Classification and identification of central nervous system glia

Pongrac, Marta

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ODJEL ZA BIOTEHNOLOGIJU

Preddiplomski sveučilišni studij

„Biotehnologija i istraživanje lijekova“

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UNIVERSITY OF RIJEKA
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„Biotechnology and drug research"

BACHELOR THESIS

CLASSIFICATION AND IDENTIFICATION OF CENTRAL NERVOUS SYSTEM GLIA

Marta Pongrac

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SAŽETAK

Glija je vrlo heterogena populacija sastavljena od vrlo različitih stanica koje zajedno sudjeluju u održavanju homeostaze središnjeg živčanog sustava (SŽS). Dugo se smatralo da se radi o acelularnom vezivnom tkivu, dok je danas to gledište u potpunosti odbačeno te je sve više istraživanja usmjereno prema glijalnim stanicama. Zahvaljujući tehnološkom napretku, otkrivaju se nove posebitosti u funkciji tih stanica te njihovoj interakciji kako s neuronima, tako i međusobno. Tijekom razvoja SŽS, radijalne glije vode migraciju neurona, NG2 stanice primaju informacije od neurona i inhibiraju aksonalni rast, oligodendrociti formiraju mijelin, astrociti formiraju krvno-moždanu barijeru (KMB) i reguliraju optimalne uvjete poput pH, ionskog gradijenta, reapsorpcije neurotransmitera. Nadalje, ependimalne stanice formiraju cerebrospinalni fluid (CSF) i štite SŽS, a mikroglija uklanjaju neželjene stanice. U patološkim uvjetima glijalne stanice djeluju s imunosnim sustavom i otpuštaju neurotrofičke faktore. Međutim, mnogo još preostaje neobjašnjeno, a već i sama identifikacija i karakterizacija glija predstavljaju izazov. Glijalne stanice se razlikuju po morfologiji, biokemiji i funkcijama. Za njihovu identifikaciju se najčešće koriste eksprimirane molekule na staničnoj membrani, proteini citoskeleta ili signalne molekule poput faktora rasta, neurotrofičkih faktora, hormona i citokina koje otpuštaju. Identifikacija nailazi na probleme jer ne postoji dovoljno markera koji mogu sa sigurnošću odrediti (pod)tipove glija zbog toga što mnoge markere ko-eksprimiraju različite stanice živčanog sustava. Ko-ekspresija i heterogenost često vodi prema svrstavanju (pod)tipova glija u istu kategoriju. Cilj ovog rada je sakupiti istraživanja provedena na glijalnim stanicama SŽS i usporediti različite stavove i pronalaske kroz povijest uz kritički osvrt. Ovaj rad se usredotočuje na karakterizaciju i identifikaciju tipova glijalnih stanica te usporedbi međusobnih sličnosti i razlika. Označavanje glijalnih stanica pomoću antitijela, kao najpopularnija metoda identifikacije je analizirana prema korištenim markerima poput GFAP, S100ß, Nestin, NG2, Iba1 i sl. te su navedene njihove prednosti i nedostatci. Kako su glije ključan dio središnjeg živčanog sustava u zdravim i patološkim uvjetima, više istraživanja treba biti usmjereno prema njima. Istraživanja bi se trebala proširiti i na ljude i opisati posebne vrste glija primata, još nepoznatih funkcija.

KLJUČNE RIJEČI

Astrociti, oligodendrociti, mikroglija, ependimalne stanice, NG2 glija

SUMMARY

Glia is a very heterogeneous cell population comprised of very different cells that work together to maintain central nervous system (CNS) homeostasis. Long believed role of glia being the acellular connective tissue has been overturned, and they have become focus of intense research. Due to the technological advances, new functions are being discovered, as well as interactions of glia with neurons and the glia between themselves. During CNS development, radial glia guide neuronal migration, NG2 receive neuronal inputs and inhibit axonal growth, oligodendrocytes myelinate axons, astrocytes form the blood-brain-barrier (BBB) and regulate optimal conditions, such as pH, ion gradient, neurotransmitter reuptake. Moreover, ependymal cells form cerebrospinal fluid (CSF) and protect CNS, while microglia remove unwanted cells. In pathological conditions, glia interact with immune system and release neurotrophic factors. However, many issues remain to be addressed, because identification and characterization of glia still represents a challenge. Glia differ in morphology, biochemistry and functions. They are most commonly identified by expressed molecules on cell membrane, cytoskeletal proteins or signal molecules, such as growth factors, neurotrophic factors, hormones or cytokines they release. Identification presents a problem because there is an insufficient amount of markers that can specifically identify a particular glial (sub)type because the markers are often co-expressed by several cell types of the nervous system. Co-expression and heterogeneity of glial (sub)types often lead to assignment of the same category. The aim of this work is to bring together the research conducted on CNS glia and to compare different opinions and findings throughout the history, critically remarking the research. This work focuses on characterization and identification of neuroglia types and pointing out the similarities and differences among them. Most commonly used immunostaining markers, for example, GFAP, S100β, Nestin, NG2 and Iba1 are analyzed with their advantages and weaknesses regarding specificity. As the glia are vital part of CNS in both healthy and pathological conditions, more research should be directed to them. Research should also involve humans and depict unique primate glia, with still unknown functions.

KEY WORDS

Astrocytes, oligodendrocytes, microglia, ependymal cells, NG2 glia

Contents

1. NEUROGLIA AT A GLANCE

From the first time R. Virchow described neuroglia in 1856, it was set against the neurocentric concept of nervous elements:

> '*Hitherto, gentlemen, in considering the nervous system, I have only spoken of the really nervous parts of it. But if we would study the nervous system in its real relations in the body, it is extremely important to have a knowledge of that substance also which lies between the proper nervous parts, holds them together and gives the whole its form in a greater or less degree'* [1].

He publicly broadcasted that glia is a connective tissue, but also highlighted the importance of studying the nervous system for the real relations. Even though the focus has been on neurons, a change in the perspective has been made and recently more research has been oriented towards the glia. As a result, glia were found to be remarkably heterogeneous cell population with variety of functions, and still a lot remains to be unveiled.

(Neuro)glia are non-neuronal CNS cell lineages of different origin, morphology, physiological and functional features [2]. They are often described as non-excitable nervous cells, but exceptions exist. The main function of neuroglia could be summarized as maintenance of the homeostasis of the nervous system.

Neuroglia can be divided into two main categories: central nervous system (CNS) and peripheral nervous system (PNS) glia. The CNS glia is subdivided into microglia and macroglia (Figure 1). Microglia originate from the yolk-sac progenitors in mesoderm that populate the brain during development [3]. They have defensive role in the nervous system and detect and phagocytose damaged neurons, synapses, and plaques [4]. Macroglia originate from precursors in the neurogenic region of the

ectoderm. Neural stem cells give rise to radial glia or neurons, depending on the extracellular signals [5]. Radial glia further differentiate into astrocytes, ependymal cells and NG2 glia, which precede oligodendrocytes. Astrocytes support neurons structurally, metabolically and trophically. Furthermore, they take part in formation of the blood-brain barrier, absorption of neurotransmitters and extracellular fluid homeostasis. They participate in synapse formation, maturation and activity and also form glial scar as a result of brain infection or injury [6]. Ependymal cells protect the CNS by forming a cellular barrier between blood and cerebrospinal fluid. They form a CSF stream with plentiful cilia, filter molecules and debris [7]. NG2 cells are abundant in proteoglycan NG2. Some cells positive for this proteoglycan are positive for glial fibrillary acidic protein (GFAP), while other NG2 positive cells are positive for ganglioside receptors, present in neurons. Therefore, proteoglycan NG2 was named "*nerve/glial antigen 2"* for being in cells with properties of both neurons and glia, while NG2 cells got their name for the proteoglycan. NG2 cells have shown potential to differentiate into several types of glial cells, but also into neurons [8]. They receive inputs from neurons and inhibit axonal growth and support formation of glial scar [9] [10]. Oligodendrocytes produce myelin sheaths to increase the velocity of impulses that propagate along axons. Because of (enormous) heterogeneity of glia, researchers hold different opinions and approaches to identify and classify the glia (Figure 1). The classification of the macroglia types is still under debate. Some researchers classify ependymal cells as the individual macroglia subcategory, while others classify ependymal cells as subtype of astrocytes [11]. NG2 cells are also in some articles considered to be macroglia category, while in others are positioned alongside oligodendrocytes, as their precursors. They show various vital functions in the CNS, requesting for further examination.

