i$ were measured using the genetically encoded Ca^{2+} probe GCaMP6f, which after binding Ca^{2+} changes its conformation and increases fluorescence level. The measurements were carried out in the cortex of both control mice and those following ischemia. In post-ischemic cortex cells were measured within the lesion border.

Table 3: Used concentrations of non-specific and specific glutamate receptor agonists.

Agonist	Concentration [µM]
Glutamate	500
NMDA	100
AMPA	100
Kainic acid	100

All chemicals were purchased from (Sigma–Aldrich St. Louis, MO, USA). NMDA, N-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

In addition, we pre-treated the slices with 1 µM tetrodotoxin (TTX) for 30s to block neuronal activity and to eliminate its impact on Ca^{2+} responses. We also pre-treated the slices with 1 µM thapsigargin (TG; a non-competitive inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase, SERCA) for 10 minutes to avoid Ca^{2+} elevations triggered by mGluR activation. Furthermore, the glutamate measurements were performed in aCSF without extracellular $CaCl_2$ in order to eliminate Ca^{2+} entry from the extracellular space through iGluRs.

In order to verify the iGluR agonist responses we used selective glutamate receptor antagonists to block different types of receptors. We perfused the slices for 40s with the antagonist and subsequently applied the antagonist together with the agonist for 40s using the perfusion.

Table 4: Used concentrations of glutamate receptor antagonists.

Antagonist	Type of receptor	Concentration [µM]
MK-801	NMDA	10
NBQX	AMPA/kainate	30

All chemicals were purchased from (Sigma–Aldrich St. Louis, MO, USA). MK-801, dizocilpine; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione; NMDA, N-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

The fluorescence intensity was recorded using Olympus software FV10-ASW 4.1 (Olympus Life Science, Waltham, MA, USA) as time series of images and then analyzed in FIJI (Fiji is just imageJ, General public license), where the fluorescent intensities were exported for individual cells and further analyzed. The values were correlated depending on photobleaching and the fluorescence levels of tdTomato. In the final analyses changes in fluorescent intensity above 120% of the base line were considered as a response.

6.7 Statistics

The results are presented as means ± S.E.M. (standard error of the mean) for *n* cells or *n* animals used depending on data analysed. Unpaired t-test was used to determine significant differences between the experimental groups. Values of */# $p<0.05$ were considered significant, **/## $p<0.01$ very significant and ***/### $p<0.001$ extremely significant.

7. Results

7.1 Gene expression profiling

To analyze NG2-glial glutamate receptors on mRNA level we performed single cell RT-qPCR analyzing cells from the cortex of ten control animals and three animals 7 days after MCAo using 3-month-old Cspg4/tdTomato mice. We selected solely tdTomato$^+$ cells and from total 95 genes analyzed, we have chosen genes encoding glutamate receptor subunits and genes encoding markers of NG2-glia and oligodendrocytes.

7.1.1. Discriminating between NG2-glia and oligodendrocytes

NG2-glia expressing Cspg4 and thus expressing tdTomato may differentiate into oligodendrocytes in-between tamoxifen administration and tissue isolation. This may result in detecting cells with a characteristic oligodendrocyte phenotype among the population of our selected tdTomato+ cells, since these oligodendroglial cells maturated from NG2-glia after tamoxifen administration. Using principal component analysis

(PCA) we identified three populations of tdTomato+ cells in the uninjured cortex. The first population of tdTomato+ cells was characterized by the strong expression of *Pdgfrb*, the well-known marker of pericytes (Zehendner et al., 2015). Since we focused solely on oligodendrocyte lineage cells in this study, *Pdgfrb*-positive pericytes were excluded from further analyses. The second and third populations of tdTomato+ cells were identified as oligodendrocytes (n = 82 cells/8 mice) and NG2 glial cells (n = 150 cells/6 mice) based on differential expression of respective cell-type specific markers *Mbp* (myelin basic protein), *Cldn11* (myelin oligodendrocyte-specific protein) and *Cspg4*, *Pdgfra* (Figure 15).

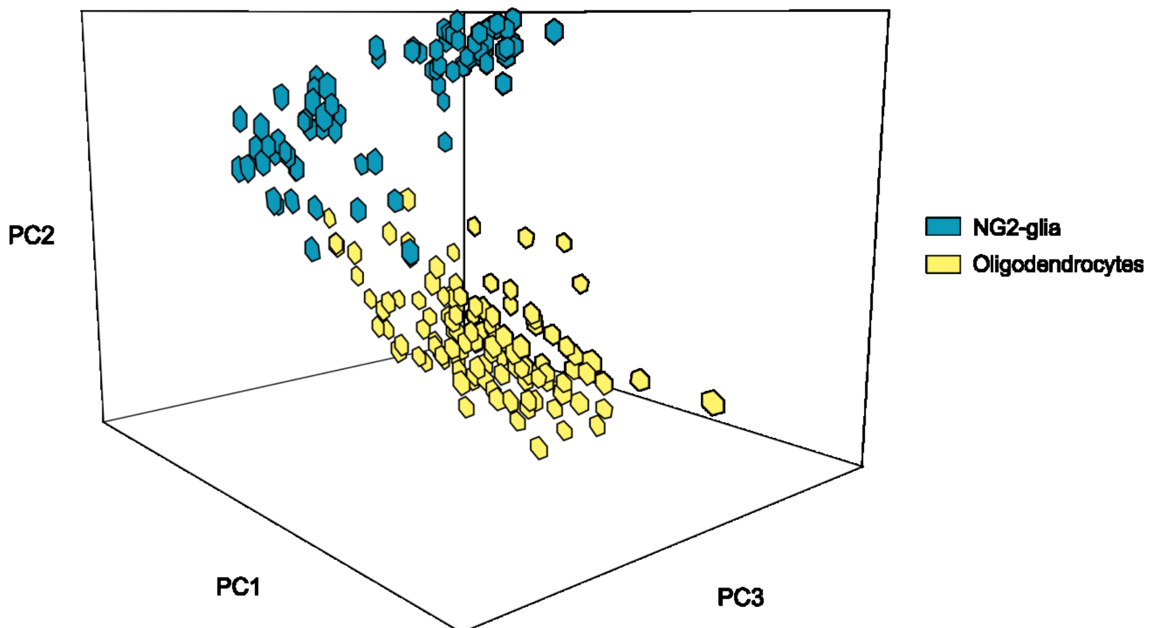

Figure 15: PCA analysis of tdTomato+ cells isolated from the cortex of control and ischemic mice. PC1-3, principal component 1-3

In controls our selected population of NG2-glia (n = 59 cells) expressed *Cspg4* in 96.6 ± 2.3 %, *Pdgfra* in 100.0 ± 0.0 %. The selected NG2-glia did not express the oligodendroglial markers *Mbp* and *Cldn11*. Interestingly, we detected the expression of *Cnp*, the well-known oligodendroglial marker, in 90.5 ± 6.7 % of NG2 cells. However, *Cnp* mRNA level was 60.9 ± 1.2-fold higher in oligodendrocytes compared to NG2 cells as shown similarly in ischemia (Figure 17) confirming *Cnp* as an oligodendroglial marker. On the other hand the selected oligodendrocyte population (n = 46 cells) expressed *Mbp*, *Cldn11* and *Cnp* genes in 100.0 ± 0.0 % of cells, only 2.2 ± 1.5 % of these cells expressed *Cspg4* (moreover, *Cspg4* expression in NG2-glia was 3.3 times

higher) and none expressed *Pdgfra* (Figure 16). The *Cnp* expression level was 60.9 times higher in the oligodendrocyte population than in the NG2-glial population. Using this division we separated 59 NG2-glial cells and 46 oligodendrocytes, which were further analyzed.

