Novel Mechanisms to Modulate Microglial Functional Phenotypes in Health and Disease

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Novel Mechanisms to Modulate Microglial Functional Phenotypes in Health and Disease

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Affidavit

I declare that the presented dissertation has been written independently and with no other sources and aids than quoted.

Berlin, May 20th, 2020

To the kindest and most supportive person in my life, my mother
Haifaa El Dabbagh

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Contents

Publications 7

Abstract 8

Zusammenfassung 10

1 Introduction 12

1.1 Microglia play critical role in maintaining brain homeostasis 13

1.1.1 Electrophysiology of microglia 14

1.1.2 Microglial functional phenotypes 16

1.1.3 Purinergic signalling in microglia 20

1.2 The VGF-derived peptides as possible modulators of microglial functional phenotypes 22

1.2.1 VGF and its biologically active derived peptides 23

1.2.2 TLQP21 nomenclature, receptors and functions 26

1.3 Neurofibromatosis type 1 disease and microglia 28

1.3.1 Neurofibromin 1 and neurofibromin 1 gene deletion 28

1.3.2 Neurofibromin 1 mutations and microglia 30

1.4 Extracellular components and microglia 31

1.4.1 Role of tenascin C in brain 31

1.4.2 Tenascin C and microglia 33

2 Aim of the study 34

3 Project I: The VGF-derived peptide TLQP21 impairs purinergic control of chemotaxis and phagocytosis in mouse microglia 36

3.1 Summary 37

3.2 Original publication 37

3.3 Supplementary Figures 50

3.4 Limitation 52

3.5 Follow up experiments and outlook 52

3.5.1 Elucidating the role of VGF in Alzheimer's disease through microglia 52

3.5.2 Elucidating the role of VGF/TLQP21 in glioblastoma multiforme 56

4 Project II: Neurofibromatosis type 1 - mutant microglia exhibit a sexually-dimorphic cAMP-dependent impairment of purinergic function 59

4.1 Summary 60

4.2 Original publication 60

4.3 Supplementary Figures 74

4.4 Follow up experiments and outlook 78

5 Project III: Tenascin C regulates multiple microglial functions involving TLR4 signaling and HDAC1 79

5.1 Original publication 79

5.2 Supplementary Figures 94

6 Discussion 102

6.1 Effect of the VGF-derived peptide TLQP21 on microglia 103

6.1.1 The C3aR1-dependant effect of TLQP21 on microglia 104

6.1.2 TLQP21 impairs purinergic signaling in microglia 105

6.2 Male neurofibromatosis 1 +/- microglia exhibit sexually dimorphic cyclic AMP-associated defects 106

6.3 Tenascin C regulates multiple functions of microglia 108

7 Conclusion 111

8 Bibliography 112

9 List of Figures 123

10 List of Abbreviations 124

11 Curriculum Vitae 126

12 Doctorate credits from MDC-Berlin PhD office 128

Publications

This cumulative dissertation is based on the following publications:

- **Elmadany, N.**, Logiacco, F., Buonfiglioli, A., Haage, U., Wright-Jin, E., Schattenberg, A., Papawassiliou, R., Kettenmann, H., Semtner, M., and Gutmann, D., 2020. Neurofibromatosis type 1 - mutant microglia exhibit a sexually-dimorphic cAMP-dependent impairment of purinergic function. Neurobiology of disease, 2020 Jul 28:105030.
- **Elmadany, N.**, Sassi, F., Wendt, S., Logiacco, F., Visser, F., Haage, V., Hernandez, DP., Mertins, P., Hambardzumyan, D., Wolf, S., Kettenmann, H., and Semtner, M., 2020. The VGF-derived peptide TLQP21 impairs purinergic control of chemotaxis and phagocytosis in mouse microglia. The Journal of Neuroscience, 2020 Apr 22;40(17):3320-3331.
- Haage, V., **Elmadany, N.**, Roll, L., Faissner, A., Gutmann, D.H., Semtner, M., and Kettenmann, H., 2019. Tenascin C regulates multiple microglial functions involving TLR4 signaling and HDAC1. Brain, behavior, and immunity, 2019 Oct;81:470-483.

Other publications:

- Gharaibeh, L., **Elmadany, N.**, Alwosaibai, K., and Alshaer, W., 2020. Notch 1 in cancer therapy: possible clinical implications and challenges. MOLPHARM-MR-2020-000006. Accepted.
- **Elmadany, N.**, Khalil, E., Vaccari, L., Birarda, G., Yousef, I., and Abu-Dahab, R., 2018. Antiproliferative activity of the combination of doxorubicin/quercetin on MCF7 breast cancer cell line: A combined study using colorimetric assay and synchrotron infrared microspectroscopy. Infrared Physics & Technology, 95:141-147.
- Ali, M.A., Yousef, A.M.F., **Elmadany, N.N.**, Nailya, R., Bulatova, N.M.A., Yousef, M.A., and Al Majdalawi, K.Z., 2016. Influence of genotype and haplotype of MDR1 (C3435T, G2677A/T, C1236T) on the incidence of breast cancer-a case-control study in Jordan. Asian Pacific Journal of Cancer Prevention, 2016, 17(1):261-266.

Abstract

Microglia are resident innate immune cells that maintain homeostasis and sense a range of pathophysiological impairments within the central nervous system (CNS). Based on their involvement in brain disease progression, microglia-targeted therapy has emerged as a potential approach for reducing the burden of CNS disease. Microglia activation requires the activation of different signal pathways via neurotransmitters, neuropeptides and/ or other extracellular molecules, which each can be targeted. The neuropeptide VGF (non-acronym) is secreted by neurons and hydrolysed into biologically active peptides. One of these peptides is TLQP21, which binds to the complement receptors C1qbp and C3aR1. Although both receptors are expressed in microglia, the effect of TLQP21 on microglial cells has not been elucidated. The goal of the first study in the present dissertation was to determine whether TLQP21 might be a useful target in modulating microglial function. I demonstrate that exogenous application of TLQP21 stimulates microglial outward rectifying potassium (K^+) currents, intracellular calcium (Ca^{2+}) release, phagocytosis, and migration in C3aR-dependent manner. Interestingly, C3aR1 receptors were only expressed in microglia *in vitro*, but not *in situ*; suggesting that their expression might vary between different microglial activation states. Furthermore, *in vitro* and *in situ* activation of C1qbp leads to TLQP21 interference with metabotropic purinergic signalling (P2Y) in microglia, thereby, decreasing P2Y12-mediated activation of K^+ conductance, microglia migration and laser lesion-induced processes outgrowth, as well as P2Y6-mediated phagocytic activity.

Next, I examined other possible P2Y regulators in microglia. Neurofibromin is a known downstream effector of tyrosine kinase receptors and G protein-coupled receptors that indirectly regulates cyclic AMP (cAMP) levels through purinergic receptors. Mutations on this protein results in Neurofibromatosis type 1 disease (NF1). In the second study, a mouse model of NF1 with heterozygous neurofibromin knockout (*Nf1 +/-*) was employed. Interestingly, only male *Nf1 +/-* microglia showed impaired ATP-induced P2Y-mediated membrane currents and P2Y-dependent laser lesion-induced process movement *in situ*. Moreover, I found that the P2Y-control of microglial phagocytosis was only affected in male *Nf1 +/-* mice. In contrast, basal phagocytic activity was reduced in both male and female *Nf1 +/-* mice. Studying the neurofibromin downstream signaling intermediate, cAMP, revealed that male *Nf1 +/-* mice exhibited defects in cAMP regulation. Pharmacological blockade of phosphodiesterase III enzyme rescued these defects.

The extracellular matrix (ECM) contains other factors that likely regulate the activity of microglia. In my third project, I studied Tenascin C (TNC), an ECM glycoprotein that activates Toll-like receptor 4 (Tlr4) expressed in microglia using TNC KO and Tlr4 KO mice. I found that TNC regulates microglial phagocytic activity *in situ* at an early postnatal age (P4) partially via Tlr4 activation. Furthermore, TNC regulates pro-inflammatory cytokine/chemokine production, chemotaxis and phagocytosis in Tlr4-dependent manner *in vitro*. Interestingly, the effect of TNC on microglia was linked to the expression of histone-deacetylase 1 (HDAC1) in microglia, which itself was induced by TNC. The use of MS-275 (HDAC1 inhibitor) attenuated TNC-induced microglia proinflammatory cytokines.

In summary, the present dissertation demonstrates that TLQP21, NF1, and TNC are critical modulators of microglial function, suggesting that they might serve as promising targets to correct microglial dysfunction in the setting of CNS disease.

Zusammenfassung

Als residente angeborene Immunzellen des ZNS sind Mikroglia wichtig für die Gewebehomöostase und erkennen jede Art von pathologischer Dysfunktion. Die zielgerichtete Mikroglia-Therapie hat sich zu einem viel versprechenden Ansatz entwickelt. Für die Aktivierung der Mikroglia über extrazelluläre Moleküle wie Pathogene, Neurotransmitter oder Neuropeptide sind verschiedene Signalwege erforderlich. Das Neuropeptid VGF wird von Neuronen sezerniert und im Golgi-Apparat zu biologisch aktiven Peptiden hydrolysiert. TLQP21 ist ein von VGF abgeleitetes Peptid, das mit metabolischen und neurologischen Störungen assoziiert ist, und die Komplementrezeptoren C1qbp und C3aR bindet, die von Mikrogliazellen exprimiert werden. Die Wirkung von TLQP21 auf Mikroglia ist jedoch noch unbekannt. In der vorgestellten Dissertation demonstriere ich, dass die exogene Anwendung von TLQP21 auswärts rektifizierende Kalium(K^+)-Ströme, intrazelluläre Calcium(Ca^{2+})-Freisetzung, Phagozytose und Migration in Abhängigkeit von C3aR stimuliert. C3aRs werden von Mikroglia nur *in vitro* was darauf hindeutet, dass ihre Expression zwischen verschiedenen mikroglialen Zuständen variieren. Durch die Aktivierung von C1qbp *in vitro* und *in situ* interferierte TLQP21 mit den metabotropen purinergen Signalen (P2Y) in Mikroglia und verringerte die P2Y12-vermittelte Aktivierung der K^+-Leitfähigkeit, die Migration, die durch Laserläsion Prozessauswüchse sowie die P2Y6-vermittelte phagozytische Aktivität induzierten.