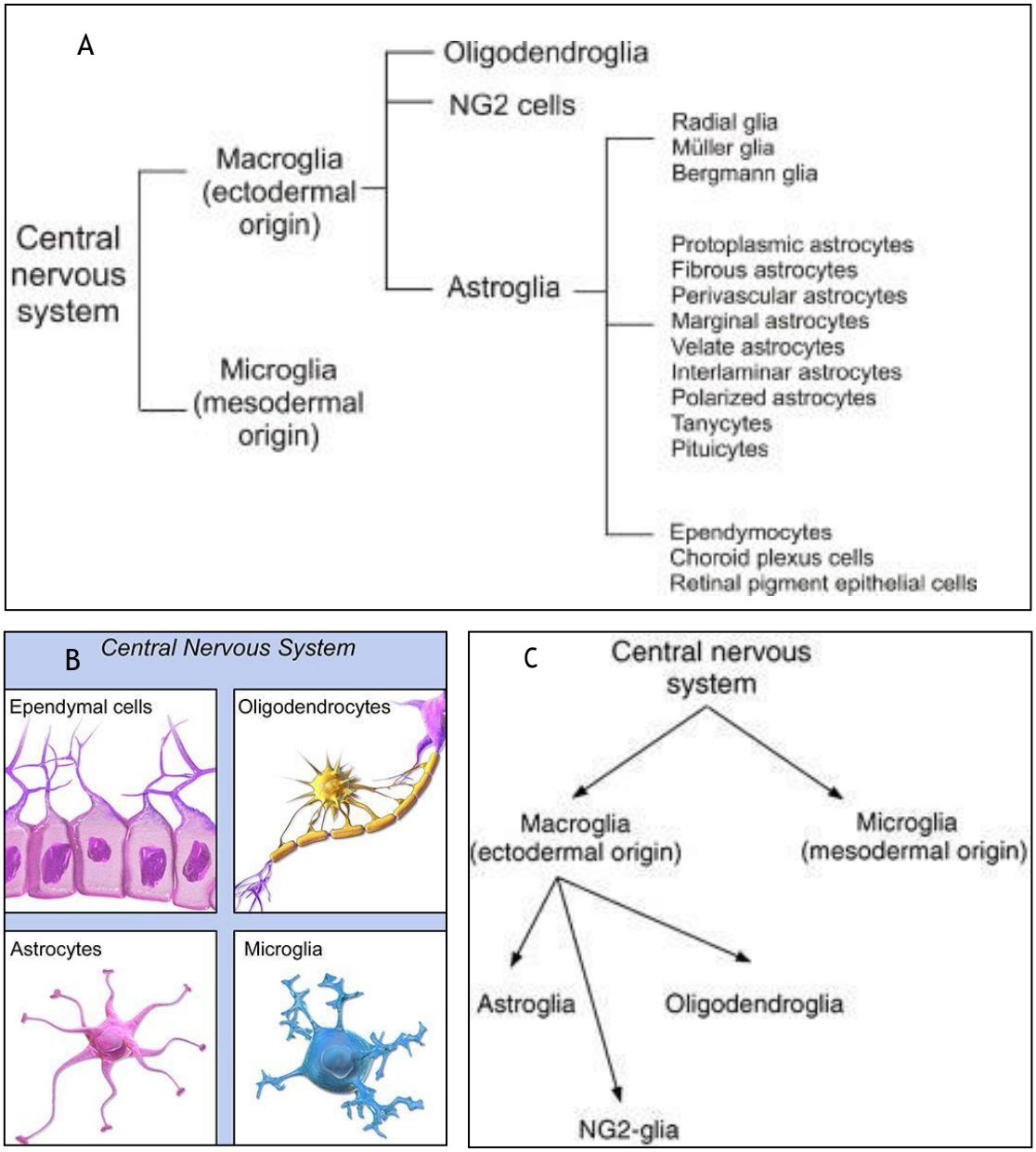

Figure 1. Classification of CNS glia. A shows NG2 as individual macroglia category, while ependymal cells are classified as a subtype of astrocytes (image taken and modified from *[2]*). B shows ependymal cells as individual category (image taken and modified from *[12]*). C shows NG2 as an individual category (image taken and modified from *[13]*).

As neuroglia perform very diverse functions and are involved in various neural activities, their division is based on their structural and biochemical characteristics. Drawback to identification of glia is that a number of molecules are co-expressed on different glial cells but also by

neurons. This potentially leads to misinterpretation and considerably reduces the number of reliable molecular markers.

2. NEUROGLIA EVOLUTION

Glial appearance began most likely when central nervous system (CNS) began to centralize and started to form ganglia, as well as peripheral sensory organs.

The first organisms in which the proto-neuroglia was found were Nematodes. Roundworm *Caenorhabditis elegans*' nervous system is made of 302 neurons and 50 supportive cells of ectodermal origin and six supportive cells of mesodermal origin. 46 of the supportive cells are associated with sensory organs. The remaining four cells are known to compose cephalic sheath and associate with neural ring. These supportive cells are the proto-astrocytes [2]. The ancestral glial cells in *C. elegans* enwrap synapses, are possibly involved in ion homeostasis, and contribute to nervous system development; however, the functions still are not fully elucidated [2] [14].

It wasn't until Arthropoda that neuroglia became indispensable for neuronal survival (Figure 2). In the *Drosophila melanogaster*, glial cells comprise approximately 10% of total number of CNS cells. They are divided into classes of wrapping glia of the PNS and surface glia that form hemolymph-brain barrier. Glial cells regulate ion control, keep the neurotransmitter and metabolic homeostasis, ensheath axons and mount astrogliotic reactions to phagocytosis in case of pathologic state [2] [14].

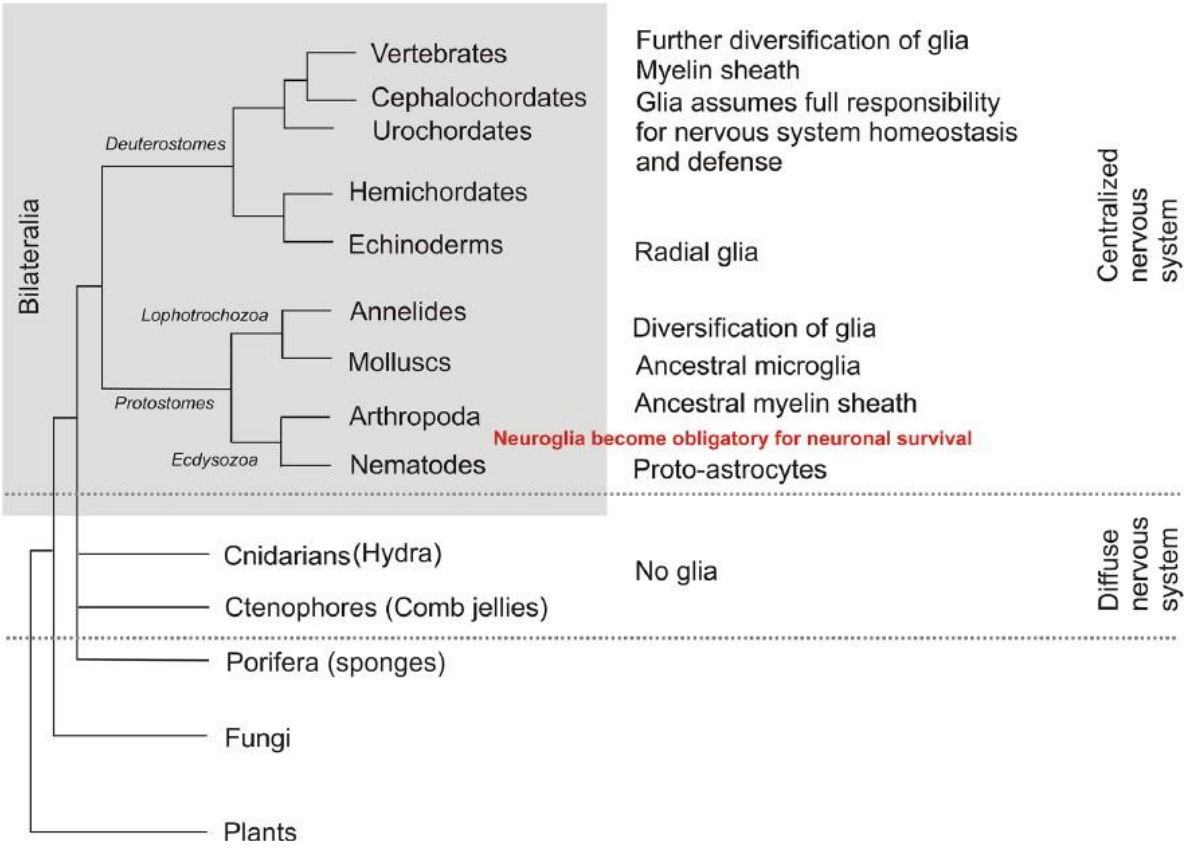

Figure 2. Evolution of glia (image taken from *[2]*).

In addition to the already developed ancestral microglia, ancestral myelin sheath and further glial diversification in Echinoderms, radial glia occurred, which led to brain gaining the layered structure. By an increase of the brain thickness, the brain parenchymal glia started to develop and gradually became predominant type of astrocytes that maintain the homeostasis of mature brain in higher vertebrates. Radial glia, on the other hand, in higher vertebrates are present only during embryonic development and not in the mature brain [14] [2].

As the brain size enlarged, glia grew in quantity and diversity. In mammals, radial glia are limited to embryonic development and are present in radial-like cells in retina, in cerebellum, in hypothalamus and in subventricular zone (SVZ). Human astrocytes are the most complex comparing to rodents, dogs or felines. The human protoplasmic astrocytes are more than two times larger than rats', process 10 times more

information and possess twofold complexity of arborisation. Humans also have several unique types of astroglia [2] [14].

Quantity of glia varies considerably among species. By increase in the cerebral cortex mass, glial number increases, even though the trend is not applicable to all species. Vertebrates follow the glia to neuron ratio positively by enlargement of brain. The adult human brain averages 1.5 kg and has 86 billion neurons and 85 billion non-neuronal cells, including glial cells, pericytes, fibroblasts and endothelial cells [15].

3. GLIOGENESIS

Gliogenesis is the process by which glial cells are derived from multipotent neural stem cells (NSC) during the embryonic development. NSC, also known as neuroepithelial cells (Figure 3); develop into radial glia, one of first specialized cells in the CNS, distinct by their bipolar shape [16]. Neuronal and glial lineages appear by asymmetric division of radial glia, as a result of extrinsic signals [5].

During the embryonic development of the CNS, radial glia are the leading population that encodes positional cues required for neuron formation, guidance, survival and maintenance [17]. They differentiate into ependymal cells, astrocytes, NG2 glia leading to oligodendrocytes. Astrocytes promote NSC differentiation by BMP ligands and signaling. Ependymal cells can also modulate BMP signaling to promote generation of NG2 cells [18]. Additionaly, astrocytes express Notch receptors [19]. Notch signals inhibit neuronal differentiation but enhance glial generation and differentiation [5].