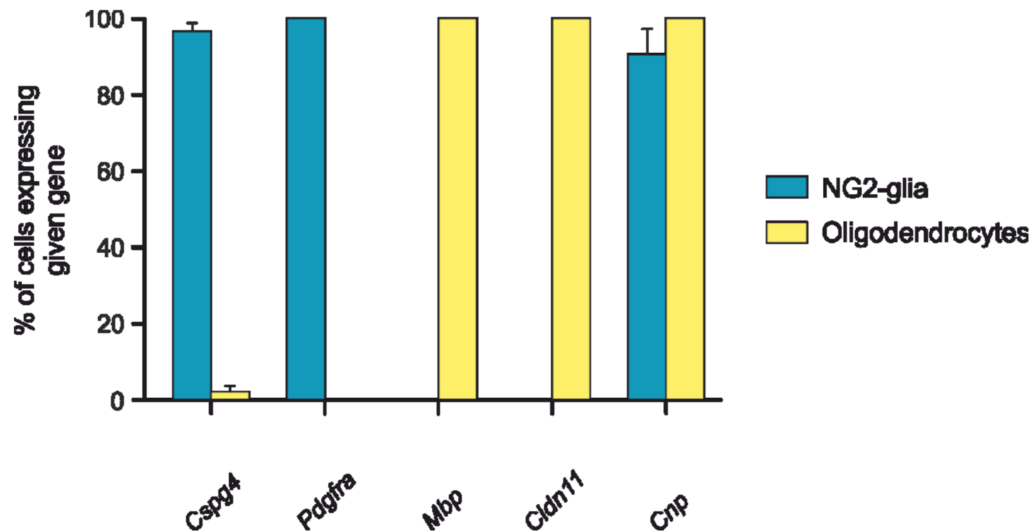

Figure 16: Expression of NG2-glial and oligodendroglial markers in cells isolated from the cortex of control mice. *Cspg4*, chondroitin sulfate proteoglycan 4; *Pdgfra*, platelet-derived growth factor receptor-alpha; *Mbp*, myelin basic protein; *Cldn11*, myelin oligodendrocyte-specific protein; *Cnp*, 2',3'-Cyclic-nucleotide 3'-phosphodiesterase.

We applied the same separation technique within cells from ischemic mice. The selected NG2-glial population (n = 91 cells) expressed *Cspg4* in 90.1 ± 3.0 %, *Pdgfra* in 97.8 ± 2.3 %, *Mbp* in 36.3 ± 4.3 %, *Cldn11* in 60.4 ± 9.5 % and *Cnp* in 92.3 ± 1.0 % of cells. The oligodendrocytes analyzed (n = 36 cells) expressed *Cspg4* in 16.7 ± 3.7 %, *Pdgfra* in 2.8 ± 3.0 %, *Mbp* in 97.2 ± 1.8 %, *Cldn11* in 97.2 ± 3.0 % and *Cnp* in 100.0 ± 0.0 % (Figure 17).

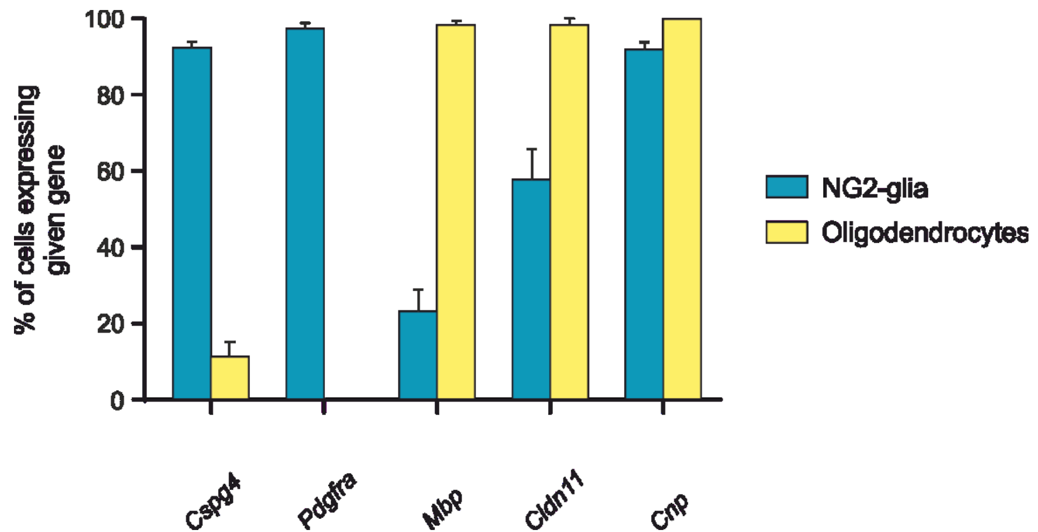

Figure 17: Expression of NG2-glial and oligodendroglial markers in cells isolated from the cortex of ischemic mice. *Cspg4*, chondroitin sulfate proteoglycan 4; *Pdgfra*, platelet-derived growth factor receptor-alpha; *Mbp*, myelin basic protein; *Cldn11*, myelin oligodendrocyte-specific protein; *Cnp*, 2',3'-Cyclic-nucleotide 3'-phosphodiesterase.

Some of the selected NG2-glia expressed also oligodendroglial markers. However, the levels of expression still clearly divided the two populations. In oligodendrocytes the expression level of *Mbp* was 61.3 ± 1.3 times higher than in NG2-glia, the expression level of *Cldn11* was 13.7 ± 1.2 times higher and the expression level of *Cnp* was 17.9 ± 1.2 times higher than in the NG2-glial population. The expression level of *Cspg4* was 4.8 ± 1.1 times higher in NG2-glia compared to oligodendrocytes (Figure 18).

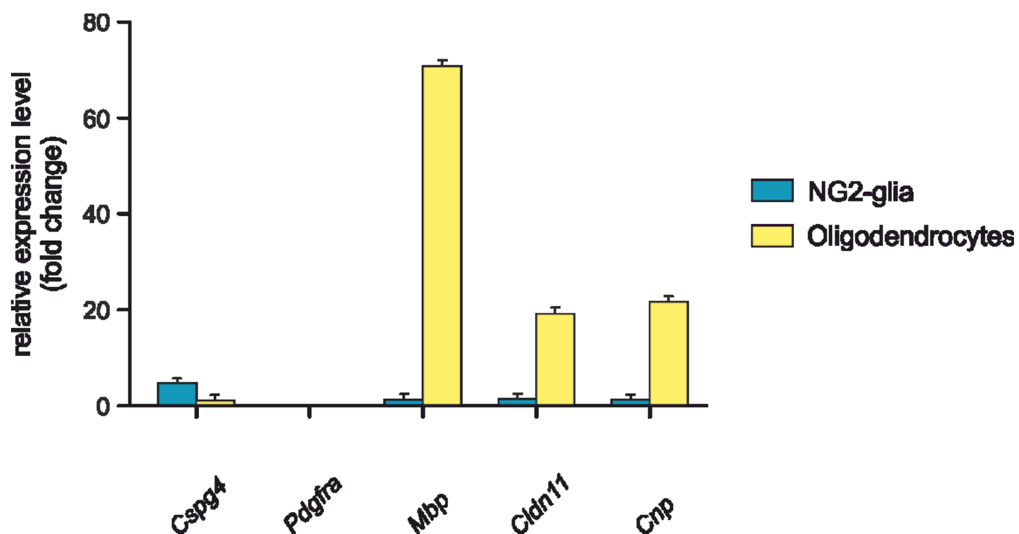

Figure 18: Relative expression levels of NG2-glial and oligodendroglial markers in cells isolated from the cortex of ischemic mice. The results are expressed relative to the cell population with lower expression level . *Cspg4*, chondroitin sulfate proteoglycan 4; *Pdgfra*,

platelet-derived growth factor receptor-alpha; *Mbp*, myelin basic protein; *Cldn11*, myelin oligodendrocyte-specific protein; *Cnp*, 2',3'-Cyclic-nucleotide 3'-phosphodiesterase.