Um mögliche Regulatoren von P2Y in Mikroglia in einem Krankheitskontext zu untersuchen, verwendete ich in einem anderen Projekt ein transgenes Mausmodell für Neurofibromatose Typ 1 (*Nf1*+/-). Interessanterweise zeigten männliche *Nf1*+/- -Mikroglia eine Verringerung der ATP-induzierten P2Y-vermittelten Membranströme und P2Y-abhängige Laserläsion-induzierte Akkumulation mikroglialer Prozesse *in situ*, die weiblichen jedoch nicht. Darüber hinaus war die P2Y-Kontrolle der mikroglialen Phagozytose nur bei männlichen *Nf1*+/- Mäusen betroffen. Allerdings war die basale Phagozytoseaktivität sowohl bei männlichen als auch bei weiblichen *Nf1*+/- Mäusen reduziert. Durch Untersuchung des nachgeschalteten Botenstoffs, des zyklischen AMPs (cAMP), es wurde herausgefunden, dass männliche *Nf1*+/- Mäuse einen Defekt in der cAMP-Regulation aufweisen. Eine pharmakologische Blockade der Phosphodiesterase korrigierte die männlichen Nf1+/- Mikroglia-cAMP-Defekte.

Als nächsten Schritt wandte ich mich der Frage zu, wie die extrazelluläre Matrix (ECM) Mikroglia beeinflussen könnte. TNC ist ein ECM-Glykoprotein, das Toll-like-Rezeptor 4 (TLR4) aktiviert. Es wurden TNC KO und TLR4 KO Mausmodelle verwendet, aus denen entweder primäre Mikrogliakulturen oder akute Hirnschnitte generiert wurden, um die Unterscheide der mikroglialen Aktivitäten zu studieren. TNC reguliert die phagozytäre Aktivität der Mikroglia *in situ* in einem frühen postnatalen Alter (P4) teilweise über die TLR4-Aktivierung. Die proinflammatorische Zytokin-/Chemokin-Produktion, Chemotaxis und *in-vitro*-Phagozytose wird hingegen in TLR4-abhängiger Weise reguliert. Darüber hinaus ist die Wirkung von TNC auf Mikroglia mit der Expression der Histon-Deacetylase 1 (HDAC1) in Mikroglia verbunden. Durch Verwendung des HDAC1-Inhibitors MS-275 konnte die TNC-induzierte Freisetzung von pro-inflammatorischen Zytokinen in Mikrogliazellen reduziert werden.

Zusammengenommen zeige ich hier, dass TLQP21, NF1 und TNC Modulatoren der mikroglialen Funktionen sind und daher vielversprechende Ziele zur Korrektur mikroglialer Funktionsstörungen in Pathologien darstellen können.

1 Introduction

Historically, the brain has been considered as an immune-privileged organ. This concept is evolving with the wide array of research highlighting the activity of microglia in the brain, and the infiltration of peripheral immune cells in brain diseases. Microglial cells were first described by Pio del Rio-Hortega (Figure 1) in a landmark publication “Cytology and Cellular Pathology of the Nervous System” (del Rio-Hortega, 1932). Thereafter, several studies demonstrated the role of microglia in shaping CNS, maintaining homeostasis, as well as sensing and responding to any pathological stimuli (Kettenmann et al., 2011, Prinz et al., 2019). In addition, the contribution of microglia to the disease progression was demonstrated; such in neurodegenerative diseases (Hickman et al., 2018, Bachiller et al., 2018), and brain tumors (Roesch et al., 2018, Gutmann and Kettenmann, 2019). Consequently, targeting microglia has emerged as a promising immunotherapeutic approach.

The present thesis covers molecular aspects on mouse microglia that might have therapeutic potential. The addressed microglial targets are either receptors or intracellular signal pathways that were modulated within the course of the present dissertation, and accordingly led to changes in microglial functions. Furthermore, pharmacological blockage of certain targets was implied to confirm our findings and elaborate the mechanisms.

In the following subsections, microglial functional phenotypes, the involved signal pathways, the compounds used as modulators of microglia, as well as the explored disease model are introduced.

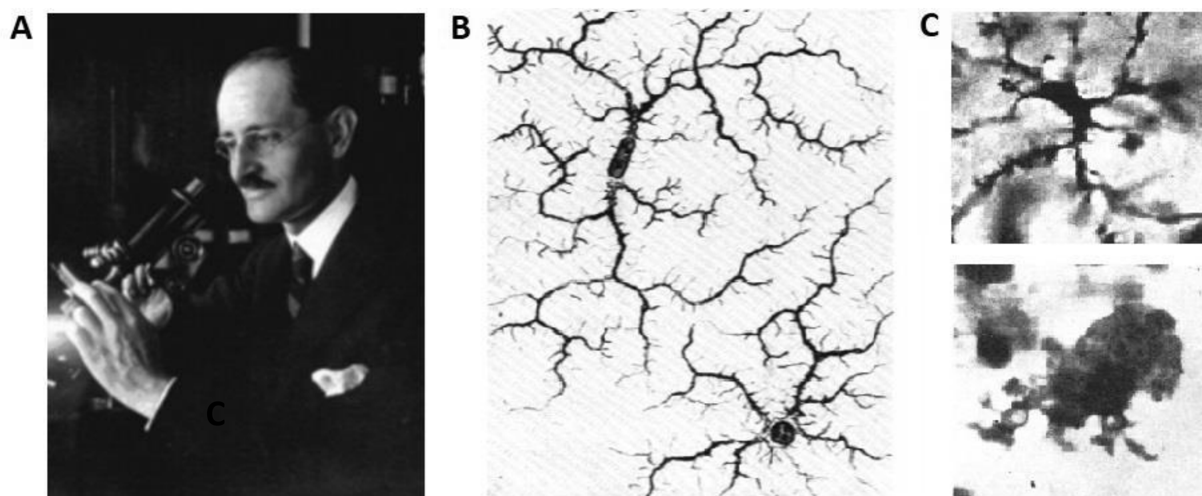

Figure 1. Discovery of microglia. (**A**) Pio del-Rio Hortega (1882-1945). (**B**) One of the first sketches of microglial cells drawn by Hortega. (**C**) Morphology of microglia as ramified (top) and amoeboid (bottom) cells. Adapted from (Kettenmann et al., 2011).

1.1 Microglia play a critical role in maintaining brain homeostasis

Microglia are the tissue-resident innate immune cells in the CNS. They evolve from runt-related transcription factor 1 (Runx1)-positive myeloid progenitors that migrate from the yolk sac into the primitive brain during embryogenesis before embryonic day E8 (Figure 2). Microglial cells are first observed in mouse embryo from ~ E 10 (Kettenmann et al., 2011, Thion et al., 2018, Smolders et al., 2019). Since microglia are not considered hematopoietic cells, they are not replenished by circulating monocytes, rather persevered throughout adulthood with self-renewal capacity (Eyo and Wu, 2019). These cells represent 5-20% of total non-neuronal glial cells in the brain and display a wide range of morphological plasticity depending on their activation state, which ranges from ramified (highly-branched) to amoeboid (less-branched) cells with large soma (Low and Ginhoux, 2018). They were initially considered as the CNS-resident phagocytes; however, recent sophisticated genomics and functional data revealed a critical role of microglia in maintaining brain homeostasis beyond phagocytosis.

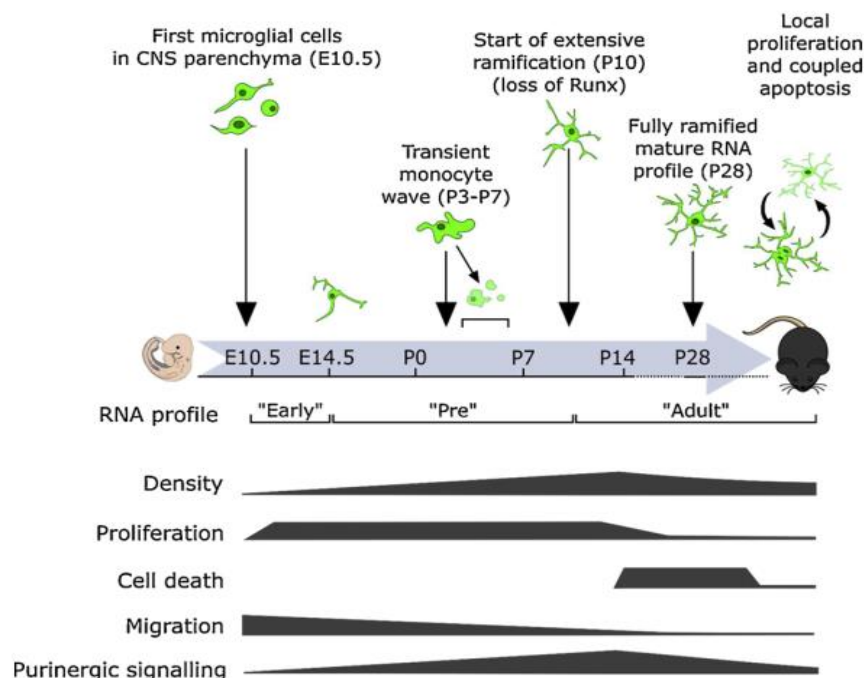

Figure 2. Microglia in mouse brain development. Microglia progenitors derived from the embryonic yolk sac as early as E9.5 and differentiated in the brain parenchyma into microglia at E10.5. These embryonic microglia colonize the whole CNS and maintain themselves via local proliferation. Based on the differential gene expression during development, microglia are defined as: early microglia (E10.5-E14), pre-microglia (E14-P9) and adult microglia (>P9), and characterized by more complex ramification with age. Purinergic signaling that controls important microglia functions including process movement and surveying process reaches threshold at early adulthood. Adapted from (Smolders et al., 2019). E: embryonic day, P: postnatal day.

1.1.1 Electrophysiology of microglia

Activation of cell surface receptors and ion channels changes microglial membrane voltage and subsequently shapes microglial functional phenotypes (Kettenmann et al., 2011). Most of the studies that described the membrane properties of microglial cells were performed *in vitro* on amoeboid cultured microglia. However, this model is not precise enough to study the different activation states and complex processes' ramification of microglia (Kettenmann et al., 2011). The utilization of transgenic mice with labelled microglia, has enabled the recognition of microglia in brain slices under more physiological condition *in situ*. A vivid instance of this, is MacGreen mouse line, in which a 3.5-kb enhanced green fluorescent protein (EGFP) reporter gene and the promoter of the colony stimulating factor-1 (CSF-1) receptor were inserted as a transgene (Chen et al., 2015). Since only microglia express CSF1 in healthy brain, they are specifically labelled and can be distinguished in brain slices from MacGreen mice. Thus, cell physiological techniques like patch-clamp, two-photon microscopy, and Ca^{2+} imaging have become feasible and provided more precise data *in vivo*, *in situ* and *ex vivo* (Izquierdo et al., 2019). In the present studies, I investigated the electrophysiological properties of microglia *in vitro* as well as *in situ* using MacGreen mice.