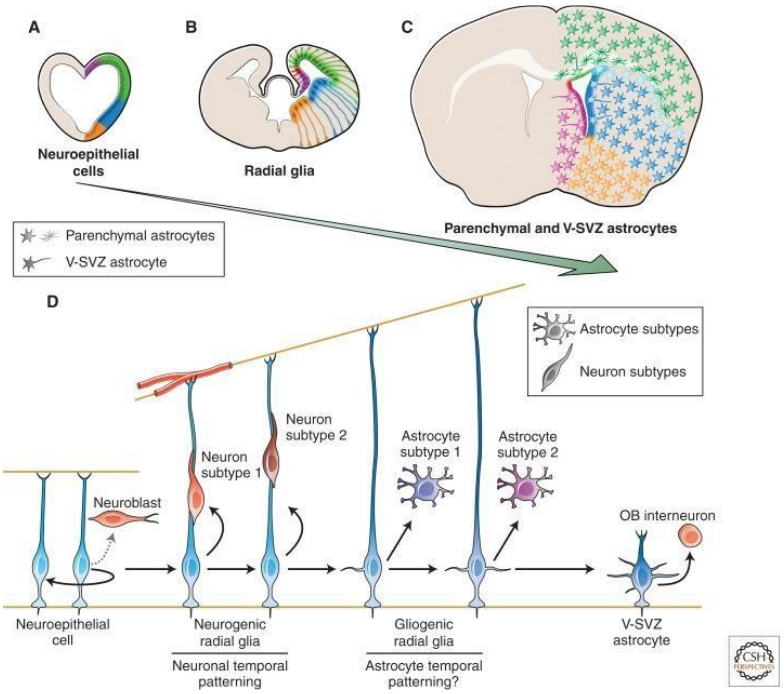

Figure 3. Development of V-SVZ astrocytes (image taken from *[17]).*

4. TRIPARTITE SYNAPSE

It is widely accepted that signal transmission in the nervous system occurs through synapses. In the terminal parts of pre-synaptic axon, numerous synaptic vesicles are filled with neurotransmitters. Neurotransmitters are released into the synaptic cleft, where they travel to the dendrites of the post-synaptic neurons. Special receptors and channels of the post-synaptic neurons uptake the signaling molecules.

Current research shows that glial cells also take part in synaptic neurotransmission. Astroglia regulate synaptic connectivity, strength and plasticity and provide for spatial specificity of synaptic inputs. Astrocyte processes create a physical barrier, contain transporters that remove neurotransmitters from synaptic cleft (glutamate, GABA and adenosine, etc.), and modulate the action potential-evoked synaptic transmission [2]. For example, reuptake of glutamate is particularly important because it is an excitatory neurotransmitter that in higher concentration damages or kills neurons, among other cells. Nevertheless, neurons need glutamate for various functions, but cannot synthetize it *de novo*. Astrocytes reuptake large quantities of glutamate from the cleft and release it in different ways. The most important is the glutamate-glutamine shuttle (Figure 4). Conversion of glutamate to glutamine, at the cost of one ATP, involves enzyme glutamine synthetase (GS). Glutamine is transported to neurons by specific transporters and hydrolyzed back into glutamate in presynaptic neuron [20].

The mechanical, electrical stimuli, photo stimulation or bradykinin addition that cause Ca^{2+} rise in cultured astrocytes may contribute to neuronal glutamate release, leading to Ca^{2+} elevation in neurons. Additionally, astrocytes cover nerve terminals and can respond to synaptic activation [21]. They release synaptically active molecules to synaptic clefts, such as glutamate, ATP, adenosine, GABA and D-serine [22]. Release of neuroligands from glia is known as gliotransmission. Furthermore,

astrocytes can release growth factors, cytokines like tumor necrosis factor alpha (TNF-α), neuroactive steroids such as estradiol, progesterone and metabolites that can effect synapses [22]. Astrocytes are actively involved in the processing the information flow along pre-synaptic and post-synaptic neurons. They respond to synaptic activity, regulate synaptic transmission and synaptic plasticity, display tripartite means of synaptic information flow.

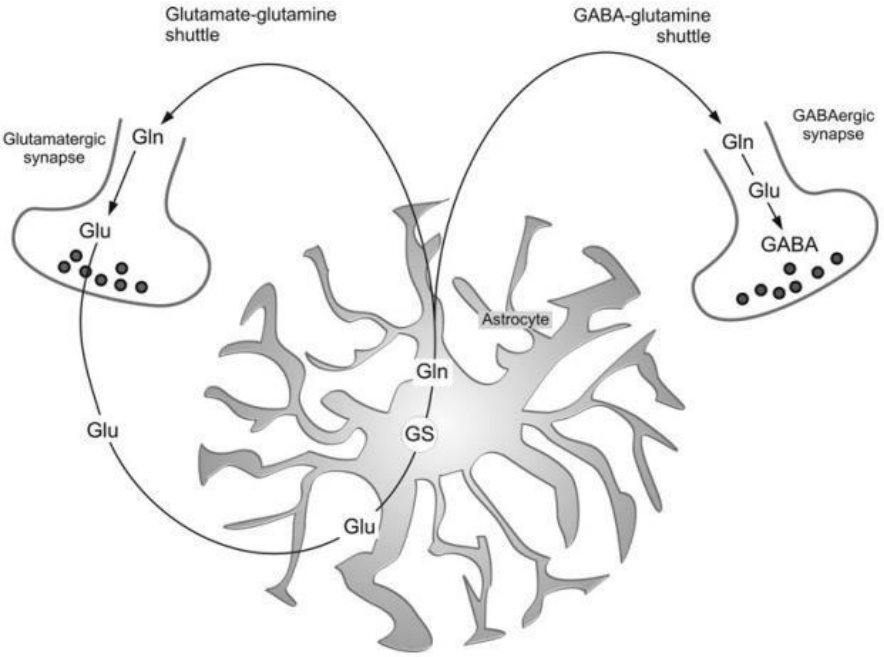

Figure 4. Glutamate-glutamine and GABA-glutamine shuttle (image taken from *[20]*).

5. FUNDAMENTS OF NEUROGLIA

5.1. RADIAL GLIA

Earlier research depicted radial glia as the cells with long processes that extend through apicobasal axis of neuroepithelium and that direct neuronal migration. Later in the development, they differentiate into both neuronal and glial lineages.

At the beginning of 2000s, the knowledge about radial glia increased when Merkle and Tramontin tested the hypothesis that radial glia produce adult NSC in the mouse SVZ using a Cre-lox technique to label striatal radial glia at P0. They investigated maturation of SVZ and found that at P0, the striatal ventricular zone (VZ) was comprised mainly from radial glia and a few immature ependymal cells. They stained the cells for GFAP and RC2 in the period from P0 to P60 and confirmed that loss of radial cells correlates to the appearance of astrocytes. From P4 to P10, radial glia developed into striatal and SVZ astrocytes, while at P4 neuroblasts were present in SVZ and rostral migratory stream. At P10 in the olfactory bulb, granular and periglomerular neurons were spotted. Ependymal cells were found in lateral ventricular wall and oligodendrocytes in the striatum and corpus callosum. Adult neural stem cells, type II astrocytes and transit-amplifying cells were confirmed. These cells were present up to P150 mice, confirming that radial glia act as neural progenitor cells during development, as well as neural stem cells in adult CNS [23].

During the early developmental stages of CNS, radial glia can be immunohistochemically marked (Figure 5) with antibodies against Vimentin, Nestin, WT1, Pax6, SOX2, HES1 and HES5, TN-C and N-cadherin, GFAP, GLAST, BLBP [24] [25]. While Vimentin and Nestin are more selective and specific for radial glia, WT1 stains other precursor cells. Glial fibrillary acidic protein (GFAP), astrocyte-specific glutamate transporter (GLAST) and BLBP are expressed in both radial glia and astrocytes. Nestin is expressed

both in NSC and radial glia [26], while intermediate filament proximal protein RC2 is unique antigen expressed only by radial glia [16].