7.1.2. Glutamate receptors

After dividing the populations of NG2-glia and oligodendrocytes we focused on genes encoding glutamate receptor subunits in both groups separately by comparing numbers of tdTomato+ cells expressing genes that encode AMPA, NMDA, kainate receptor subunits and genes encoding mGluR subtypes in control and ischemic animals. The results for NG2-glia and oligodendrocytes are summarized in Table 5 and 6, respectively.

Table 5: Glutamate receptor subunit expression in NG2-glia in the cortex of control mice and those 7 days after FCI.

Receptor type	Protein	Gene	Percentage of cells expressing given gene [%]	
			Control	D7
AMPA	GluA1	***Gria1***	27.1 ± 8.9	24.2 ± 0.8
	GluA2	***Gria2***	100.0 ± 0.0	95.6 ± 0.9 ***
	GluA3	***Gria3***	100.0 ± 0.0	95.6 ± 2.3 **
	GluA4	***Gria4***	89.8 ± 3.9	84.6 ± 2.9
Kainate	GluK1	***Grik1***	44.1 ± 6.2	30.8 ± 6.1
	GluK2	***Grik2***	91.5 ± 5.3	87.9 ± 1.3
	GluK3	***Grik3***	54.2 ± 3.9	80.2 ± 6.4 *
	GluK4	***Grik4***	45.8 ± 6.3	61.5 ± 6.7
	GluK5	***Grik5***	81.4 ± 5.9	89.0 ± 4.8
NMDA	GluN1	***Grin1***	28.8 ± 4.3	25.3 ± 2.1
	GluN2A	***Grin2a***	0.0 ± 0.0	1.1 ± 1.1
	GluN2B	***Grin2b***	3.4 ± 2.3	1.1 ± 1.1
	GluN2C	***Grin2c***	0.0 ± 0.0	1.1 ± 1.2
	GluN2D	***Grin2d***	45.8 ± 6.2	19.8 ± 3.3
	GluN3A	***Grin3a***	88.1 ± 5.4	82.4 ± 4.2
	GluN3B	***Grin3b***	0.0 ± 0.0	2.2 ± 1.0 ***
Metabotropic	mGlu1	***Grm1***	0.0 ± 0.0	0.0 ± 0.0
	mGlu2	***Grm2***	0.0 ± 0.0	0.0 ± 0.0
	mGlu3	***Grm3***	10.2 ± 3.7	7.7 ± 1.0
	mGlu4	***Grm4***	1.7 ± 2.1	0.0 ± 0.0
	mGlu5	***Grm5***	55.9 ± 9.9	54.9 ± 4.9
	mGlu6	***Grm6***	28.8 ± 9.0	56.0 ± 5.7
	mGlu7	***Grm7***	38.9 ± 5.7	18.7 ± 2.2
	mGlu8	***Grm8***	5.1 ± 3.2	2.2 ± 2.1

Significant changes are highlighted in yellow. FCI, focal cerebral ischemia

Table 6: Glutamate receptor subunit expression in oligodendrocytes in the cortex of control mice and those 7 days after FCI.

Receptor type	Protein	Gene	Percentage of cells expressing given gene [%]	
			Control	D7
AMPA	GluA1	***Gria1***	2.2 ± 2.4	0.0 ± 0.0
	GluA2	***Gria2***	47.8 ± 18.9	80.6 ± 9.2
	GluA3	***Gria3***	13.0 ± 5.6	61.1 ± 5.3 ***
	GluA4	***Gria4***	65.2 ± 10.3	80.6 ± 9.5
Kainate	GluK1	***Grik1***	4.4 ± 3.6	2.8 ± 2.2
	GluK2	***Grik2***	80.4 ± 8.9	77.8 ± 2.2
	GluK3	***Grik3***	13.0 ± 8.8	63.9 ± 11.1 *
	GluK4	***Grik4***	17.4 ± 7.4	30.6 ± 3.3
	GluK5	***Grik5***	43.5 ± 9.6	91.7 ± 3.4 *
NMDA	GluN1	***Grin1***	0.0 ± 0.0	13.9 ± 10.3 *
	GluN2A	***Grin2a***	2.2 ± 2.4	0.0 ± 0.0
	GluN2B	***Grin2b***	0.0 ± 0.0	0.0 ± 0.0
	GluN2C	***Grin2c***	0.0 ± 0.0	0.0 ± 0.0
	GluN2D	***Grin2d***	0.0 ± 0.0	2.8 ± 3.7
	GluN3A	***Grin3a***	6.5 ± 4.4	11.1 ± 6.5
	GluN3B	***Grin3b***	0.0 ± 0.0	0.0 ± 0.0
Metabotropic	mGlu1	***Grm1***	4.4 ± 2.9	5.6 ± 7.4
	mGlu2	***Grm2***	0.0 ± 0.0	0.0 ± 0.0
	mGlu3	***Grm3***	71.7 ± 10.7	22.2 ± 9.7 *
	mGlu4	***Grm4***	0.0 ± 0.0	0.0 ± 0.0
	mGlu5	***Grm5***	0.0 ± 0.0	2.8 ± 2.2 *
	mGlu6	***Grm6***	63.0 ± 8.5	55.6 ± 8.6
	mGlu7	***Grm7***	30.4 ± 5.5	25.0 ± 12.5
	mGlu8	***Grm8***	0.0 ± 0.0	8.3 ± 3.4 **

Significant changes are highlighted in yellow. FCI, focal cerebral ischemia

Overall after FCI we observed more frequent changes in the numbers of cells expressing glutamate receptor subunits in oligodendrocytes than in NG2-glia. The only common significant result is the increase in the percentage of cells expressing *Grik3* after FCI in both cell populations. Notably, the level of expression of all glutamate receptor subunit encoding genes did not change after FCI in any of the cell populations.

7.2 Calcium imaging

To analyze functional properties of glutamate receptors and their changes after FCI in NG2-glia we used two-photon fluorescent calcium imaging. The experiments were performed using 3-month-old Cspg4/GCaMP6f/tdTomato mice. Based on morphology we excluded pericytes from the analyses and analyzed only with NG2-glia and cells derived therefrom. Consequently we measured changes in GCaMP6f fluorescence intensities of the cell somas. We analyzed two parameters, the percentage of cells responding to glutamate receptor agonists (n = number of animals used) and the average magnitude of these responses, i.e. the changes of $[Ca^{2+}]_i$, which we processed by averaging the areas under the response curves excluding cells that did not return to baseline.

7.2.1. Glutamate

Initially, we applied glutamate as a physiological glutamate receptor agonist. After glutamate application we observed an increase in $[Ca^{2+}]_i$ represented by raise in fluorescence intensities in both control and ischemic mice. Some of the cells showed a biphasic type of response, possibly indicating the fast ionotropic response and the slower metabotropic response (Figure 19).