Ion channels and membrane potential of microglia. The patch-clamp technique, which was pioneered by Bert Sakmann and Erwin Neher, enabled studying of CNS ion channels and detecting membrane potentials (Schilling and Eder, 2013). With the aid of patch-clamp, microglia in brain slices from adult mice were shown to have resting membrane potentials (Vm) of around –40 mV (Schilling and Eder, 2015). Two-pore domain halothane-inhibited K^+ channel type 1 (THIK-1) was reported to maintain the negative Vm of microglia (Madry et al., 2018). Later, THIK-1 was found to be potentiated by the ATP/ADP activation of the metabotropic purinoreceptors P2RY12, Gi-coupled P2 receptors (G_iPCR) which are highly expressed on microglial processes (Izquierdo et al., 2019). As summarized in Figure 3, THIK-1 channel regulates Vm in health and consequently promotes the inflammasome assembly and interleukin-1β (IL-1ß) cytokine release, while P2RY12 induces inflammatory cytokines release and directed process movements upon tissue damage or infection via its G_iPCR downstream (Madry et al., 2018). Cultured microglia maintain the negative Vm via voltage-gated K^+ (Kv1.3) channels, inward rectifier K^+ channels (Kir), Cl^- channels and several other channels (Newell and Schlichter, 2005). Kir channel displays a pronounced inward rectification and a high

inactivation at more negative Vm (Kettenmann et al., 2011). Moreover, Kir is considered as a marker for active microglial cells, with minimum expression in resting microglia (Kettenmann et al., 1990, Boucsein et al., 2000). Furthermore, Kir channel regulates microglial Ca^{2+} signaling by increasing the electric driving force for Ca^{2+} entry (Franchini et al., 2004).

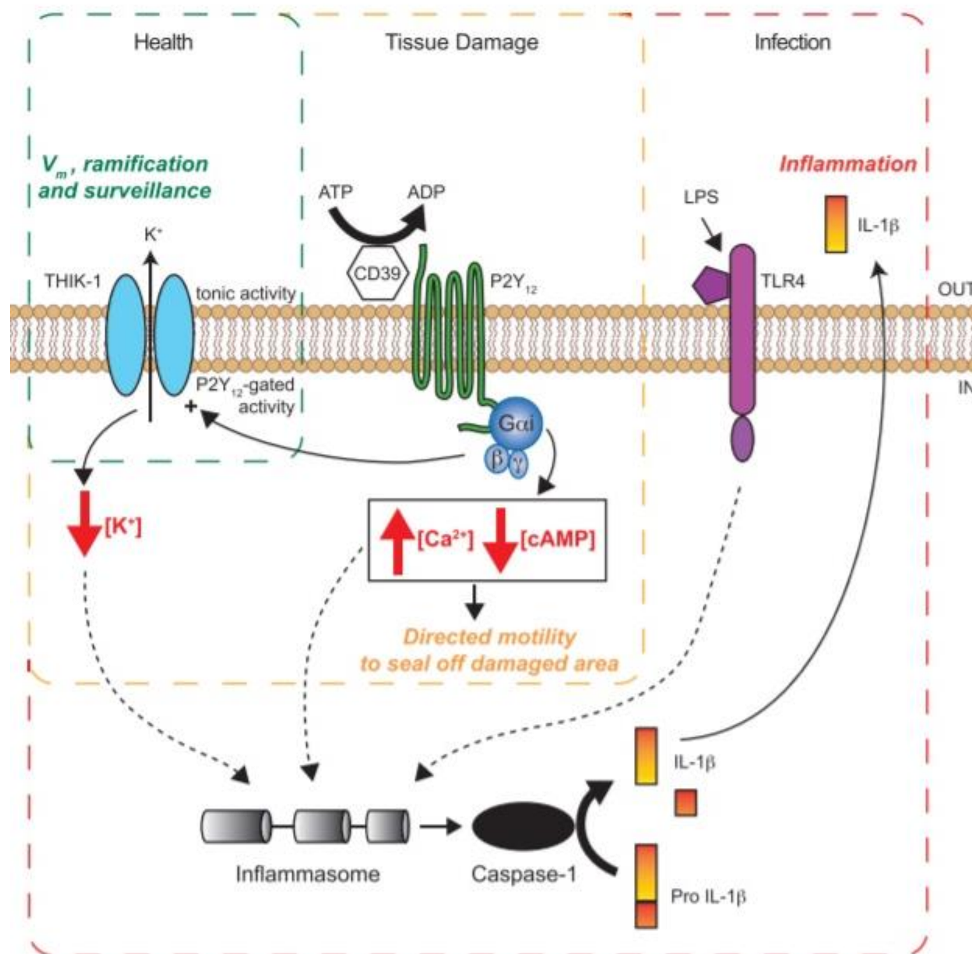

Figure 3. Membrane ion channels and receptors shape the functions of microglia. THIK-1 support microglial ramification and surveillance via maintaining a negative Vm. P2RY12 receptors potentiates THIK-1 activity in health, whereas they induce inflammatory cytokines release and directed process movements upon tissue damage or infection. THIK-1: Two-pore domain halothane-inhibited K^+ channel type 1, Vm: resting membrane potential. Taken from (Madry et al., 2018).

Delayed (outward) rectifier K channel belongs to another class of the large family voltage-gated (Kv) channel. Wu *et al.* found that the expression of Kv1.1 and Kv1.2 is critical for microglial production of proinflammatory cytokines until P14 (Wu et al., 2009), while Di Lucente *et al.*, and Pannasch *et al.*, documented that knocking out Kv1.5 or blocking Kv1.3 interfered with the outward rectifying K^+ current and the subsequent microglial activation

in vitro and *in vivo* (Kettenmann et al., 2011, Pannasch et al., 2006, Di Lucente et al., 2018). However, several studies demonstrated that Kv1.3 is the main voltage-gated K^+ channel in microglia which is highly upregulated in active microglia and provokes outwardly rectifying K^+ currents in stimulated microglia (Nörenberg et al., 1992, Walz and Bekar, 2001, Wu et al., 2009, Kettenmann et al., 2011, Schilling et al., 2000). These Kv1.3 channels together with Kir and Ca^{2+}-dependent K^+ channels regulate intracellular Ca^{2+} movements via oscillatory alterations in membrane potential (Di Lucente et al., 2018, Lund et al., 2006).

Calcium signaling in microglia. Intracellular Ca^{2+} signaling is crucial for the immune function of microglia. The primary route for Ca^{2+} entry is via store operated Ca^{2+}-permeable and ligand-gated channels, whereas endoplasmic reticulum and mitochondria are considered the intracellular Ca^{2+} stores. About 22% of microglia display spontaneous Ca^{2+} transients infrequently (Eichhoff et al., 2011). The baseline of spontaneous Ca^{2+} events is increased with age and under pathophysiological conditions (Brawek and Garaschuk, 2014, Olmedillas Del Moral et al., 2019). Upon the release of purines accompanied neuronal damage, ATP/UDP activates G protein-coupled metabotropic purinoreceptors (P2Y) which steers many of microglial functions (Kettenmann et al., 2011). Extracellular ATP activates P2RY12, whereas UDP activates P2R6, and ultimately trigger the release of Ca^{2+} from the intracellular stores through phospholipase C (PLC) activation (more details about P2Y are in the subsection “purinergic signaling in microglia”). Subsequently, intracellular Ca^{2+} elevation induces microglial functions; including the release of pro- and anti-inflammatory cytokines, process movement and phagocytosis (Olmedillas Del Moral et al., 2019).

1.1.2 Microglia functional phenotypes

During development, microglia were demonstrated to contribute in shaping CNS through synaptic pruning (Prinz et al., 2019), whereas throughout adulthood, they scan the brain continuously using their ramifications to detect any changes in brain environment (Madry et al., 2018). They acquire ramified/resting morphology with small soma and highly branched elongated processes to cover about 50 µm in diameter of the surrounding area in human and mouse brains (Illes et al., 2020). Under pathological conditions, microglia respond first to any brain injury by extending their processes towards the lesion, changing their morphology to more amoeboid, releasing pro-inflammatory cytokines, chemokines and other mediators, and

eventually migrate towards the affected region to remove cellular debris or infected cells by phagocytosis (Illes et al., 2020). It has become clear that microglia display a high level of morphological plasticity and the ramified/amoeboid activity-dependent morphology is not all or none, but rather includes many intermediate stages (Illes et al., 2020). Depending on the surrounding environment and the released immune mediators, such as colony-stimulating factor (CSF), interferon-γ (INF-γ) and lipopolysaccharide (LPS), microglia change from ramified into more amoeboid morphology (Savage et al., 2019, Illes et al., 2020).

Microglia are the brain surveillants under physiological and pathological conditions. At resting conditions, microglial processes were found in random constant motions. The turnover of protrusions and retractions was estimated to has the speed of 4.1 mm/min (Tvrdik and Kalani, 2017). This dynamic nature of microglia supports the surveillance activity. Microglial cells are equipped with sensomes of chemokine receptors (Cx3cr1, Cxcr2, Cxcr4, and Ccr5), purinergic receptors (P2RX4, P2RX7, P2RY6, P2RY12, and P2RY13), interferon-induced transmembrane proteins (Ifitm2, Ifitm3, and Ifitm6), Fc receptors (Fcer1g and Fcgr3), Toll-like receptors (Tlr2 and Tlr7), and Siglecs (Siglech and Siglec3/Cd33). These receptors collectively facilitate the recognition of misfolded proteins, pathogens, chemokines and cytokines, metabolites, and any changes in extracellular matrix or the pH (Prinz et al., 2019). If any brain pathology is detected, microglia respond accordingly. Moreover, it has been reported that microglial processes protrude and create connections with neuronal synapses in durations dependent on the neuronal activity (Nimmerjahn et al., 2005, Tvrdik and Kalani, 2017). This dynamic crosstalk which includes microglia, neurons and astrocytes raised the concept of ‘quadpartite synapses’ (Illes et al., 2020). However, once a cellular damage is detected, the multidimensional process movement is turned into directed movement towards the site of injury (Pozner et al., 2015).