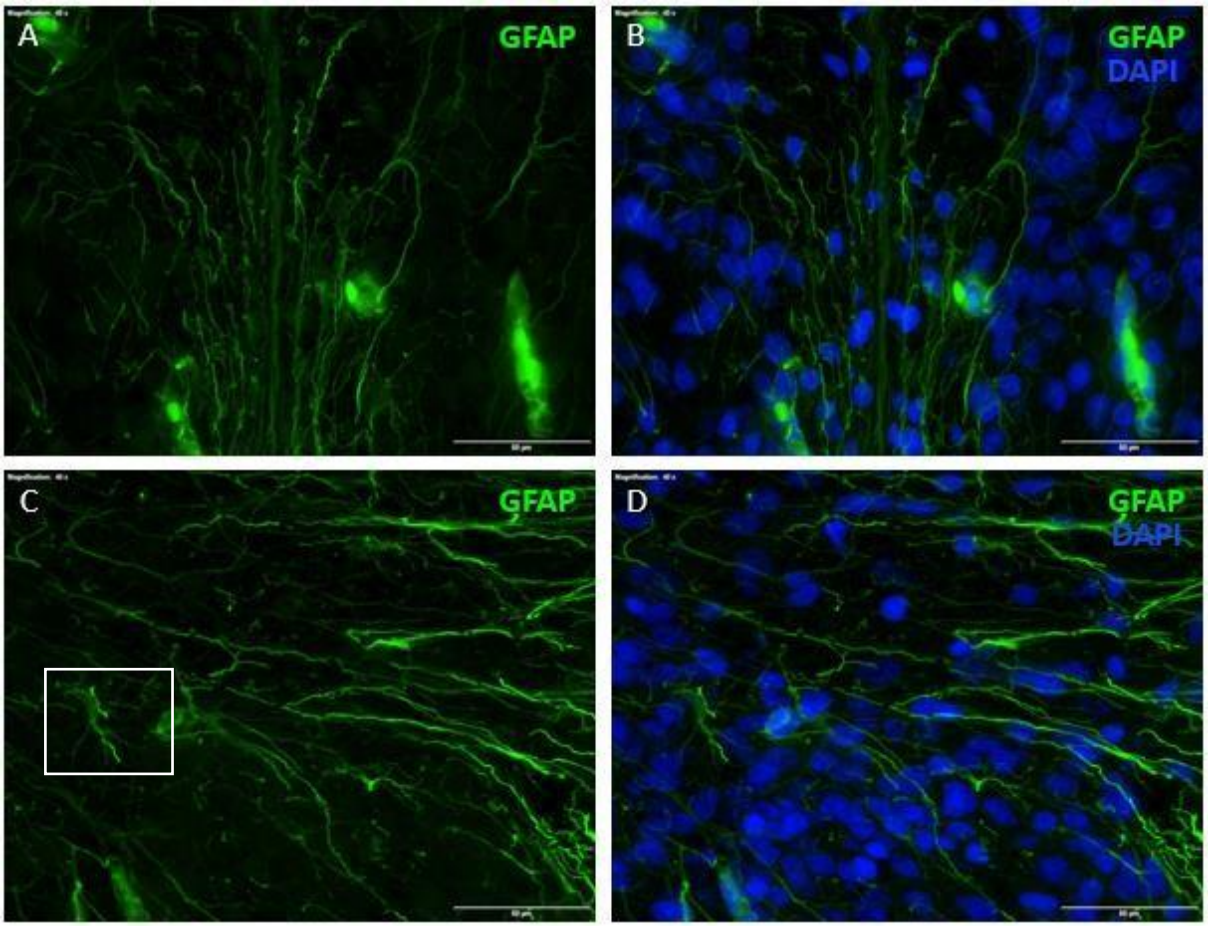

Figure 5. Immunohistochemistry of postnatal (P18) opossum spinal cord radial glia stained for GFAP (left column), GFAP and DAPI (right column). A-B Radial glia in ventral column of spinal cord. C-D Radial glia in lateral column of spinal cord. In both spinal cord locations, radial glia show bipolar morphology. Some cells show branching, implicating the developing astroglia (inset). Scale bar, 50μm. (Source: Laboratory for Molecular Neurobiology, neuroreg.uniri.hr).

5.2. NG2 CELLS

NG2 glia, synantocytes or polydendrocytes, were relatively recently discovered (in 1980s). They are CNS parenchymal cells, specific for expressing a single membrane-spanning chondroitin sulphate proteoglycan NG2 with large extracellular domain and short cytoplasmic tail [27].

Cells positive for NG2 are multipotent progenitors present in developing and mature CNS. Small stellate cells positive for NG2 are different from neurons and astrocytes. Fraction of those cells are positive for GFAP, but later in their development, they become negative for NG2. Other portion of the NG2-positive stellate cells have ganglioside receptors, also present in neurons, on which the tetanus toxin binds. These neurons lose the expression of NG2 once they are fully developed. Therefore, $NG2^+$ cells show properties of both neurons and glia, explaining the origin of their name [28].

NG2 glia from early postnatal rat optic nerves have shown potential to reprogram into multipotent stem cells. Cultivation of cells required fetal calf serum (FCS) or bone morphogenetic proteins (BMPs) and the resulting cells were population of type II astrocytes. Further treatment with basic fibroblast growth factor (bFGF) gave rise to neurospheres, which contained neural stem cells. Neurospheres may differentiate into neurons, astrocytes and oligodendrocytes [29].

NG2 cells express platelet-derived growth factor alpha (PDGFRα), as well as antigens on the surface, A2B5 and NG2 proteoglycan [25]. Localization of NG2 cells with antibodies has shown opulent NG2 distribution throughout the whole brain and spinal cord, which led to NG2 known as the "fifth neural cell type" [30] [27] [31] [32]. This claim put NG2 cells to separate category next to astrocytes and oligodendrocytes, microglia and neurons.

5.2.1. NG2 IN NEURAL NETWORKS

NG2 and astrocytes hold similarities. Morphologically, both cell types are stellate, but they diverge in structure and arborisation of their processes. NG2 cell processes are finer and branch closer to the body, while astrocyte have higher density of processes and more abundant collateral processes [8].

NG2 domains are similar to astrocyte domains. NG2 domains interconnect with astrocytic domains to communicate. They diverge in interactions with neurons, which suggests individual communication with neural networks [8]. For instance, astrocytes uptake glutamate at synapses, but NG2 do not because they lack glutamate transporters. On the other hand, both cell types express AMPA-type glutamate receptors [33]. Receptors recognize neural activity and result in elevation of intracellular calcium levels [8].

NG2 associate with neurons at axons, Ranvier nodes and near synapses [8]. NG2 glia in hippocampus obtain synaptic input from neurons [34]. Glutamatergic and GABAergic synaptic communication amid NG2 and neurons was found in cerebellum and cerebral cortex [35]. Additionally, in the corpus callosum were found physical synapses amid NG2 and unmyelinated axons [36].

5.2.2. NG2 AND GLIAL SCAR

Defensive mechanism of glial cells to CNS injury is to proliferate and form glial scar. Glial scar acts as biochemical and physical barrier between damaged and healthy tissue. Astrocytes form glial scar with help of other myelin-associated cells. Following the damage at lesion site, number of NG2 positive cells rapidly raises (Figure 6) [10].

In vitro studies have shown that neurons avoided surface that was coated in NG2 proteoglycan. The neurite outgrowth decreased when NG2

was present in the substrate [37]. This shows that NG2 contributes to the formation of the barrier that inhibits axonal regrowth.

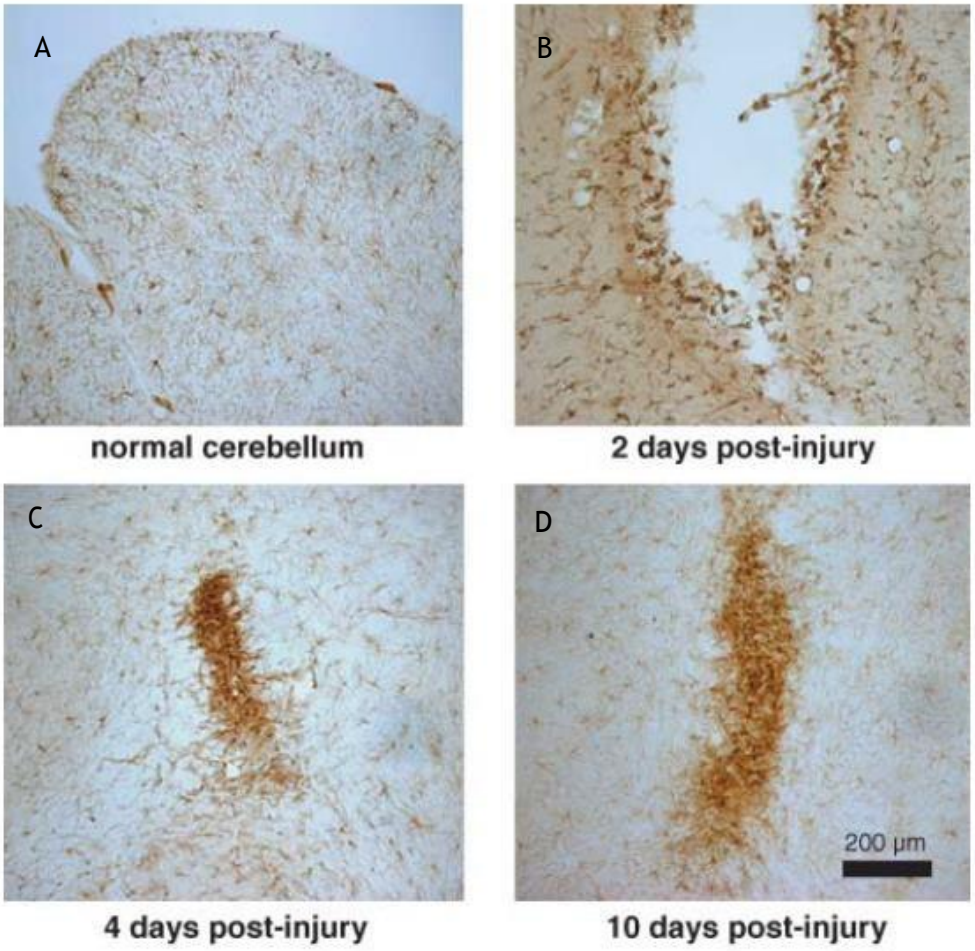

Figure 6. Following the injury to adult rat cerebellum tissue, NG2 cells arrive to lesion site. Tissue was labeled with anti-NG2 at different days. C-D show NG2 cell-formed glial scar (image taken and modified from [38]).

5.3. OLIGODENDROCYTES

Oligodendrocytes are CNS glial cells that produce myelin sheath that enwraps axons. Signal transduction is necessary for the proper function of the brain. Evolutionally, organisms have developed in different ways to increase the transduction of action potential and communication between neurons and effector organs. In invertebrates, the diameter of axons grew in more evolved species. Larger axons enable faster propagation of action potential. However, by increase of the brain size and quantity of neurons, larger neurons are not the most favorable option. This led to development of myelin sheath in vertebrates [2]. Oligodendrocytes spirally wrap the neuronal axon segments to provide structural stability and insulation. They are also called internodes because at each end of myelin segment are Ranvier nodes. The fatty layer, myelin sheath, allows faster velocity of nerve impulses and minimizes the axonal diameter in the CNS connective tracts [2].