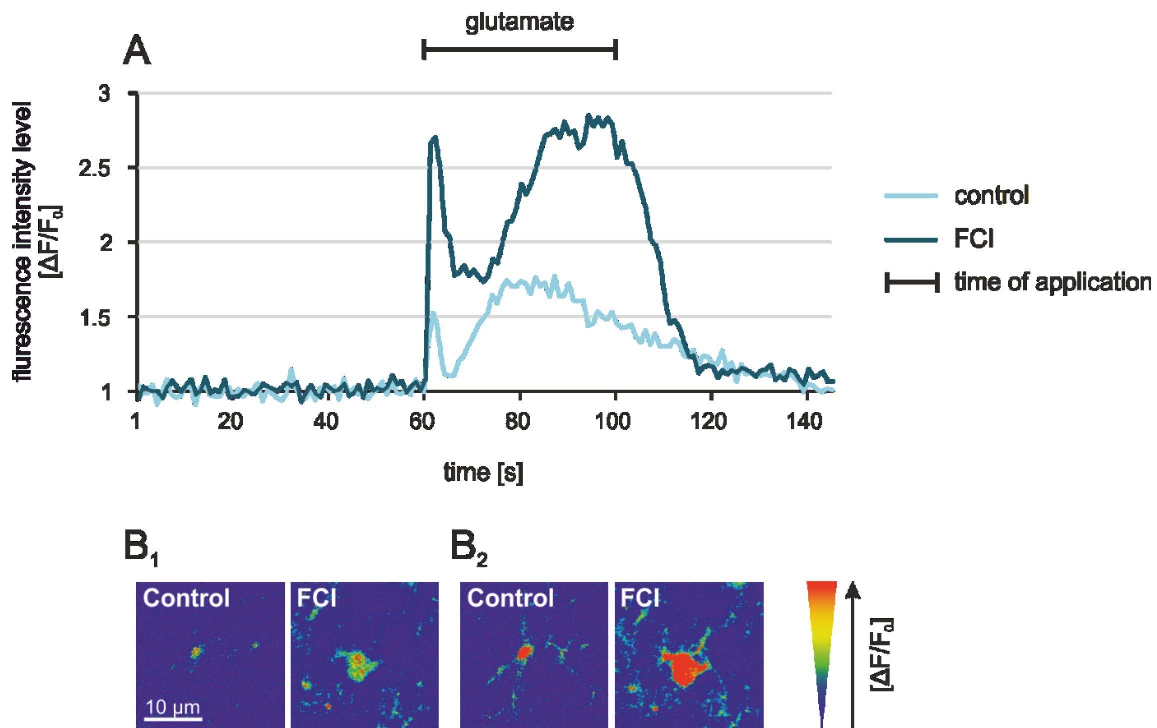

Figure 19: [Ca^{2+}]$_i$ changes evoked by the application of glutamate in tdTomato+ cells in the cortex of a control mouse and an ischemic animal. A – Graph illustrating the response of one cell from the cortex of a control animal and one cell from the cortex of an animal after FCI. Note the biphasic form of the responses. B – Images from two-photon microscope of cells prior to (B_1) and during glutamate application (B_2) showing the increase in fluorescence. Scale bar – 10 µm. Control, cell from control mice; FCI, cell from mice after focal cerebral ischemia

Next, we applied glutamate following 30s preincubation of control slices with TTX in order to block neuronal Na^+ channels, thus eliminate any influence of neuronal activity on the responses of tdTomato+ cells whatsoever. The percentage of cells responding to glutamate application alone (58.6 ± 8.2 %, n = 5 animals) and to glutamate co-applied with TTX (57.3 ± 7.2 %, n = 5 animals) was not significantly different as well as the average [Ca^{2+}]$_i$ increase after glutamate application (22.5 ± 2.1, n = 76 cells) and its application with TTX (21.1 ± 2.9, n = 42 cells). (Figure 20)

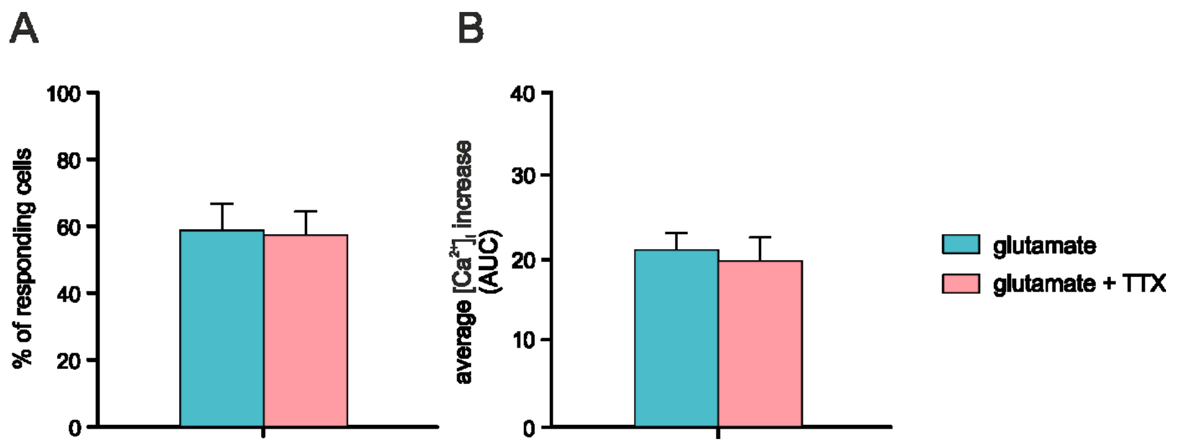

Figure 20: Calcium responses in tdTomato+ cells to glutamate application with TTX. A – Percentage of responding cells. B – Average $[Ca^{2+}]_i$ increase. TTX, tetrodotoxin; AUC, area under curve

As glutamate alone evokes responses mediated by ionotropic as well as metabotropic glutamate receptors, we carried out two types of experiments to separate these two components. Firstly, we preformed the entire experiment using aCSF without $CaCl_2$ to prevent Ca^{2+} ion influx from the extracellular space and thus exclude the iGluR mediated component of the responses. Secondly, we used TG to minimize mGluR evoked responses. The results in Figure 21 suggest that after FCI the number of cells expressing functional glutamate receptors significantly increases as the percentage of responding cells was significantly higher after FCI (82.6 ± 1.6 %, n = 4 animals) when compared to controls (58.6 ± 8.2 %, n = 5 animals). Interestingly, in controls glutamate application without $CaCl_2$ (52.0 ± 6.1 %, n = 4 animals) and application together with TG (59.2 ± 5.9 %, n = 4 animals) did not result in any change in the percentage of responding cells. However, after FCI we observed significantly lower percentage of responding cells after glutamate application without $CaCl_2$ (61.5 ± 5.5 %, n = 3 animals) as well as after its application with TG (67.2 ± 4.8 %, n = 3 animals). The results are illustrated in Figure 21.