Microglia orchestrate neuroinflammation in response to any changes in the brain environment. After sensing any changes in the CNS environment, microglia undergo changes in morphology and activities, and orchestrate neuroinflammation by releasing pro- and anti-inflammatory immune mediators. Accordingly, the active state of microglia used to be described as either M1 (pro-inflammatory) phenotype that favors neuronal damage and apoptosis, or the alternative M2 (anti-inflammatory) phenotype that protects neurons and promotes recovery; depending on the overall microglial response to any danger. For the past

decade, it has become clearer that M1/M2 polarization is not an accurate description of microglial behavior. Cutting-edge technologies such as single cell sequences, epigenomic and whole-genome transcriptomic, proteomics, Cytometry by time-of-flight and two-photon imaging revealed intermediate phenotypes of microglia with differential gene expression and functional activities (Ransohoff, 2016). Therefore, recent studies, including ours, avert the use of M1/M2 polarization concept.

As the innate sentinel cells, microglia express pattern recognition receptors (PRR); examples of PRRs are Tlrs, that recognized any damage-associated or pathogen-associated molecular patterns (DAMP or PAMP; respectively), endogenous alarmins, or misfolded protein. Examples of PAMP is LPS that is released from gram -ve bacteria and activates microglial Tlr4, and repeats of viral or bacterial nucleic acid. In response to Tlr activation, microglia release pro-inflammatory cytokines like tumor necrosis factor alpha (TNF-α), IL-1ß, IL-6, IL-18 and IL-23, chemokines, reactive oxygen/nitrogen species, and proteases (Bianchi, 2007, Illes et al., 2020, Kettenmann et al., 2011). However, microglia-mediated neuroinflammation contributes to the progression of neuroinflammatory and neurodegenerative diseases such as Alzheimer disease (AD), Parkinson disease (PD), and Multiple Sclerosis (MS) (Liddelow et al., 2017). In contrast, tumor associated microglia (TAMs) acquire immunosuppressive phenotypes that favors a cold tumor microenvironment, in which the immune cells are unable to fight tumor cells (Chen and Hambardzumyan, 2018).

Microglial phagocytosis as a function to shape the CNS and to restore brain homeostasis. As mentioned, microglial phagocytosis starts early during development and early postnatally to permit synaptic pruning (Prinz et al., 2019). The unneeded synapses are tagged by the complement proteins C3aR which are then recognized by microglia (Schafer et al., 2012). This process of postnatal synaptic pruning and shaping of the neuronal circuits is dependent on the neuronal firing activity and the microglial phagocytic cascade (Illes et al., 2020). Synapses expressing CD47 were found to prevent excessive microglial pruning via binding microglial SIRPα to initiate ‘’don’t eat me’’ signal (Prinz et al., 2019). In addition, microglia phagocytose microbes and foreign materials, as well as clear apoptotic, surplus, and the dead cells that expose phosphatidylserine via a variety of receptors, including TREM2 and P2Y purinergic receptors; mainly P2RY6 (Prinz et al., 2019, Kettenmann et al., 2011, Illes et al., 2020).

A summary of microglial activation states and functional phenotypes is shown in Figure 4.

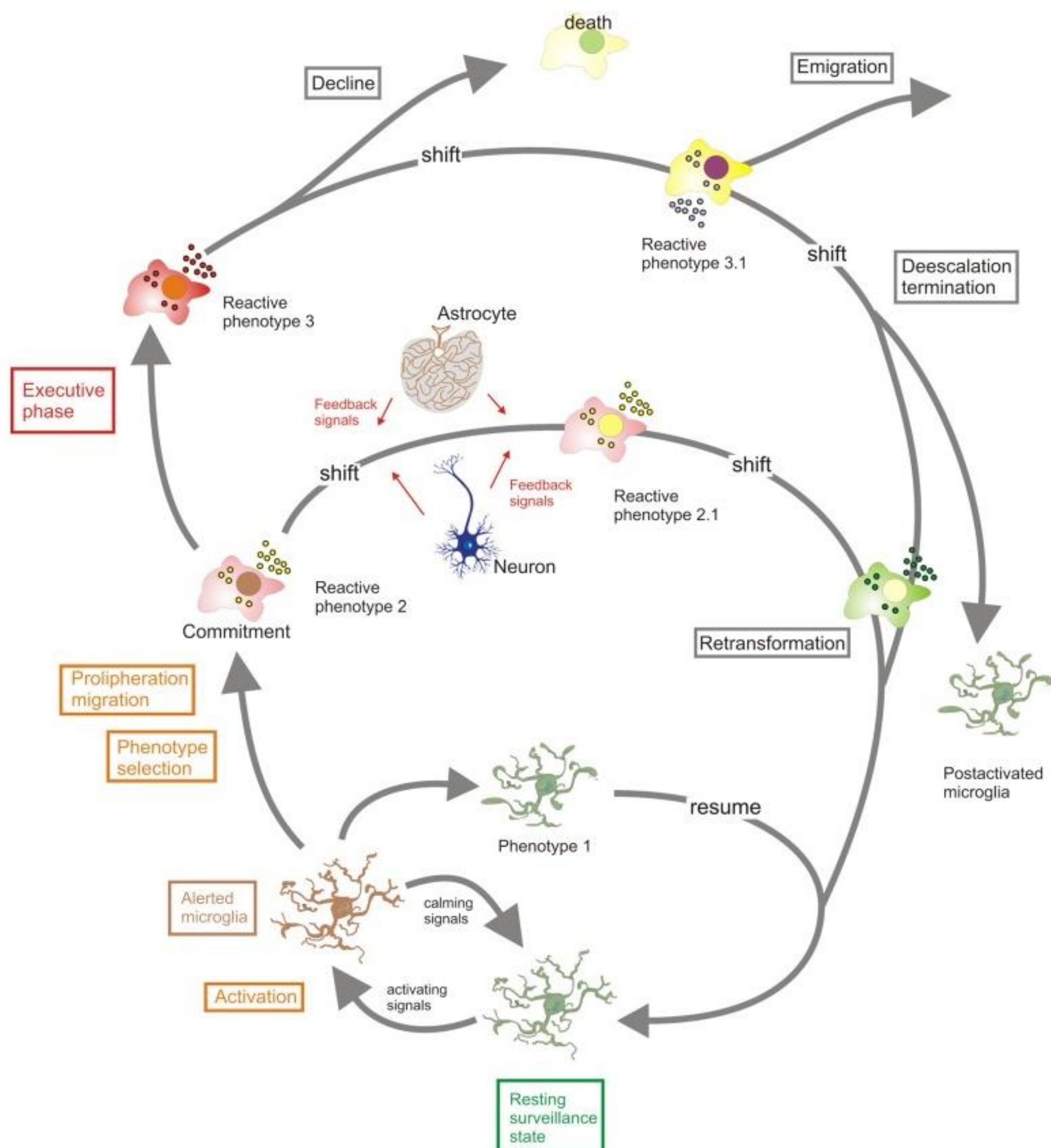

Figure 4. Summary of microglial functional phenotypes. At resting, microglia constantly scan the brain environment for exogenous or endogenous signals of a threat. Once microglia detect "activating" signals or lose "calming" inputs, they become alerted. Depending on the stimuli microglia either commit to an amoeboid active phenotype or resume a ramified morphology at rest. Microglial responses include migration, phagocytosis of pathogens or damaged cells, releasing immune mediators, crosstalk within the synapses, in addition to repair-orientated activities. Due to the impressive level of microglial plasticity, they might shift from one active state with specific responses to another, return to a resting state, or stay experienced to possible re-challenging stimuli. Taken from (Kettenmann et al., 2011).

1.1.3 Purinergic signalling in microglia

In the CNS, purines and pyrimidines act as extracellular signaling molecules that activate variety of signal pathways (Burnstock and Verkhratsky, 2010, Zarrinmayeh and Territo, 2020). Accompanied by neural damage, abundant amounts of ATP (has a purine nitrogenous base) is released and bind to microglial receptors called purinoreceptors (Kettenmann et al., 2011). Other extracellular nucleosides and nucleotides that activate purinergic signaling in microglia are adenosine, UTP and UDP (Madry and Attwell, 2015). Microglia express a wide array of purinoceptors which are stratified into metabotropic P1 adenosine receptors, metabotropic P2Y purinoceptors, and ionotropic P2X purinoceptors. While P1 and P2Y receptors are seven-transmembrane spanning GPCRs, P2X receptors are ligand-gated cationic ($Na^+/K^+/Ca^{2+}$) channels (Zarrinmayeh and Territo, 2020). There are different subtypes of these purinoreceptors that pertinently modulate microglial functions. The most well-studied ones are the ionotropic receptors P2RX4 and P2RX7, and the metabotropic receptors P2RY6, P2RY12 and P2RY13 (Kettenmann et al., 2011, Calovi et al., 2019) (Figure 5).

P2X signaling in microglia. Starting with microglial electrophysiology, ATP activation of P2X receptors on cultured microglia results in biphasic currents: a P2X-driven cationic depolarizing inward K^+ current and a P2Y/G protein-dependent outward K^+ current. (Calovi et al., 2019, Egan et al., 2006). Additionally, pore-forming P2RX7 receptors have a substantial role in increasing Ca^{2+} permeability and the subsequent elevation of $[Ca^{2+}]_i$ levels (Egan et al., 2006).

Shedding light on microglial functions, activation of P2RX4 was reported to switch on the pro-inflammatory phenotype of microglia in relation to neuropathic pain and brain injury (Gong et al., 2009, Roth et al., 2014, Zhang et al., 2017). Furthermore, the pro-inflammatory role of P2RX7 receptors; ubiquitously expressed in microglia, has attracted huge attention. Monif *et al.*, demonstrated that the sole overexpression of microglial P2RX7 receptors (in absence of any extra stimuli) activated cultured microglia from rat hippocampus (Monif et al., 2009). Interestingly, P2RX7 activation has been documented mainly in pathology and linked to increasing Ca^{2+} influx and the subsequent membrane blebbing and microvesicle release (Turola et al., 2012). However, a recent study demonstrated that membrane shedding and cytokines-induced microvesicle release can be independent to P2RX7 activation (Colombo et al., 2018). In addition, activation of P2RX7 receptor provokes inflammasome

assembly and pro-inflammatory cytokines release (such as IL-1β and IL-18), produces reactive nitrogen and oxygen species, activates caspases, and induces apoptosis and cytotoxicity (Seeland et al., 2015, Savio et al., 2018). Blocking P2RX7 has been used in pre-clinical studies as an emerged target in the neuroinflammatory diseases (Karasawa and Kawate, 2016, Savio et al., 2018).