Oligodendrocytes undergo specific stepwise morphological and functional changes from progenitors to myelinating oligodendrocytes. Specific markers have been identified for each maturation step (Figure 7). NG2 migrate to their final position according to signals such as PDGFRα, fibroblast growth factor (FGF), hepatocyte growth factor, chemotropic molecule netrins, secreted semaphorins, chemokine CXCL1, among others. Migration process is under control by extracellular matrix proteins and cell surface molecules [39]. When NG2 differentiate into oligodendrocytes, PDGFRα, A2B5 and NG2 proteoglycan get downregulated. Migrating NG2 and mature oligodendrocytes express Olig genes. Olig1 and Olig2 are basic helix-loop-helix (bHLH) transcription factors, known for oligodendrocyte specification, differentiation, maturation and myelination. Olig1 induces abundance of NG2 expressing cells [40]. Myelin-oligodendrocyte glycoprotein (MOG) is used to detect maturation of oligodendrocytes. It is expressed on oligodendrocyte surface 1-2 days after the rest of oligodendrocyte markers [41]. Myelin basic protein (MBP) also marks

mature oligodendrocytes. It is a structural compound of myelin, which is largely present at the beginning of myelination [42]. Furthermore, oligodendrocyte-specific protein (OSP) is also present in oligodendrocytes and opulent in myelin [43]. Finally, transcription factor group SoxE induces precursors to differentiate into oligodendrocytes. SoxE group involves Sox8, Sox9 and Sox10 transcription factors, where each has distinct role in oligodendrocyte fate. Sox9 is important for oligodendrocyte specification and Sox10 guides neural stem cells differentiation into oligodendrocytes and regulates expression of MBP and PDGFRα [44].

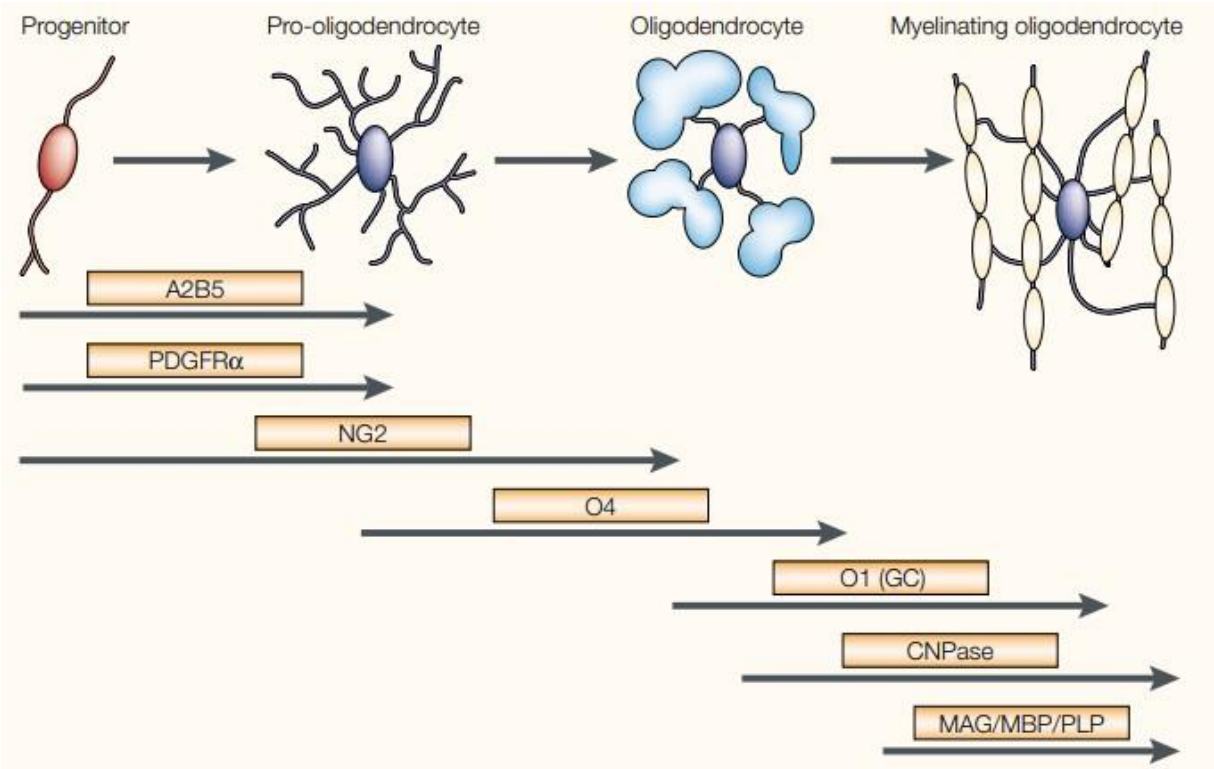

Figure 7. Oligodendrocyte markers during development (image taken from *[25]*).

5.4. ASTROCYTES

The second most abundant population of glial cells in the central nervous system, astrocytes, are highly heterogeneous cells and tightly integrated in neural networks. They sustain neurotransmission, metabolic, water and ion homeostasis and help to form and control the blood-brain barrier. They also contribute to sleep cycle regulation, female sexual maturity and other [2].

Astrocytes are present throughout the healthy CNS. Protoplasmic astrocytes are situated in grey matter and do not overlap between each other. Most of the distal processes interlock and form gap junctions [45]. The protoplasmic fine processes are evenly distributed in grey matter. Territorial domains of protoplasmic astrocytes are called neurovascular or gliovascular units depending on the type of cells that predominantly appear in. Both protoplasmic and fibrillary astrocyte processes are in contact with Ranvier nodes. Membranes of the astroglia are connected to neighboring capillaries through an astroglial perivascular process. Astrocytes initiate contact and thereby provide for morphological integration of neuronal elements within the neurovascular units [2].

In pathological conditions, astrocytes can become reactive. After an injury or distress of the CNS, astrocyte quantity increases greatly, while neuronal quantity recedes. This phenomenon is called astrogliosis and is accompanied by morphological changes that contribute to formation of scar tissue [46].

Heterogeneity in astroglial cell group is a result of their diversity and complexity, leading to different functions and potential for specialization. Astrocyte cell group, according to Parpura and Verkhratski, can be extended into fibrous, protoplasmic astrocytes, but also Müller glia, Bergmann glia, tanycytes, pituicytes, perivascular, marginal and velate astrocytes. Humans and hominid species have a few more unique astrocyte types. Moreover, they claim that radial glia, ependymal cells, choroid plexus cells and retinal

pigment epithelial cells also belong to astrocyte category (Figure 1A) [2]. Other researchers like Wolburg, Mack and Reichenbach oppose the claims, by categorizing ependymocytes, choroid plexus cells, tanycytes, Müller cells and retinal pigment epithelial cells as a glial subtype of ependymal cells. They support their opinion on morphology and contacts of the cell processes [47].

5.4.1. HUMAN- AND PRIMATE-SPECIFIC ASTROCYTES

Human cortex comprises of several subtypes of astroglia that are not present in rodents - interlaminar astrocytes, polarized astrocytes and varicose projection astrocytes [14].

Interlaminar astrocytes have spheroid cell bodies and very long unbranched processes, which end in the neuropil or on the capillaries. While function is still studied, research has shown that their long processes upkeep calcium waves in humans, integrate cortical column activity and serve as non-synaptic pathway for long-distanced signaling [48].

Polarized astrocytes are situated deep in the cortex. They have long, unbranched processes or processes with one branch. Cell morphology is similar to neurons, but they show extent GFAP labelling. They are unipolar and show bare synaptic contacts, serve as long-distance communication pathway across cortex [48].

Varicose projection astrocytes have shorter and straighter processes than protoplasmic astrocytes. They exhibit up to five long processes that evenly divide varicosities. Their function still needs to be revealed [49].

5.4.2. MOLECULAR MARKERS

Identification and characterization of astrocytes in healthy and pathological conditions is often done with immunohistochemical techniques. Astrocytes express GFAP (Figure 8), which belongs to group of intermediate filament proteins that assist to architectural functions of astrocytes. GFAP labels most reactive astrocytes at pathological lesions and is essential in astrogliosis [50]. However, GFAP does not label all non-reactive astrocytes present in healthy tissue [46]. It also does not distinguish the fibrous from protoplasmic astroglia and is not exclusive to astrocytes as it can be expressed in various tissues. GFAP labels main stem branches, but is not present in fine processes [51]. Immunohistochemical staining with GS and S100β is also a method of identifying astrocytes. S100β protein localizes in both the nucleus and the cytoplasm [52]. It is expressed in mature astrocytes which ensheath blood vessels, but also in ependymal cells [53]. GS is an enzyme that is present in astrocytes and used in glutamate to glutamine conversion. Sox9 marker has recently been used for identification of astrocytes. It is expressed in astrocyte nuclei in adult brain outside of the neurogenic regions [54]. Even though astrocytes can be labelled with various markers, not many of them are specific for astrocytes. Identification of astrocytes should be confirmed by multiple-staining method.