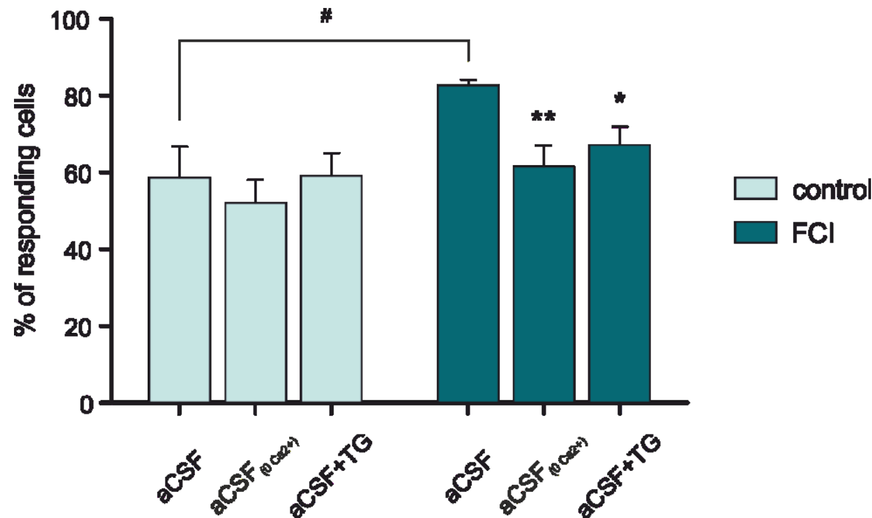

Figure 21: Percentage of tdTomato+ cells responding to glutamate application in aCSF, aCSF without $CaCl_2$ and aCSF with TG in cortical slices from control and ischemic animals. Significant differences compared to controls are marked by #; compared to aCSF by *. aCSF, experiments in artificial cerebrospinal fluid; $aCSF_{(0\ Ca2+)}$, experiments in aCSF without $CaCl_2$; aCSF+TG, experiments in aCSF with thapsigargin; control, measurements in cortex of control mice; FCI, measurements in cortex of mice after focal cerebral ischemia

Nevertheless, when analyzing the changes in $[Ca^{2+}]_i$ we observed more radical differences in both controls and animals after FCI. Comparing control and ischemic values we observed significant increases in the average $[Ca^{2+}]_i$ elevation in ischemic cells after glutamate application (control, 22,5 ± 2.1, n = 76 cells; ischemia, 33.6 ± 2.2, n = 210 cells) and after application with TG (control, 14.9 ± 1.6, n = 76 cells; ischemia, 31.7 ± 3.3, n = 70 cells). The changes after glutamate application in aCSF without $CaCl_2$ were not significantly different comparing control (10.3 ± 0.9, n = 62 cells) and ischemic animals (12.7 ± 1.0, n = 97 cells). Additionally, when comparing values to glutamate application in controls, we detected significantly smaller $[Ca^{2+}]_i$ increases after glutamate application without $CaCl_2$ and its co-application with TG, however in ischemic animals the increases were smaller only in the absence of $CaCl_{2;}$ after co-application with TG they did not change. In summary, iGluR mediated Ca^{2+} entry was increased after FCI, while the metabotropic component of the glutamate evoked response did not change. The results are illustrated in Figure 22.

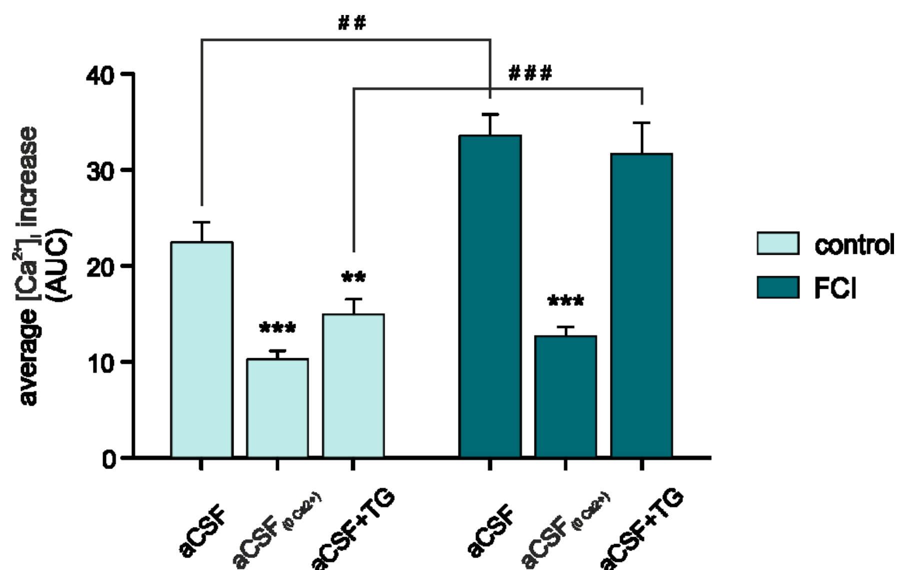

Figure 22: Average $[Ca^{2+}]_i$ elevations after glutamate application in aCSF, aCSF without $CaCl_2$ and aCSF with TG in cortical tdTomato+ cells from control and ischemic animals. Significant differences compared to controls are marked by #; compared to aCSF by *. aCSF, experiments in artificial cerebrospinal fluid; $aCSF_{(0\ Ca2+)}$, experiments in aCSF without $CaCl_2$; aCSF+TG, experiments in aCSF with thapsigargin; control, measurements in cortex of control mice; FCI, measurements in cortex of mice after focal cerebral ischemia

7.2.3. Specific agonists

We used specific iGluR agonists (NMDA, AMPA, kainic acid) to elucidate which types of iGluRs are functionally present in NG2-glia and participate in the glutamate evoked responses. Furthermore, we applied the agonists together with specific iGluR antagonists (MK-801, NBQX) to confirm that observed responses are specifically triggered by NMDA or AMPA/kainate receptor activation.

The application of NMDA resulted in a very low percentage of responding cells in both control animals (11.6 ± 1.9 %, n = 3 animals) and animals after FCI (4.9 ± 1.7 %, n = 3 animals). Moreover, when using the specific NMDA receptor antagonist MK-801, we did not detect any significant changes in the percentage of responding cells neither in controls (8.2 ± 1.7 %, n = 3 animals) nor in ischemic animals (6.7 ± 4.1 %, n = 2 animals). Hence, we considered the observed NMDA evoked responses as negligible.

On the other hand we observed a high percentage of responding cells after the application of AMPA in control (75.7 ± 4.2 %, n = 4 animals) as well as in ischemia

(73.3 ± 5.3 %, n = 4 animals), the values not being significantly different. However, when comparing the changes in $[Ca^{2+}]_i$, we detected significantly increased Ca^{2+} entry in cortical tdTomato+ cells from animals after FCI (46.6 ± 2.7, n = 154 cells) compared to control cortical slices (34.4 ± 2.4, n = 76 cells). The results are illustrated and summarized in Figure 23.