P2Y signaling in microglia. Studies on microglial P2Y receptors unveiled a major correlation with P2RY6-mediated phagocytosis, and P2RY12-induced chemotaxis and process movement (Calovi et al., 2019, Lou et al., 2016, Inoue et al., 2009, Diaz-Aparicio et al., 2020). Moreover, P2RY12 and P2RY13 have been associated with microglial generation of pro-inflammatory cytokines including IL-1β, IL-6 and TNF-α (Liu et al., 2017), neuronal hyperexcitability (Tatsumi et al., 2015), and neuropathic pain (Niu et al., 2017, Tatsumi et al., 2015).

P2Y6 receptors are coupled to G_q/G_{11} proteins with the subsequent cytosolic PLC/IP3 signaling that regulate cytosolic Ca^{2+} mobilization (Abbracchio et al., 2006). These receptors are sensitive to uracil nucleotides like UTP and UDP (Kettenmann et al., 2011), and blocked selectively by the MRS 2578 compound (Quintas et al., 2014). UDP-activated P2RY6 was reported to mediate both *in vitro* and *in vivo* phagocytosis (Neher et al., 2014). Moreover, P2RY6 activation was found to induce NO synthesis *in vitro* and astrogliosis (Calovi et al., 2019).

In contrast to P2RY6 receptors, P2RY12 receptors are coupled to $G_{i/o}$ proteins with the subsequent inhibition of adenylyl cyclase and modulation of ion channels (Abbracchio et al., 2006). The P2RY12 receptors are activated by adenine nucleotides like ATP and ADP (Kettenmann et al., 2011), and blocked by ARC69931 compound (Hidetoshi et al., 2012). Knocking out P2RY12 gene on microglial cells *in vitro* or *in vivo* severely inhibits microglial migration, proliferation, and process extension towards microlesions (Kettenmann et al., 2011). The P2RY12-mediated microglial chemotaxis involves the activation of PLC-dependent intracellular Ca^{2+} release and phosphatidylinositol 3´-kinase (PI3K) and Akt signalling cascades. Moreover, P2RY12 activates integrin-1 signaling cascade to allow better control of microglial process movement (Kettenmann et al., 2011). However, a recent study by Madry *et al.,* revealed that P2RY12 receptors only potentiate THIK-1-induced microglial ramification and surveillance (Madry et al., 2018). The inhibition of P2RY12 does not influence membrane potential, ramification, or surveillance, but rather suppresses microglial process outgrowth to

reach the site of injury (Madry et al., 2018). On the other hand, blocking THIK-1 depolarizes microglia membrane potential, suppresses K^+-dependent inflammasome assembly and the subsequent IL-1ß release, and decreases ramification and surveillance (Madry et al., 2018).

Knowing that purinergic receptors are the steering tools that define microglia phenotypes, agonists/antagonists of these receptors may profoundly modulate microglial functions as well as microglial involvement in a disease context.

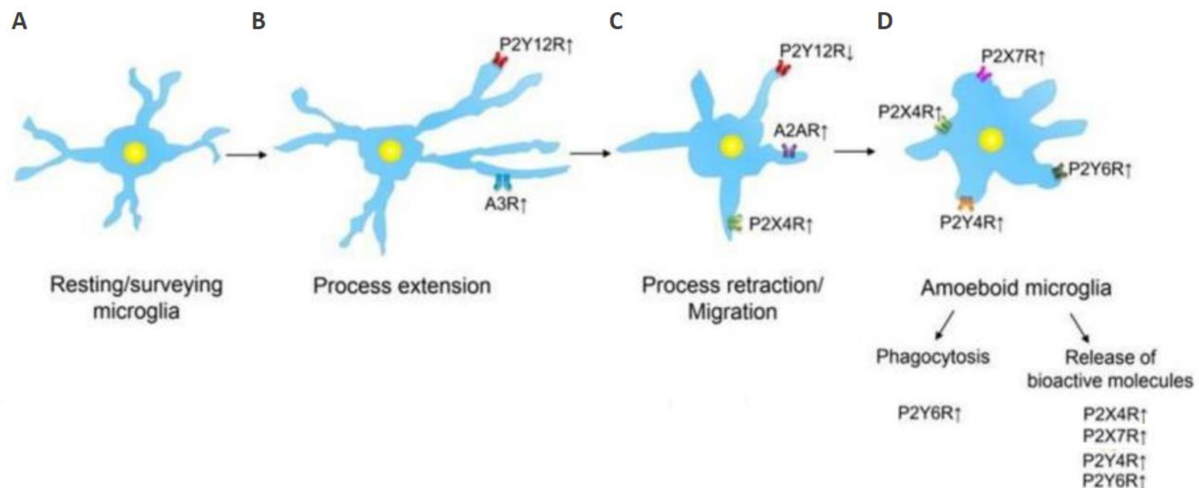

Figure 5. Role of purinergic receptors in modulating microglial phenotype at the different activation states. (**A**) Ramified microglia at resting state scan the surrounding area of the brain to detect any pathological conditions or microbial attack. (**B**) When neural damage occurs, the released ATP activates microglial P2RY12, and induces process extension. Both P2RY12 and A3Rs are upregulated at this phase and cooperate in steering process extension. (**C**) Subsequently, the expression of P2RY12 is downregulated, whereas A2RA and P2RX4 receptors are upregulated; leading to process retraction and closer migration of microglia to the injury site where they become more amoeboid. (**D**) After complete retraction of the processes, microglia evolve into an active amoeboid phenotype. On this phenotype, activation of P2RY6 mediates phagocytosis, while activation of P2RY4 promotes pinocytosis. Additionally, P2RX7 mediates the release of pro-inflammatory cytokines, chemokines, growth factors, proteases, reactive nitrogen/ oxygen species, cannabinoids, and other excitotoxic molecules. Furthermore, activation of P2RX4 induces the release of brain-derived neurotrophic factor in spinal cord microglia. Upwardly and downwardly directed arrows indicate either upregulation or downregulation of the receptors; respectively. Adapted from (Illes et al., 2020).

1.2 The VGF-derived peptides as possible modulators of microglial functional phenotypes

The mRNA of VGF (non-acronym) was first detected by Andrea Levi in 1985 (Levi et al., 1985), while he was inducing the differentiation of rat pheochromocytoma PC12 cells into neurons; using different concentrations of the nerve growth factor (NGF). The induced mRNA

hybridized with the cDNA clone Vgf8a from a 5000 clones' library, and was found to encode a 90 KD polypeptide (Levi et al., 1985). The original name of this polypeptide was "*Plate V Nerve Growth Factor Inducible Gene"* before it was recognized as VGF. VGF gene is located on 7q22.1 position and translated into 2.5 Kbp transcript that encodes a protein of 615 amino acids in human and 617 amino acids in rodents (Bartolomucci et al., 2011). VGF is mainly expressed in the CNS, but also in the peripheral nervous system and endocrine tissues. According to the human protein atlas, VGF RNAs are enriched in the brain and the pituitary gland (Figure 6). Therefore, in the present study, I focused on the cerebral cortex as the region of interest for all the VGF-related experiments. Post-translation, VGF polypeptide undergoes proteolytic processing by prohormone convertase (PC) enzymes which leads to the production of biologically active VGF-derived peptides (Ferri et al., 1992, Trani et al., 2002)

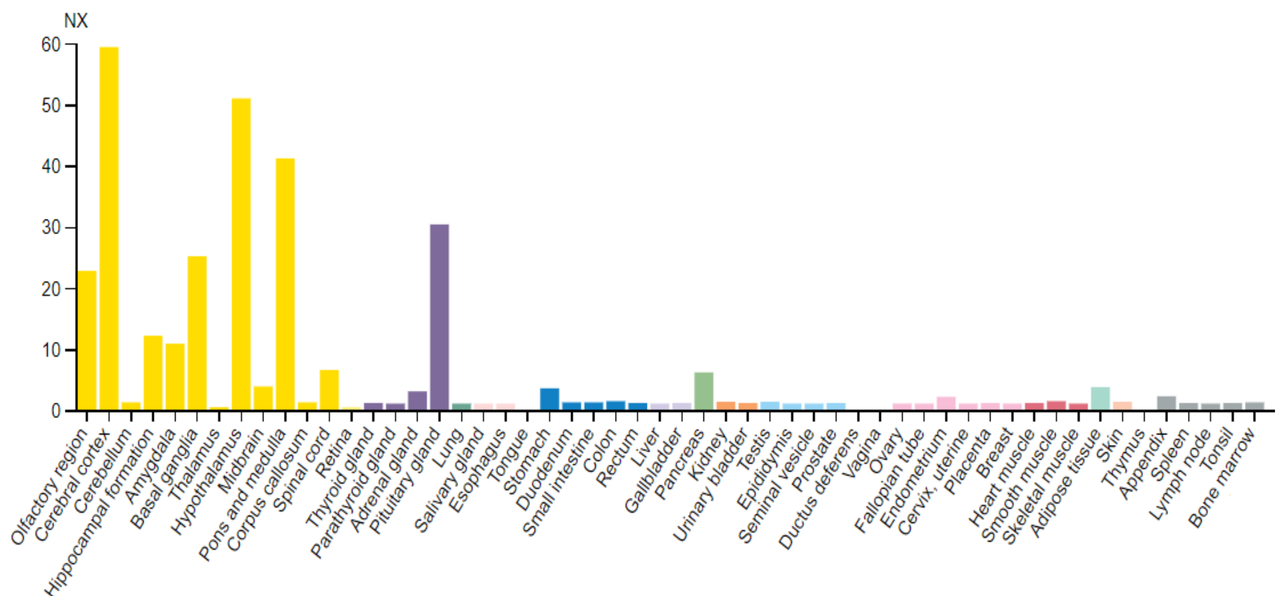

Figure 6. RNA expression of VGF in the human body. The expression of VGF was detected in the different body organs; using consensus Normalized eXpression (NX) levels for 55 tissue types and 6 blood cell types from three transcriptomics datasets (GTEx, HPA, and FANTOM5). The highest expression was detected in the brain and pituitary gland. Color-coding refers to tissue groups, each consisting of tissues with common functional features.

https://www.proteinatlas.org/ENSG00000128564-VGF/tissue

1.2.1 VGF and its biologically active derived peptides

Ferri and other colleagues demonstrated that VGF is directly proteolyzed into more than ten biologically active derived peptides (Figure 7) with prominent activities at the c-

terminal domain in terms of biological function (Ferri et al., 1992, Trani et al., 2002, Bresciani et al., 2020). Trani *et al.* described the post-translational proteolytic processing of VGF by prohormone convertase (PC) enzymes (Trani et al., 2002). They demonstrated that PC 1/3 and PC2 target the cleavage site at NAPP129, whereas only PC1/3 cleaves VGF at the amino acid sequence Arg-Pro-Arg on position 555, and generate TLQP21 peptide (Trani et al., 2002).