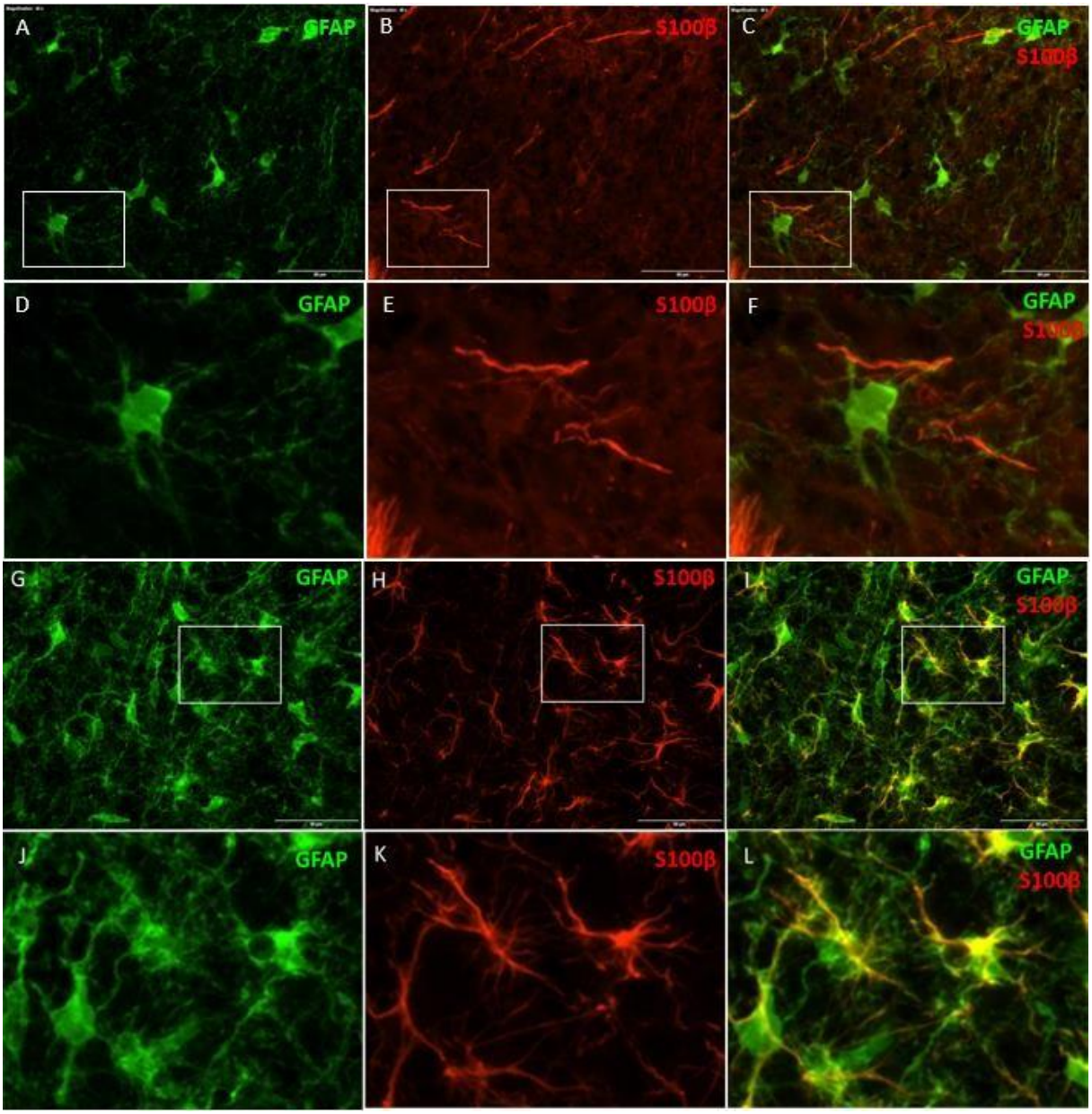

Figure 8. Immunohistochemistry of neonatal (P0) rat spinal cord astrocytes stained for GFAP (left column) and S100β (middle column). A-F In resting astrocytes these two markers don't overlap while in reactive phenotype (24 hours after dissection, G-L) GFAP expression is upregulated of and co-expressed with S100β. Scale bar, 50μm. (Source: Laboratory for Molecular Neurobiology, neuroreg.uniri.hr).

5.5. EPENDYMAL CELLS

Ependymal cells are considered as separate macroglia cell type derived from neuroectoderm. The choroid plexus cells, the ependymal cells and the parenchyma form cerebrospinal fluid (CSF) in the cerebral ventricles. The numerous cilia of ependymal cells spread into the ventricular cavities and move systematically to generate a CSF stream. Along the stream, they filter molecules and transport the debris. Ependymal cells form barrier to protect the CNS from harmful molecules in the CSF [7].

Radial glia cells are ependymal cell progenitors, which was confirmed by fluorescent tagging of radial cells. Double-labelling with radial glia marker GLAST and ependymal cell marker S100β at P2 and P6 has shown that nearly all cells at P2 expressed GLAST, while by P6 most expressed S100β, meaning radial glia differentiate into ependymal cells [7].

Vimentin, intermediate filament protein is expressed in fibroblasts, radial glia, astrocytes and ependymal cells. It is expressed earlier than GFAP in the cell development, which is why it is used as early glia differentiation marker. GFAP is also a marker of ependymal cells, as well as astroglia [55]. In ependymal cells, GFAP is lightly expressed, in contract to vivid expression in astrocytes. Ependymal cells can also be stained with S100β marker, which is primary astrocytic marker (Figure 9).

Ependymal cells and astrocytes are rich in water transport protein aquaporin 4. They are abundant in the latero-basal domains and lightly in the apical domain in the ependymal cells, while in parenchymal astrocytes they are located in the vascular feet [56]. The protein caveolin-1 is an endocytosis-linked protein present both in ependymal cells and in reactive astrocytes and it has similar roles. In ependymal cells, it reuptakes some molecules from CSF and in astrocytes it serves for endocytosis and sorting molecules [56].

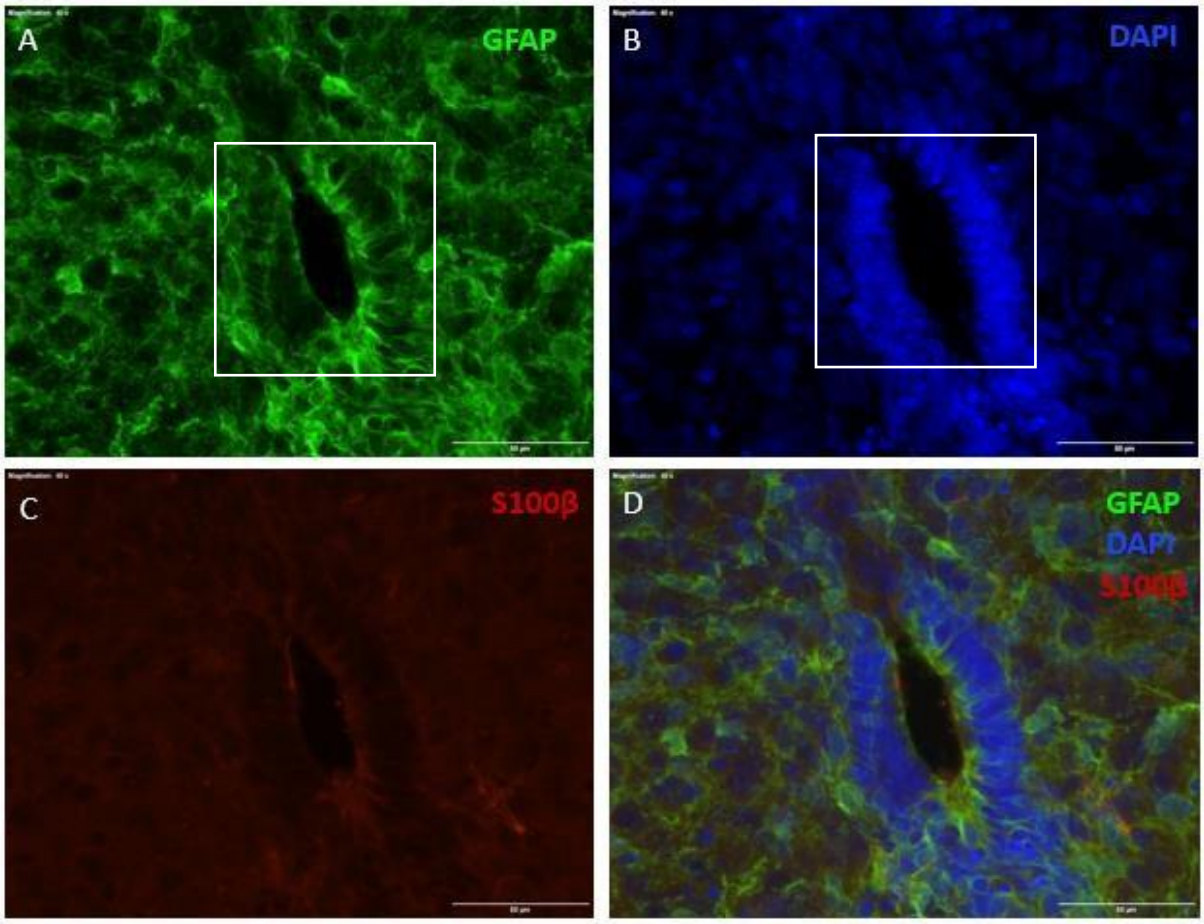

Figure 9. Immunohistochemistry of neonatal (P0) rat spinal cord central canal for ependymal cells stained with GFAP (A), DAPI (B), S100ß (C) and merged image with all markers (D). Ependymal cells show lighter GFAP expression than astrocytes. Comparing the inset from image A to number of cell nuclei stained in the same position (B), it is likely that ependymal cells are present greatly around the central canal. C shows that both astrocytes and ependymal cells can be stained with S100β. Scale bar, 50μm. (Source: Laboratory for Molecular Neurobiology, neuroreg.uniri.hr).

5.6. MICROGLIA

Microglial cells are derived from their progenitors situated in extra-embryonic yolk sac. They colonize neuroepithelium around E9.5. Embryonically-derived microglia proliferate during late gestation, post-natal development and in pathological conditions due to inflammation in adulthood (Figure 10) [57]. Microglia are part of the innate immune system and act as first responders to the CNS injury. They also have a role in plastic remodeling of neuronal tissue. Microglia release TNF-α, provide support to cells and assist synaptic repair. TNF-α signals lymphocytes and macrophages and promotes production of oligodendrocytes [58].