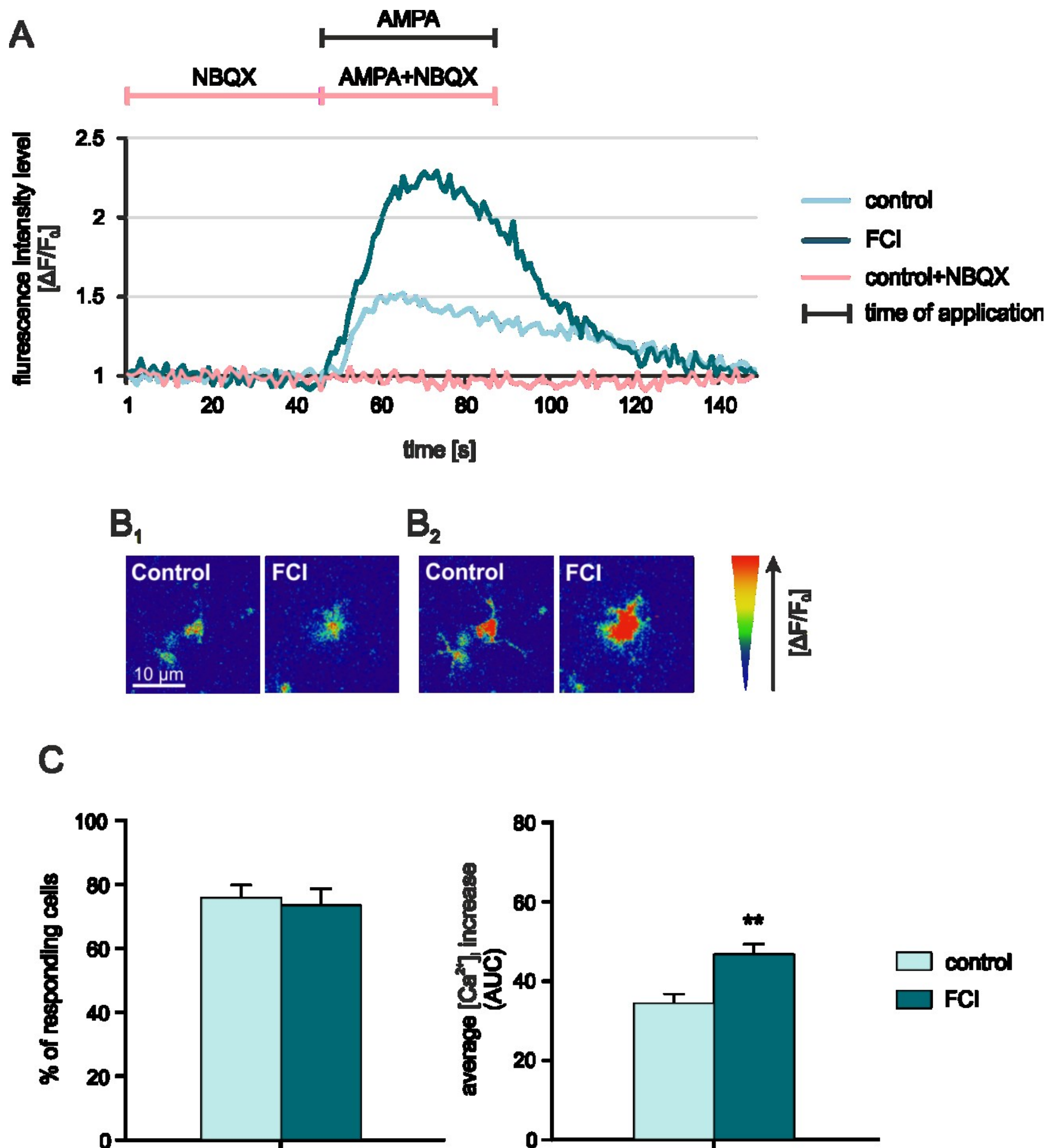

Figure 23: $[Ca^{2+}]_i$ changes evoked by the application of AMPA in tdTomato+ cells in the cortex of a control mouse and an ischemic animal. A – Graph illustrating the response of one cell from the cortex of a control animal and one cell from the cortex of an animal after FCI. B – Images from two-photon microscope of cells prior to (B_1) and during AMPA application (B_2) showing the increase of fluorescence. Scale bar – 10 µm. C – Graph depicting the percentage of

responding cells and an average $[Ca^{2+}]_i$, increase between tdTomato+ cells from control and ischemic mice. AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NBXQ, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione; control, cells from control mice; FCI, cells from mice after focal cerebral ischemia

We obtained similar results after the application of kainic acid where the percentage of responding cells in controls (75.4 ± 8.3 %, n = 3 animals) and in ischemia (69.1 ± 6.4 %, n = 4 animals) were not significantly different as well. However, when comparing the changes in $[Ca^{2+}]_i$, we detected a significant increase in cells from the cortex of animals after FCI (65.0 ± 4.9, n = 75 cells) compared to controls (36.3 ± 3.1, n = 49 cells). The results are illustrated and summarized in Figure 24.

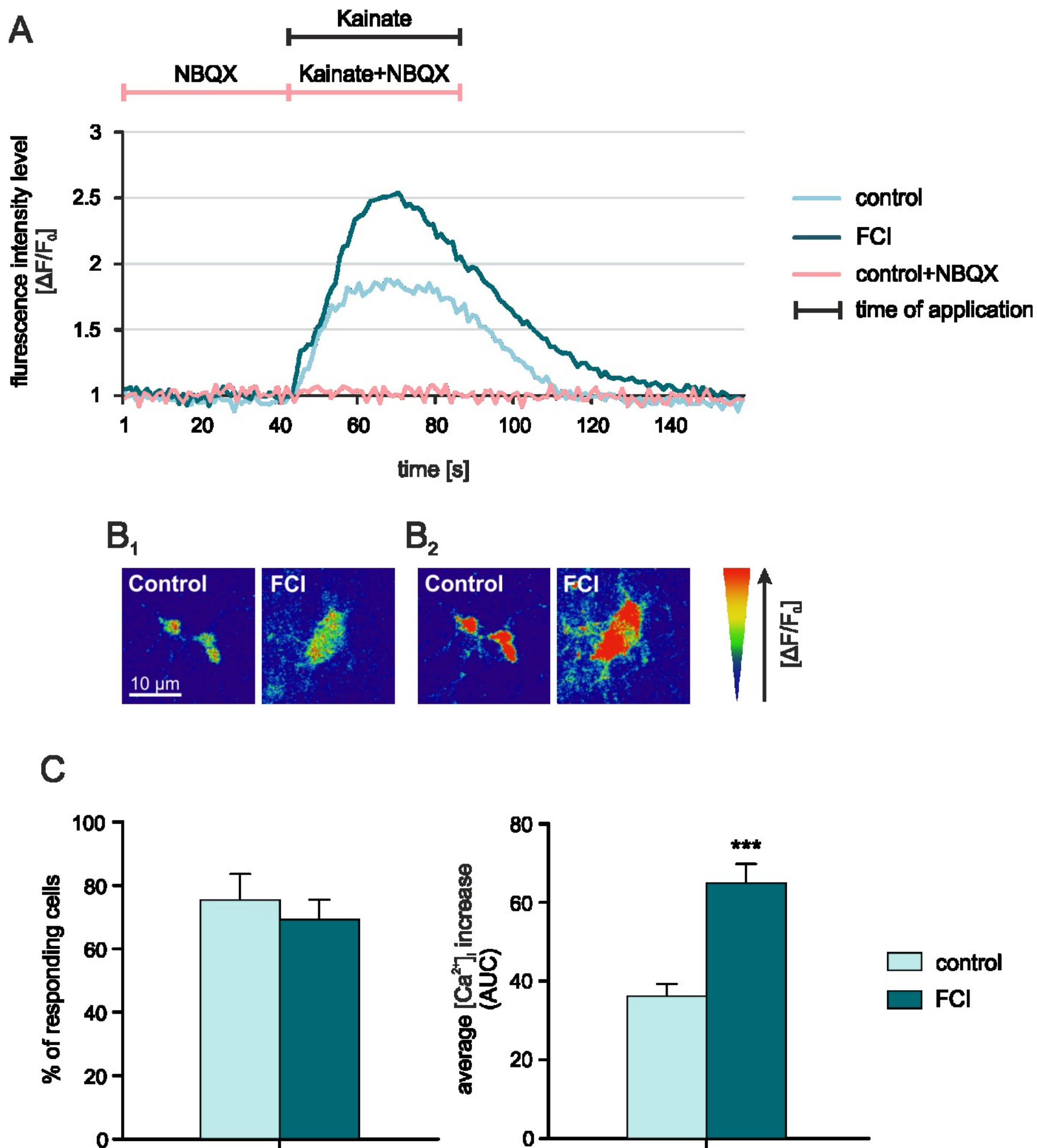

Figure 24: $[Ca^{2+}]_i$ changes evoked by the application of kainate in tdTomato+ cells in the cortex of a control mouse and an ischemic animal. A – Graph illustrating the response of one cell from the cortex of a control animal and one cell from the cortex of an animal after FCI. B – Images from two-photon microscope of cells prior to (B_1) and during kainate application (B_2) showing the increase of fluorescence. Scale bar – 10 µm. C – Graph depicting of the percentage of responding cells and average $[Ca^{2+}]_i$, increase between tdTomato+ cells from control and ischemic mice. NBXQ, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione; control, cells from control mice; FCI, cells from mice after focal cerebral ischemia