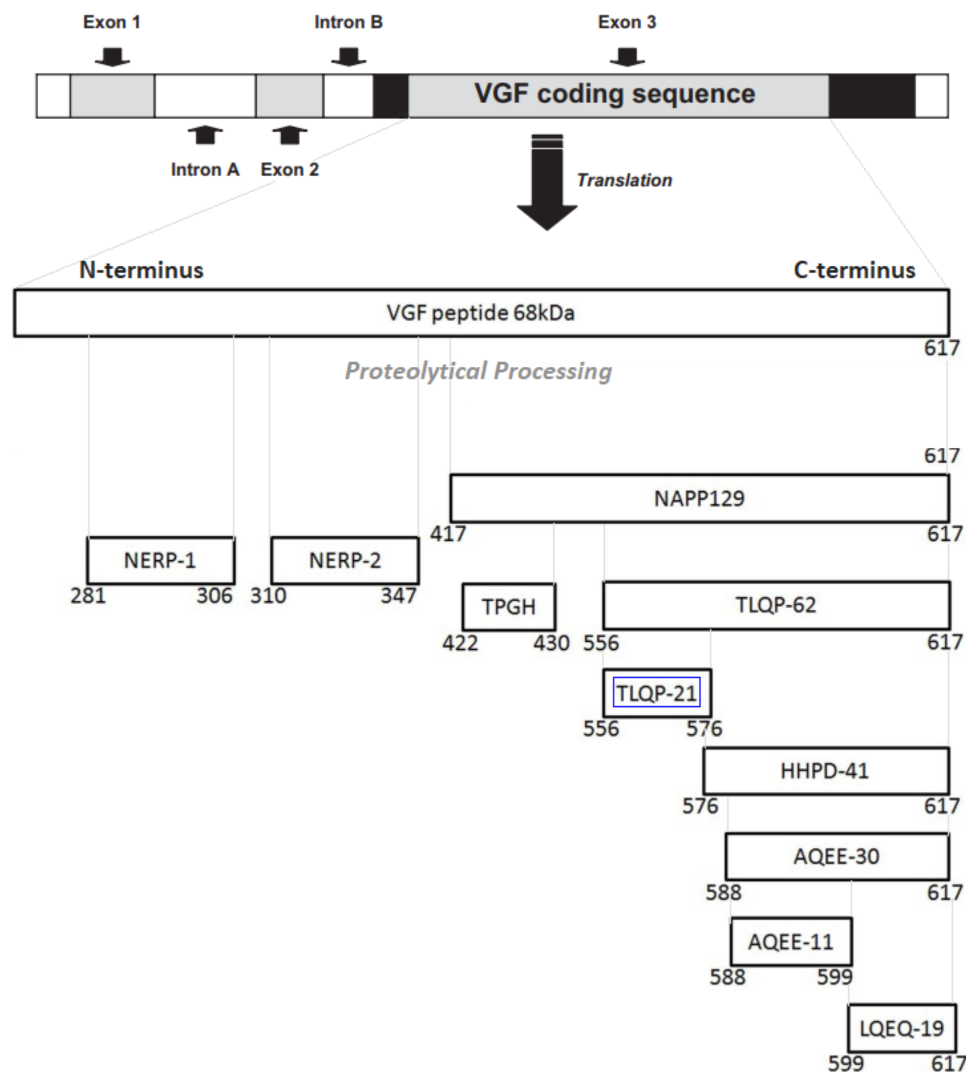

Figure 7. Schematic representation of VGF translation and processing into the VGF-derived peptides. VGF is a single copy gene located on 7q22.1 position and contains three exons and two introns. Exon 3 is translated into VGF polypeptide which undergoes proteolytical processing into biologically active VGF-derived peptides. The nomenclature of these peptides indicates the first four amino acids from the N-terminal region, each letter represents the corresponding amino acids, whereas the number represented the sequence length (Levi et al., 2004). Highlighted in blue the VGF-derived peptide TLQP21 which is the focus of one of the studies in the present dissertation. The Figure is adapted from (Jethwa and Ebling, 2008) and (Bresciani et al., 2020).

There is a group of VGF-derived peptides at the c-terminus that displays neuronal bioactivities; including neuroendocrine regulatory peptides (NERP)-1 and -2, TLQP21, TLQP62, HHPD41, LQEQ19, AQEE11, and AQEE30 (Lewis et al., 2015). Lewis *et al.*, mentioned in their review "Neuroendocrine role for VGF" that AQEE30 and TLQP62 induce neurogenesis and enhance the neuronal firing in the hippocampus, whereas AQEE11, AQEE30, LQEQ19 and HHPD41 have a stimulatory effect on sympathetic neurons and induce penile erection in male, and NERP2 and TLQP21 regulate metabolism and energy balance (Lewis et al., 2015). Many other studies showed that VGF itself has been associated with neuronal differentiation, synaptogenesis, synaptic plasticity associated with learning, and memory formation and consolidation, in addition to energy balance and reproduction (Thakker-Varia and Alder, 2009, Sakamoto et al., 2015, Alder et al., 2003, Bartolomucci et al., 2007, Salton et al., 2000). However, there is no clear consensus whether these effects are related to the polypeptide VGF itself, a particular VGF-derived peptide, or both.

Several studies showed that the expression level of VGF mRNA is associated with the prognosis of neurological diseases. The Array Collection studies in the SMRI genomics database revealed that the transcription of VGF mRNA is downregulated in the frontal cortex of bipolar disorder and schizophrenic patients (Thakker-Varia and Alder, 2009). Another group found that VGF mRNA level is elevated when they used SUN N8075 to ameliorate endoplasmic reticulum stress. They described the mechanism as SUN N8075 inhibited endoplasmic reticulum stress-induced neuronal cells apoptosis via VGF expression (Shimazawa et al., 2010). Moreover, some other studies reported a reduction in VGF expression in depression and neurodegenerative diseases (Cattaneo et al., 2010b, Cattaneo et al., 2010a). In a recent study by Beckmann *et al.* in 2020, overexpression of VGF in 5xFAD mice (A mouse strain recapitulating features of AD) decreased the ß amyloid plaques load in the cortex and hippocampus, and rescued the associated memory impairment and neuropathology (Beckmann et al., 2020). On the other hand, Wang *et al.* demonstrated that VGF supports Glioblastoma Multiforme (GBM) progression by promoting the growth and stemness of both stem-like and differentiated tumor cells (Wang et al., 2018). As a result, overexpression of VGF increased the tumor size (Wang et al., 2018). Collectively, these findings indicate that VGF and its peptides might be promising therapies for brain diseases.

1.2.2 TLQP21 nomenclature, receptors and functions

TLQP21 is a VGF-derived peptide that is highlighted the most in VGF-related studies due to its functions centrally and peripherally. According to Levi nomenclature (Levi et al., 2004), TLQP21 consists of 21 amino acids, the first four from the N-terminus are threonine, leucine, glutamine and proline. The full structure of TLQP21 is TLQPPASSRRRHFHHALPPAR, in which each letter represents the corresponding amino acids (Rivolta et al., 2017).

TLQP-21 activates C3a and C1qBP receptors. The Complement 3a receptor 1 (C3aR1) was first identified by Hannedouche *et al.* as the TLQP21 receptor (Hannedouche et al., 2013). They applied fluo-4 calcium assay on the rat ovarian tumor cell line O-342/DDP and the Chinese hamster ovarian cell line CHO-K1 as a screening tool for TLQP21-activated targets. They found that TLQP21 increases the ATP-induced Ca^{2+} efflux in both cell lines. They then applied pertussis toxin (PTX) which inhibited the recorded signals; suggesting that TLQP21 receptor might be a G_i/G_o protein coupled receptor. After using a third cell line, Chinese hamster lung Cells (CCL39), no TLQP21 response was observed, therefore, the authors compared between the transcriptomes of the three cell lines to identify possible TLQP21 binding sites by genome-wide sequencing. Next, they silenced the genes of the possible targets; using short interfering RNA (siRNAs) screening. The one siRNA that could significantly suppress the TLQP-21-induced Ca^{2+} signal was that against C3AR1. To further confirm their findings, they repeated the experiment with the C3aR selective antagonist SB290157 which completely blocked the TLQP21-induced Ca^{2+} signals. Cero *et al.* showed that TLQP21 underwent a disorder-to-order transition and changed its conformation from random coil into a-helical conformation upon binding to C3aR1 (Cero et al., 2014). C3aR1 is a member of the GPCR superfamily as an orphan GPCR for the ligand C3a (Cero et al., 2014). It has been shown earlier in another study that the intracellular signal transduction following C3aR1 activation is established via PTX-sensitive $G\alpha_i$-proteins that subsequently mobilizes extracellular Ca^{2+} fluxes in neutrophilins (Norgauer et al., 1993).

A second TLQP21 receptor has been identified later (Chen et al., 2013). The authors equipped chemical cross-linking combined with mass spectrometry analysis to further screen possible TLQP21-activated receptors and identified C1qBP (C1q binding protein, called also gC1qR the globular heads of the C1q receptor, p32, p33), as a receptor for TLQP21. As a confirmatory

step, the authors compared the TLQP21-induced Ca^{2+} release from intracellular Ca^{2+} stores; using Ca^{2+} imaging techniques, to that in the presence of a blocker of C1qbp (siRNA/neutralizing monoclonal antibodies) on a primary culture of either marrow-derived primary macrophages or microglia. It was found that blocking C1qbp suppresses TLQP21; confirming that C1qbp is a second receptor for TLQP21. Both C3aR and C1qbp are components of the complement cascade that mediates important functions in immunity; including neutralizing microbes, opsonization and starting inflammation. C1qbp is a highly anionic cellular protein of 33 kDa which was identified as the first subcomponent of the classical pathway for the complement cascade, and demonstrated to bind to several ligands necessary for immune surveillance, as well as inflammation (Dembitzer et al., 2012).