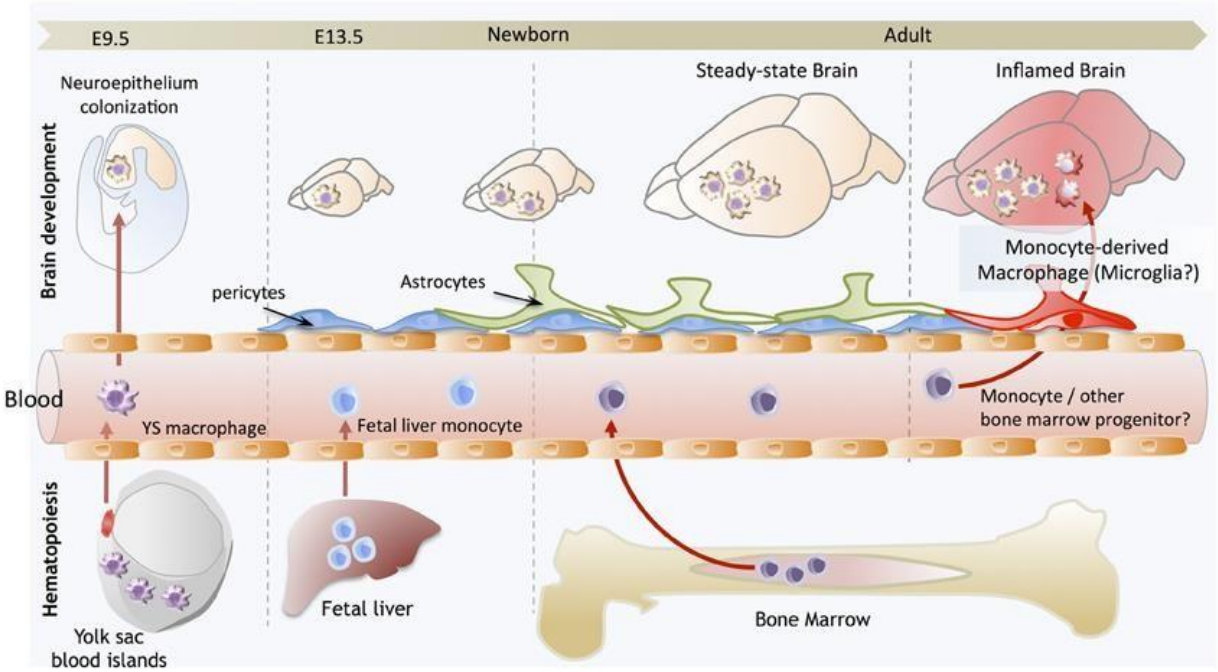

Figure 10. Microglia homeostasis and brain development (image taken from [57]).

Microglia at early developmental stages have amoeboid shape. They use blood vessels and white matter tracts to propagate through the CNS. After some point in development, microglia assume more branched morphology with compact bodies and fine processes [2].

Resting microglia in the healthy CNS have tiny soma and long and branched processes [59]. Their processes extend and cover the tissue looking for potential danger. They exchange information with glial cells and neurons by surface molecules expressed on the cells. These molecules on microglia are mostly receptors, which recognize the infection, but also bind ligands on healthy cell membranes. Ligands such as CD200 on neurons, astroglia and oligodendroglia, and CX3CL1 and CD47 on neurons bind to microglial receptors and keep them quiescent. Receptor for colony stimulating factor1 (CSFR1) is also expressed on microglia membrane and is important for cell development and survival [60].

When Toll-like receptors (TLR), scavenger receptors and other cytokine and chemokine receptors detect infection, ischemia, trauma or impaired homeostasis, microglia retract their processes, increase mobility and undergo activation. Activation makes them become amoeboid, like at early developmental stages, and release protective and/or proinfammatory factors. They recruit other immune cells and microglia at the lesion site. Microglia can proliferate in pathological conditions. They perform phagocytosis and clear out microbes, injured cells and debris. Moreover, they present antigens to T lymphocytes and evoke adaptive immune responses [61] [62] [2] [63].

Microglia identification is still challenging because they express antigens that other cell types express as well. Microglia and macrophages both express CD11b, CD68; lymphocytes and microglia LCA, LN-1 and microglia and oligodendrocytes both express GD3 [64]. Marker that is specific for microglia, yet its function remains to be explored, is transmembrane protein 119 (Tmem119). This marker is not expressed on macrophages and other immune or neural cells [65]. Furthermore, microglia are very often identified by staining with calcium binding adaptor molecule 1, Iba1 (Figure 11) [66]. It is expressed in microglia and macrophages and has functional role in membrane ruffling and the phagocytosis [67].

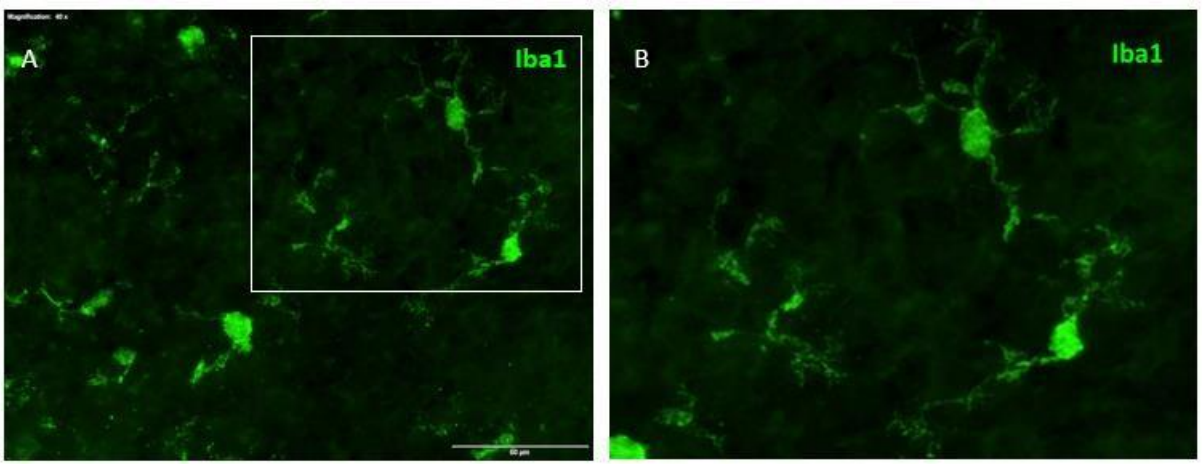

Figure 11. Immunohistochemistry of postnatal (P19) opossum cortex microglia stained for Iba1. Image B shows enlarged section of inset in image A. It is visible that microglia is not in one focal line, proving that microglia surrounds neighboring cells communicating and scavenging for distress. Scale bar, 50μm. (Source: Laboratory for Molecular Neurobiology, neuroreg.uniri.hr).

6. DISCUSSION

Neuroscience is a multidisciplinary scientific field that studies the nervous system. It aims to discover the functions of the nervous system, including how information is spread and integrated to execute complex cognitive and motor tasks, memory, perception, consciousness, behavior and learning. The huge advances in technology made possible to examine the cell structure and morphology, such as the invention of the super-resolution microscope and development of more sophisticated staining techniques. Neuronal networks and synapses were revealed, as well as the action potential that allowed fast spreading of the information.

Even though neurons and glial cells were described at the early 19th century, physiological study of neuroglia began 50 years later. As the neuroglia were considered to be acellular connective tissue of the brain, they were overshadowed by neurons and left out from the majority of researches until it was discovered that glia gave nervous system its form and held it together. More focus was directed to glia when the researchers started unveiling that glia have important roles in healthy brain function, synapses, behavior and in neurological disorders.

Glia classification presents a challenge and opposing views come from different researchers. In the first half of 20th century, neuroglia were divided into astroglia, oligodendroglia and microglia. In 1980s another glial subtype, the NG2 glia have been proposed [8]. NG2 glia have multiple roles in the CNS: they receive synaptic inputs from neurons, form glial scar and inhibit axonal growth and remyelinate axons (similar to astrocytes). During gliogenesis, NG2 cells develop into immature pro-oligodendrocytes that have numerous processes. They further develop into mature oligodendrocytes that have membrane sheaths and finally into myelinating oligodendrocytes, which is the reason some researchers put NG2 glia in the category of oligodendrocytes. Each oligodendrocyte maturation step has specific markers, which determine their development. However, terms like

NG2, OPC and pro-oligodendrocyte get associated as well as mature non-myelinating and myelinating oligodendrocytes. Therefore, the unambiguous distinction between NG2 and oligodendrocytes (and/or their precursors) is still missing. However, apart from being oligodendrocyte precursor cells, NG2 glia have shown potential to reprogram and become multipotent stem cells (similar to radial glia). NG2 can differentiate into type II astrocytes and oligodendrocytes in postnatal rat optic nerve [29]. When treated with bFGF they can become neurospheres, which can, in addition to glia, produce neurons [29].

Furthermore, astroglia have shown that they are extremely heterogeneous and in different CNS regions. They adjust to environment, express different molecules and preform many diverse functions. Most researches examine fibrous and protoplasmic astrocytes, but there are plenty of other subtypes of astroglia, whose function is not studied enough. Additionally, primates have unique astroglia subtypes, but their function remains unexplored and sets questions of functional and regional heterogeneity of astrocytes in humans.

Ependymal cells participate in CSF formation and flow, protection and homeostasis of CNS. They have been regarded as astrocytes for their plentiful similarities. Both cell types can be labeled with the same markers – GFAP, vimentin and S100β. Water transporter aquaporin 4 and endocytosis protein caveolin-1 are expressed in both cells and have similar functions. But over and above, ependymal cells and astrocytes show various differences in morphology and function, proving that identification on the sole morphology level tends to be unreliable.