Based on these results we used specific iGluR antagonists to block the responses, hence verify the activation of the specific receptors (results summarized in Figure 25). We successfully blocked the AMPA and kainate mediated responses by the AMPA/kainate receptor antagonist NBQX, the percentage of responding cells significantly decreasing in control (7.9 ± 1.3 %, n = 3 animals) as well as in ischemia (9.1 ± 0.3 %, n = 2 animals).

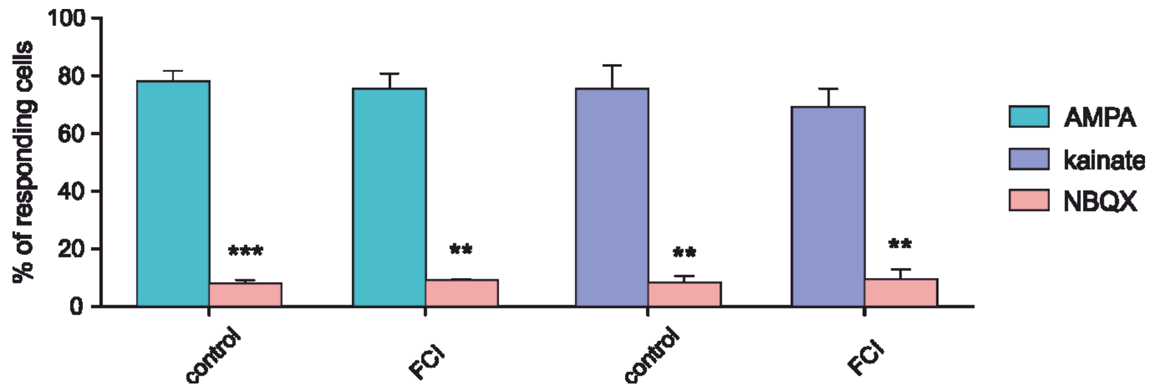

Figure 25: AMPA and kainate mediated Ca^{2+} responses of cortical tdTomato+ cells blocked by NBQX. Control, cells from control mice; FCI, cells from mice after focal cerebral ischemia; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione

8. Discussion

In our study we used two types of mice. For examining gene expression we used mice with tamoxifen-inducible Cre recombinase under the Cspg4 promotor which were crossbred with a reporter mouse strain. This allowed us to identify NG2-glia and cells derived therefrom and analyze their expression profile before and after ischemic insult focusing primarily on glutamate receptors. Furthermore, to study functional properties of glutamate receptors in NG2-glia we crossed these mice with a strain with a genetically encoded Ca^{2+} probe that allowed us to detect $[Ca^{2+}]_i$ levels.

It is not surprising that the majority of studies concerning glutamate receptors focused primarily on neurons. Since NG2-glia were recognized as a separate glial cell type relatively recently, there are not as many data regarding different types of glutamate receptors in these cells, moreover there are no data in the context of cerebral ischemia. Therefore, this diploma thesis represents the foremost study dealing with expression profile and function of glutamate receptors in NG2 glia under physiological conditions as well as following ischemia. Firstly, using single cell RT-qPCR we proved that NG2-glia and oligodendrocytes express almost all glutamate receptor subunits on mRNA level, the expression of several subunits being significantly altered after FCI (Table 5, 6). Secondly, we conducted functional studies utilizing intracellular calcium imaging, in which we detected responses to glutamate, AMPA and kainate and thus verified the functional presence of these receptors on the cell membrane of cortical NG2-glia and cells derived therefrom. Importantly, we observed significant changes in the responses after FCI indicating that NG2-glia react differently to glutamate signaling in the pathological conditions of ischemia.

To clarify the effects of ischemic injury on the expression profile and functional properties of glutamate receptors in NG2-glia we used a permanent model of FCI; micro-surgically-induced distal MCAo, which is a well reproducible animal model of stroke (Kuraoka et al., 2009). In accordance to previous works using this model, the injured area of the brain was restricted primarily to the cortical regions unlike intraluminal monofilament MCAo, which produces severe hemispheric ischemia involving thalamic and hypothalamic regions (Carmichael, 2005; Kuraoka et al., 2009).

As previously described (Claus et al., 2013), NG2-glia were densely abundant in the demarcation zone insulating the healthy and necrotic tissue (Figure 13).

Employing single cell RT-qPCR we detected NG2-glia and also cells derived therefrom as a result of persistent reporter protein expression. This resulted in detecting cells with a characteristic oligodendrocyte phenotype among the population of NG2-glia, hence we divided the NG2-glial and oligodendroglial populations based on the expression of specific markers (Dyer, Hickey, & Geisert, 1991; Redwine & Armstrong, 1998; Roussel et al., 1983; Stallcup & Beasley, 1987). After FCI some of the selected NG2-glia expressed also oligodendrocyte markers. This may be the result of increased NG2 glia proliferation and subsequent new generation of oligodendrocytes from these cells in response to oligodendrocytic cell death induced by FCI (Mandai et al. 1997; Butt et al. 2002; Simon et al. 2011). Our findings are in agreement with those detecting various developmental stages of oligodendrocytes (Marques et al., 2016; McTigue & Tripathi, 2008). However, concerning the expression levels of the markers we were able to clearly separate the two distinct glial cell types.

Regarding the expression of genes encoding glutamate receptor subunits we analyzed oligodendrocytes and NG2-glia separately. We detected AMPA and kainate receptor subunit mRNA in oligodendrocytes from the cortex of control animals as shown in previous studies (García-Barcina, 1996; Yoshioka, Bacskai, & Pleasure, 1996). In contrast to previous studies (Káradóttir et al., 2005; Salter & Fern, 2005) we detected a very low to lacking expression of mRNA of NMDA receptor subunits as was recently also shown by single-cell RNA sequencing performed by the group of Marques (Marques et al. 2016). Furthermore, we detected mGluR mRNA presence corresponding with previous works of Luyt and colleagues (Luyt, Váradi, Durant, & Molnár, 2006; Luyt, Varadi, & Molnar, 2003). After FCI the number of oligodendrocytes expressing mRNAs for several glutamate receptor subunits increased, notably mRNA coding AMPA and kainate receptor subunits. As previously mentioned, this is presumably the result of detecting more cells in various stages of oligodendrocyte development after FCI. As described by Marques and colleagues (Marques et al., 2016) the expression of glutamate receptor mRNA gradually decreases during oligodendrocyte maturation. Notably, as shown by the groups of Luyt and Marques (Luyt et al., 2006; Marques et al., 2016), the expression of mGluR mRNA changes

during the oligodendrocyte development, switching gradually from *Grm5* in NG2-glia to *Grm3* in mature oligodendrocytes. This explains our observed decrease in the percentage of oligodendrocytes expressing *Grm3* after FCI, since not all of the cells were fully maturated. Regarding NG2-glia we observed fewer statistically significant changes after FCI, the only notable change being the increase in the percentage of cells expressing *Grik3*.