Functions of TLQP21. Severini *et al.* pointed out that TLQP21 has neuroprotective functions on cerebellar granule cells (Severini et al., 2008). The authors reported that stimulation of K^+- and serum-deprived cells by TLQP21 activates mitogen-activated protein kinases (MAPK) Erk1/2 with subsequent elevation of intracellular Ca^{2+} and also phosphorylation of Akt which eventually inhibits DNA fragmentation and improves cell survival by more than 20% compared to a control (Severini et al., 2008). The level of TLQP21 was found to be reduced in the blood of patients suffering from amyotrophic lateral sclerosis; suggesting that TLQP21 is a biomarker with a neuroprotective role (Brancia et al., 2018). Furthermore, the effect of TLQP-21 goes beyond the CNS, as it regulates the contractility of the gastrointestinal tract and the gastric emptying, suppresses gastric acid secretion, increases insulin and amylase secretion from the pancreas, regulates metabolism, lowers blood pressure in rodent, and modulates pituitary hormone secretion (Lewis et al., 2015, Petrella et al., 2012, Sibilia et al., 2012).

TLQP21 and microglia. The complement cascade is a danger sensing system, therefore, receptors for complement components are expressed in the innate immune cells. Whereas VGF is minimally expressed in microglia, both C1qbp and C3aR1 are expressed in microglia (Figure 8). Nevertheless, the effect of TLQP21 has not been well-understood up to the time of designing my study. In a related context, the VGF-derived peptides AQEE30 and LQEQ19 were shown to activate MAPK p38 in microglia and induce hyperalgesia (Riedl et al., 2009). This later finding highlights a possible association between VGF and its peptides, and microglia.

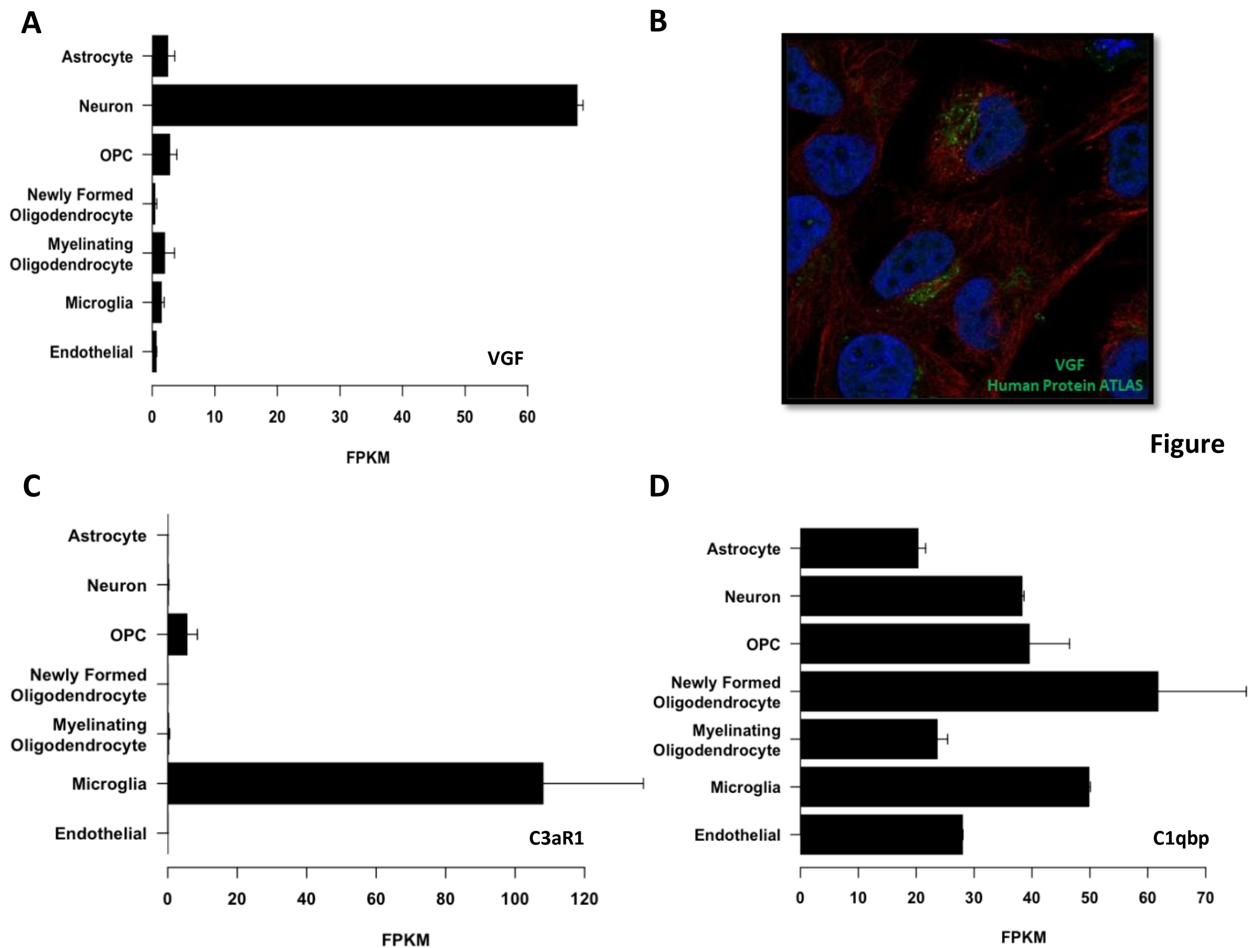

Figure 8. Expression of VGF, C3aR1 and C1qbp in microglia. (**A**) The main source of VGF in the CNS is the neuron but not microglia. (**B**) A photo obtained from the human protein atlas showing VGF in the cytosol of neurons. (**C**, **D**). the receptors of the VGF-derived peptide TLQP-21 are highly expressed in microglia, with C3aR1 showing high selectivity to microglia cells (**C**), whereas C1qbP is expressed by most of the brain cells (**D**). FPKM: Fragments Per Kilobase Million; a normalised estimation of gene expression in RNA-seq data. These data are extracted online from the brain-seq database released by Barres lab https://www.brainrnaseq.org/.

1.3 Neurofibromatosis type 1 and microglia

Neurofibromatosis type 1 was chosen as a disease model in which a knock out (KO) of intrinsic regulatory component, NF1, potentially modulates microglial functional phenotypes.

1.3.1 Neurofibromin 1 and neurofibromin 1 gene deletion

The Neurofibromin 1 (NF1) gene is located on chromosome 17, at q11.2, and encodes the neurofibromin (NF1) protein. NF1 is a GTPase-activating cytoplasmic protein expressed in neurons, astrocytes, oligodendrocytes, Schwann cells, immune cells, epithelial cells, and in

the adrenal gland (Anderson and Gutmann, 2015). NF1 is considered as a tumor suppressor gene due to the fact that it negatively regulates the RAS/MAPK signaling pathway (Figure 9) and therefore, inhibits uncontrolled cellular growth (Peltonen et al., 2017, Trovó-Marqui and Tajara, 2006). Moreover, by activating RAS signaling, NF1 regulates the release of cyclic AMP (cAMP) through protein kinase C-ζ (PKCζ) and GPCRs (Gutmann et al., 2017). The NF1 gene has a high mutation rate and is linked to an autosomal dominant disorder called neurofibromatosis type 1 (NF1, also known as von Recklinghausen syndrome). Most of NF1 patients manifest pigmentary lesions (café-au-lait macules, Lisch nodules and skinfold freckling). Some individuals suffer from skeletal abnormalities; including tibial pseudarthrosis, orbital dysplasia and scoliosis. Additionally, NF1 mutations are associated with peripheral nerve tumors (plexiform neurofibromas, spinal neurofibromas and malignant peripheral nerve sheath tumors), brain tumors (Low grade gliomas and glioblastoma), attention deficits, learning disabilities, and behavioral and social problems (Cimino and Gutmann, 2018).

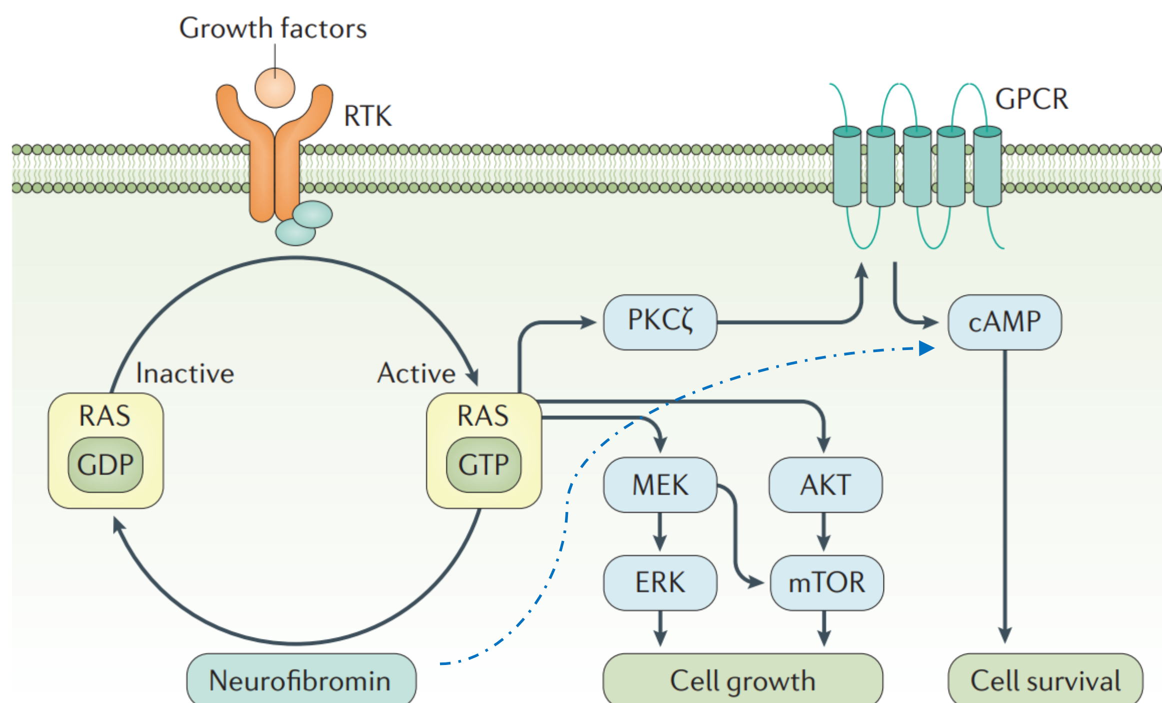

Figure 9. Activation of neurofibromin and associated signaling. Neurofibromin (NF1) is a GTPase-activating protein that accelerates the conversion of active GTP-bound RAS into the inactive GDP-bound form. RAS signalling can be initiated by receptor tyrosine kinases (RTKs) after binding growth factors. RAS activates G protein-coupled receptors to generate cyclic AMP (cAMP) through protein kinase C-ζ (PKCζ). The downstream of RAS activation includes switching on AKT–mechanistic target of rapamycin (mTOR) and MEK–extracellular signal-regulated kinase (ERK) effector pathways that are associated with cell growth and survival. Thus, loss of NF1 hyperactivates cellular proliferation increasing the risk of tumor formation. Taken from (Gutmann et al., 2017). Highlighted in blue the correlation between NF1 and cAMP which is going to be the core of one of my studies in the present dissertation.