Radial glia is another example of marker redundancy: common markers exist with neuroepithelial cells, as well as astrocytes; such as GFAP, GLAST and BLBP in astrocytes and Nestin in neuroepithelial cells. Radial glia used to be referred to as a "scaffold" for neuronal migration, but later it became evident that they in parallel generate neurons. Furthermore, some

researchers still consider radial glia is an astrocyte subtype, while it is clear that radial glia is a source for both neuronal and glial lineages.

Oligodendrocytes happen to be one of the better-characterized glial cells, but not all identification markers are oligodendrocyte-exclusive. Sox9, a transcription factor vital for oligodendrocyte specification [44] was recently used as an exclusive marker for astrocytes in adult mouse brain except for ependymal cells and adult neurogenic niches, where SOX9 is also expressed by NSC [54].

Identification markers for microglia are not that specific as well. CD11b, CD68 and Iba1 stain macrophages and GD3 stains oligodendrocytes. Microglia also have some similarities with astrocytes. They both have some similar functions in synaptic pruning and releasing the same factors, such as brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF) and insulin-like growth factor (IGF-1) [68].

Apart for not being specific and labelling different types of glia, markers can cross-react with similar antigens and bind non-specifically, bringing interference and additional identification distress.

7. CONCLUSION

Glial cells are very diverse contributors to retaining the function of the CNS in healthy and pathological conditions. Glia can be divided by their origin into macroglia and microglia. Macroglia can further be split into astrocytes and oligodendrocytes. Other neuroglia types, ependymal cells and NG2 are associated with astrocytes or oligodendrocytes.

The main basis of glial identification is immunostaining with markers for distinct molecules expressed by cells. However, many similar overlaps in marker expression can be found between glia. Labeling more glia types brings possibilities of misinterpretation, as well as nonspecific binding. Furthermore, most markers bind regionally, not labeling the whole cell. In addition, some markers are differently expressed during development and maturation. Ependymal cells can be labeled with astroglia markers, indorsing the belief they are subtype of astrocytes. But taking into consideration the function and morphology, ependymal cells undoubtedly differ from astrocytes. NG2 cells have been reflected as oligodendrocyte precursor cells. However, they have proven the ability to differentiate into other glia and neurons suggesting change of view. Microglia have some similar functions to astrocytes, yet they have completely different origin pathways.

Apart from identification, the definition and classification of neuroglia also remains superficial. While some glia subtypes are well researched, there are subtypes that have barely been looked into. Research has mostly been conducted on rodents, but this still brings up questions about humans. Primate cortex has higher heterogeneity of astrocytes than rodents'. Apart from being larger in size, there are unique astrocyte subtypes whose function is not known at all.

Although neuroglia have different morphology, biochemistry and functions, they act together and maintain homeostasis of the CNS. They each contribute in their specific way and more focus should be aimed

towards glia to understand how they interact with each other and how they interact with neurons. Cell identification should avoid being solely on the expressed molecules or by their function or development. New tools such as genetic screening should be considered for exploring the glia evolution. Furthermore, functional heterogeneity of cells could be identified with discovery of developmental gene regulatory pathways. Also, new noninvasive techniques in myelin water imaging (MWI) may be used to better characterize and monitor myelin. Genome, proteome and metabolome approaches could also help to differentiate glia subtypes.

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europass Curriculum vitae

PERSONAL INFORMATION

Marta Pongrac
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EDUCATION AND TRAINING

06/09/2004–21/06/2012

Primary school diploma

EQF level 1

Third elementary school, Čakovec (Croatia)

03/09/2012–2016

High school diploma

EQF level 4.2

Grammar school Josip Slavenski, Čakovec (Croatia)

Natural sciences and mathematics direction

03/10/2016–Present

Undergraduate study of biotechnology and drug research

University of Rijeka, Department od biotechnology, Rijeka (Croatia)

05/08/2019–08/08/2019

Biotech summer school

3 ECTS

Vrije University Brussel, Brussel (Belgium)

Lessons in Biotechnological potential of microorganisms (with workshops Synbio engineering of flourescent bacteria and Mycelium materials using filamentous fungi), Proteins and structure-function relationship (with workshops Pymol visualization of protein structure, X-ray diffration), Nanobodies (with workshops Cancer research and Nanobodies in parasitology research).

WORK EXPERIENCE

15/10/2018–15/04/2019

Promoter of natural cosmetics and dietary supplements for Sensapharm company

Sensapharm, Zagreb (Croatia)

PERSONAL SKILLS

Mother tongue(s): Croatian

Foreign language(s)

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken interaction	Spoken production	
English	B2	B2	B2	B2	B2
German	A2	A2	A1	A1	A2

Levels: A1 and A2: Basic user - B1 and B2: Independent user - C1 and C2: Proficient user
Common European Framework of Reference for Languages

Digital skills

SELF-ASSESSMENT				
Information processing	Communication	Content creation	Safety	Problem-solving
Proficient user	Independent user	Proficient user	Independent user	Independent user

Digital skills - Self-assessment grid

MS Office Software

- Word, Excel, PowerPoint, Outlook

Windows 8.1

Programming

- C++, C

Computional biochemistry (bioinformatics)

-Avogadro, GAMESS, Chimera, MacMolPlt, KinTek, PyMol, VMD

Other skills

-10 years of active learning of English language in the Čakovec Language School

-3 years of playing keyboards

- first aid course

-actively engaged in sports (cheerleading, dancing, swimming, fitness, alpinism, skiing, gymnastics)

Driving licence B

ADDITIONAL INFORMATION

Memberships

I am an active member of the Student Association of Biotechnology at the University of Rijeka, UsbRi, within which I lead the project and volunteer.

Volonterski rad

I volunteer as a member of the Student Association of Biotechnology in Rijeka on the following projects:

-Putujući znanstvenici (Travelling scientists), October 2017 - today

-Demonstration of experiments aimed to children of kindergarten and elementary school age in order to popularize science.

-Taking part in multiple classes and different schools and on organized events that bring children together.

-Kuglice dobrih želja (Good desire ornaments), December 2017

-Making Christmas tree ornaments that were sold for charity purposes.

-SOBRi (Deparment of biotechnology) Summer School, September 26 - 28, 2018

- Teaching freshman students of proper working laboratory skills such as pipetting with an automatic pipette, weighing on analytical scales, calibration of glass laboratory pots and using them in experiments.

- Solving problems that included dilution calculations, redox equations, chemical equations ...

-NatuRIs, 31.12.2017. - today

-Preparing products of natural cosmetics and educating participants as part of the project on the following events: Open Day of the Department of Biotechnology 2018, 2019, Workshop on Natural Cosmetics as part of the Scientific Corner of the Student Day Festival 2018, Event for the freshman students of the Department of Biotechnology, Conference on the Future and Prospects of the Department of Biotechnology 2018, Primary schools in Rijeka, "Baltazar na Gradini", Bilingual scientific camp...

-Student-mentor, October 2017 - today

-Helping two students as a student-mentor in mastering the materials in order to perform their responsibilities successfully during the studies.

-I volunteered the Day of the Department of Biotechnology in April 2017

-Demonstrated several experiments for students of elementary and high school age and other visitors of the event.

I volunteered on an expert-scientific interdisciplinary convention of "STEM in the Educational System: TODAY FOR THE TOMORROW", October 22, 2018.

- I have led active exhibitors and passive participants, I shared accreditations and acknowledgments about attending and listened to lectures.

Sudjelovanja u projektima, konferencijama i slično

Participated in the Case study competition "Realizer" in 2017, organized by the Foundation of the University of Rijeka and StepRi (The Science and Technology Park of the University of Rijeka).

-In the framework of the competition I led a team of three more members and we worked on the project "Ophthalmic Portfolio JGL (Jadran Galenski Laboratory)". The topic was to elaborate and write the plan of product emplacement of line of artificial tear products "Vizol S" to the markets of Germany and Poland.

-We presented the plan as a pitch presentation in front of the expert commission.

Attended a two-day school "Basic Scientific Research" on March 2-3, 2018 and February 15-16 2019, organized by the Office of Science of the Student Assembly of the University of Rijeka.

-Two-day school covered the topics about the proper structure of knowledgeable research papers, search for reliable sources of information and instructions on how to write a quality research paper.

Participated in the "Coca-Cola Youth Support Program", organized by "Coca Cola HBC Croatia".

- The program included three-day workshops on financial literacy, writing and project management, financial plan ...

Regularly attending numerous conferences organized by the Department of Biotechnology, the Student College of the University of Rijeka, scholarship fairs, introductory lectures, conferences ...

Attended a professional trip of the Department of Biotechnology in Naples, 23-25 September 2018. We visited the "Anton Dohrn Institute, Stazione Zoologica", which deals with the research in interdisciplinary fields of molecular and cell biology, biochemistry, oceanography ... We have had several lectures there and visited their labs and found out about their research and work they are dealing with.

Attended the National student body congress in Rijeka, April 26-27.

-The congress had topics of Quality assurance in higher education, student policy and regulation, as well as good practice in student representation.

STEM games 2019. in Poreč, May 7-12.

-Science Arena case study competiton, 3rd place.

Attended a professional trip of the Department of Biotechnology in Piran Marine Biology Station, National Institute of Biology Slovenia, 28-30 june, 2019. Guided visit to their laboratories and learning about their research.

Projects

As the part of the Student Association of Biotechnology in Rijeka I am the main organizer of the project NatuRIs, 31.12.2017. - today.

-The project involves the making of natural cosmetics products, short education on the ingredients used and discussion.

-We attend various events in our region, schools, etc.

The aim of the project is to teach children, young people and other citizens with the making, ingredients and application of cosmetics from completely natural ingredients without preservatives.