Employing calcium imaging we analyzed responses to various glutamate receptor agonists as elevations of $[Ca^{2+}]_i$, aimed to elucidate the participation of specific receptors in the response and determine changes after FCI. We excluded tdTomato$^+$ pericytes from the analysis based on their distinct morphology. However, we were not able to distinguish between tdTomato$^+$ oligodendrocytes and other oligodendrocyte lineage cells; hence we were forced to analyze cells representing all oligodendrocyte development stages from NG2-glia to mature oligodendrocytes. Glutamate application evoked $[Ca^{2+}]_i$ increase in most of the cells analyzed corresponding with previous works (Gallo, Patneau, Mayer, & Vaccarino, 1994; Kastritsis & McCarthy, 1993; Pende, Holtzclaw, Curtis, Russell, & Gallo, 1994). We used TTX in order to block neuronal Na^+ channels (Moore, Blaustein, Anderson, & Narahashi, 1967), thus eliminate any influence of neuronal activity on the responses whatsoever. The number of responding cells and the glutamate mediated increases in $[Ca^{2+}]_i$, did not change, thus indicating that neuronal activity does not have any influence on the oligodendrocyte lineage cell responses to glutamate. Therefore, we did not use TTX for further measurements. Interestingly, after glutamate application we observed responses with a biphasic character (Figure 19) in part of the cells, hence we presumed the first rapid $[Ca^{2+}]_i$ increase to be of iGluR origin and the second slower increase of mGluR origin. However when applying glutamate with TG, which as a non-competitive inhibitor of SERCA minimized the mGluR component of the responses (Rogers, Inesi, Wade, & Lederer, 1995), we occasionally observed a similar character of responses. The same applied to glutamate application in aCSF without $CaCl_2$, where in the absence of extracellular Ca^{2+} the iGluR component of the responses was diminished. Henceforward, we considered the character of the responses as irrelevant. Moreover, regarding our findings that the percentage of cells responding to glutamate application with TG and without $CaCl_2$ in control animals does not significantly change, we presume that functional iGluRs and mGluRs are expressed evenly among the

oligodendrocyte lineage cells, both taking a part in the glutamate response in the healthy brain. This corresponds to our results from single cell RT-qPCR and to studies proving the presence of functional mGluRs (Luyt et al., 2003) and iGluRs (Bergles et al., 2000; Ge et al., 2006; Kukley & Dietrich, 2009) in cells of the oligodendrocyte lineage. On the other hand, after FCI we observed a significant decrease in the number of responding cells following application with TG and without $CaCl_2$, which may resemble the previously mentioned higher heterogeneity of the cell population, thus meaning also higher heterogeneity in the expression of glutamate receptors. Importantly, after FCI the application of glutamate resulted in an overall higher percentage of responding cells. This may be the result of the previously mentioned death of mature oligodendrocytes and increased proliferation and differentiation of NG2-glia after FCI; hence the cell population was presumably composed of more cells in different oligodendrocyte development stages with overall higher expression of various glutamate receptors. Interestingly, after applying AMPA and kainate we did not observe an increase in the number of responding cells after FCI. A possible explanation lies in the previously mentioned “mGluR5/3 switch” during oligodendrocyte maturation (Luyt et al., 2006; Marques et al., 2016). Unlike mGluR5, mGluR3 belongs to the group II of mGluRs, which do not affect $[Ca^{2+}]_i$. FCI causes death of mature oligodendrocytes expressing mGluR3 and increased proliferation and differentiation of NG2-glia expressing mGluR5, thus the analyzed cell population presumably consisted of more cells expressing the $[Ca^{2+}]_i$ affecting mGluR5. This could clarify the observed higher number of cells responding to glutamate after FCI due to the activation of all types of glutamate receptors, while the application of AMPA or kainate activates specific iGluRs only, thus after FCI we did not observe such changes. Furthermore, glutamate uptake could also affect the number of responding cells, since it was shown that in ischemia it could be impaired or even reversed by various pathological changes (Mitani & Tanaka, 2003; Raghavendra Rao et al., 2000; Rossi et al., 2000; Swanson, Farrell, & Simon, 1995), thus being another possible factor in the observed higher number of cells responding to glutamate after FCI.

The second characteristic we studied was how much the $[Ca^{2+}]_i$ increases after various agonist applications. As our results show, cortical oligodendrocyte lineage cells in aCSF without $CaCl_2$ responded to glutamate with a significantly lower $[Ca^{2+}]_i$ elevation compared to glutamate application only, this applying to control and ischemic

mice respectively. Therefore, we could further confirm that Ca^{2+} influx through iGluRs significantly contributes to the glutamate response. Interestingly, when applying glutamate with TG we showed a lower $[Ca^{2+}]_i$ elevation particularly in cells from control mice, however, not after FCI. These results suggest that after FCI the contribution of mGluRs to the glutamate response is negligible which is in contrast with the previously described "mGluR5/3 switch". However, overall the $[Ca^{2+}]_i$ increase after glutamate application was higher after FCI, suggesting probably higher Ca^{2+} permeability of iGluRs since as stated in the results the levels of mRNA expression did not change. We confirmed the contribution of AMPA and kainate receptors to this elevated response since after application the $[Ca^{2+}]_i$ increase was significantly higher after FCI as well. We presume that after FCI the subunit composition of these receptors may change, resulting in higher Ca^{2+} permeability. The explanation could lie in the rapid downregulation of *Gria2* after ischemia as was shown in the study of Pellegrini-Giampietro et al. 1997. Not including the Ca^{2+} impermeable GluA2 subunit into the composition of AMPA receptors causes them to be Ca^{2+} permeable. However, our results do not show any notable significant changes in *Gria2* expression. Another possible solution may be provided by a possible decrease in *Gria2* mRNA editing in ischemia resulting in Ca^{2+} permeability of GluA2-containing AMPA receptors (Wright & Vissel, 2012). Interestingly, similarly to the GluA2 subunit, GluK1 and GluK2 kainate receptor subunits also undergo mRNA editing, thus the level of mRNA editing of *Grik1* and *Grik2* could also affect the glutamate response after FCI as was shown in a study by Paschen et al. 1996. These assumptions have yet to be discussed in further studies concerning specifically NG2-glia and the oligodendrocyte lineage.

In summary, we have shown that both iGluRs and mGluRs are functionally present in cells of the oligodendrocyte lineage and take part in the response to glutamate signals from other cell types. The expression and functional properties alter after FCI, mostly in an amplifying character.

9. Conclusion

In summary, we have shown that under physiological conditions NG2-glia in the adult mouse cortex express more various glutamate receptor subunits than oligodendrocytes. Furthermore, the expression profile and functional properties of glutamate receptors in NG2-glia as well as oligodendrocytes change significantly following cerebral ischemia, indicating the involvement of glutamate receptors in the reaction of these cell types to ischemic damage. We namely confirmed the contribution of AMPA and kainate receptors to such response.

The increase in the expression of certain glutamate receptor subunit after FCI was accompanied by an increased number of cells responding to glutamate and augmented Ca^{2+} entry. The latter shown to be contributed by AMPA and kainate receptors. Since Ca^{2+} influx modulates numerous Ca^{2+}-dependent effectors, our results suggest these signaling cascades to be altered during ischemia in NG2-glia and cells derived therefrom, possibly affecting their functions, such as myelination, oligodendrocyte maturation, and maintenance of neuron-glia signaling.

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