1.3.2 Neurofibromin 1 mutations and microglia

NF1 patients have a fivefold increased risk for cancer, particularly neurogenic malignancies (2000-fold risk) (Peltonen et al., 2017). One characteristic manifestation of NF1 is the formation of neurofibroma, a neuronal sheath tumor with an intimate association with cranial, peripheral or spinal nerves. Examples of NF1-associated neurogenic malignancies are astrocytomas, optic gliomas and glioblastoma multiforme (GBM) (Reilly et al., 2000, Anderson and Gutmann, 2015). Tumor associated microglia (TAMs) contribute to the tumorigenesis and metastasis of NF1-linked glioma (Brossier and Gutmann, 2015, Daginakatte and Gutmann, 2007). Daginakatte and Gutmann demonstrated that there is a paracrine crosstalk between murine heterozygous NF1-KO (*Nf1 +/-*) microglia and homozygous NF1-KO (*Nf1 −/−)* astrocyte (Daginakatte and Gutmann, 2007). The authors found that *Nf1 +/-* microglia increase *Nf1 −/−* astrocyte proliferation in astrocyte-microglia co-cultures by 1.5-fold. To determine which factors are involved in this microglial effect, the authors used Affymetrix microarray gene expression analysis and defined five transcripts that were significantly upregulated in *Nf1 +/-* microglia; jagged-1, pleiotrophin, insulin-like growth factor-1 and hepatocyte growth factor/scatter factor and hyaluronidase. Moreover, by inactivating microglia using minocycline, the authors observed a 3-fold decrease in the number of BrdU-labeled cells representing the proliferation of the optic glioma. A second study from the same lab showed that murine *Nf1 +/-* microglia have elevated levels of activated c-Jun-NH2-kinase (JNK) which is involved as well in the uncontrolled proliferation of optic nerve glioma. The inhibition of microglial JNK by SP600125 reduced the growth of the optic glioma *in vivo* (Daginakatte et al., 2008). Interestingly, microglia was reported to promote optic glioma growth in athymic mice only after being exposed to T-cells (Pan et al., 2018). The authors demonstrated that T-cell-microglia/macrophage interaction resolves the impaired microglial CCR2 and CCL5 expression in primary cultured microglia from athymic mice and consequently support the NF1-linked optic glioma growth (Pan et al., 2018).

Collectively, these findings encouraged studying the effects of heterozygous NF1 knock out on microglial functions. Furthermore, taking in consideration that some of NF1 manifestations exhibit sexual dimorphism, I discriminated male and female mice in the NF1 study.

1.4 Extracellular components, tenascin C and microglia

Microglial cells represent in average 10-15% of the cells in the CNS surrounded by neurons, glial cells, neurovascular unit and extracellular matrix (ECM). Early in 2003, Milner and Campbell demonstrated that ECM components, vitronectin and fibronectin but not laminin, activate microglia and increase the expression of microglia $\alpha_4\beta_1$, $\alpha_5\beta_1$ and Mac-1 integrins, a family of adhesion molecules that is involved in the regulation microglial immunity (Milner and Campbell, 2003). Interestingly, the interaction between ECM and microglia was found to be reciprocal. Könnecke and Bechmann highlighted in their review that microglia-derived matrix metalloproteinase (MMP)-2 and MMP-9 contribute to the composition of ECM through the interaction with dystroglycan, a transmembrane protein that anchors end-feet of the astrocytes on the basement membrane (Könnecke and Bechmann, 2013). Furthermore, most recently Crapser *et al.* (Crapser et al., 2020) demonstrated that activated microglia in 5xFAD, a mouse model for Alzheimer disease, engulf damaged perineuronal nets and modulate these ECM components that are responsible for enwrapping and stabilizing neuronal synapses. Moving closer to the extracellular space, the crosstalk between microglia and other cells through extracellular vesicles was described in several studies. Extracellular vesicles like microvesicles and exosomes contain proteins, lipids and RNAs. Their tasks include disposing unnecessary cytosolic material into the extracellular area, transfer materials between cells, and mediate long-distance intercellular communication. Microglial microvesicles enrich the ECM with proteins implied in cell adhesions at ECM, cellular metabolism and the autophago-lysosomal pathway (Drago et al., 2017). The ECM protein tenascin-C (TNC) was found inside exosomes generated by brain tumor-initiating cells to suppress T cells (Mirzaei et al., 2018). To further elucidate the association between TNC and brain immunity, one of the present studies focused on the action of TNC on microglia.

1.4.1 Expression and functions of tenascin C

TNC is a hexametric multimodular glycoprotein within the ECM and characterized by several molecular forms due to alternative splicing during translation and the subsequent protein modifications. The expression of TNC starts during embryogenesis and fades during adulthood (Whitlon et al., 1999). Furthermore, TNC levels increase rapidly and transiently in

response to pro-inflammatory cytokines released by pathological stimuli like infections, injury, fibrosis, cancer or any inflammation, *de novo* in wound healing, and in stem cell niches (Yalcin et al., 2020, Whitlon et al., 1999). In the CNS, astrocytes are the source of TNC which appears around the neurons and glial cells in the developing brain (Faissner, 1997).

TNC was first recognized as a cell adhesion modulator that shapes the cellular microenvironment (Chiquet-Ehrismann et al., 1986). However, several studies described roles of TNC in regulating cellular signaling, morphologies and functions. It was linked to cellular movement, neural blast migration and tumor metastasis (Saupe et al., 2013, Yalcin et al., 2020). Moreover, by downregulating the Wnt inhibitor Dickkopf-1, TNC induces Wnt target genes and activates Wnt signaling in tumor, thereby contributing to tumorigenesis in a mouse model exhibiting pancreatic β-cell cancer (Saupe et al., 2013). In addition, TNC was reported to inhibit bone morphogenic protein signaling, enhance fibroblast growth factor receptor (FGF), and consequently support the survival of oligodendrocyte precursor cells (Garcion et al., 2004). Moreover, by activating epidermal growth factor (EGF) receptors and extracellular signaling-related kinases 1 and 2 (ERK1/2), TNC triggers the constriction of cerebral arteries (Fujimoto et al., 2016). Chiquet-Ehrismann and Tucker reported that TNC knockout mice are characterized by behavior abnormalities, changes in brain cytoarchitecture, and disrupted inflammatory and trauma responses (Chiquet-Ehrismann and Tucker, 2011). On the other hand, TNC deficiency was associated with beneficial effects in TgCRND8 mice (an AD mouse model) in terms of lower Amyloid ß plaques load and increased postsynaptic density protein 95 (Xie et al., 2013).

TNC has several cellular receptors and binding partners in the ECM. One of the binding receptors is Tlr4 which regulates the production of pro-inflammatory cytokines in primary human macrophages (Midwood et al., 2009) (Figure 10). A study from our lab reported that TNC is involved in regulating IL-6 secretion from glioma stell cells through the activation of Tlr4 (Dzaye et al., 2016).

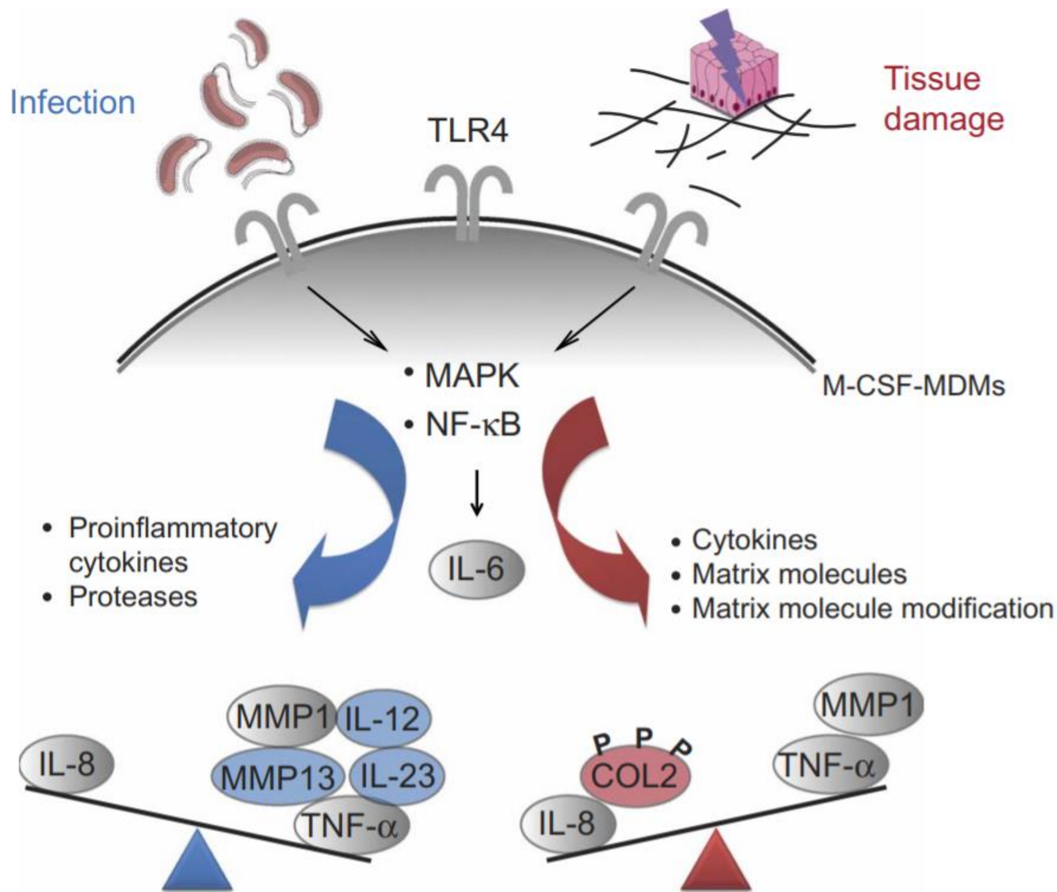

Figure 10. TLR4-mediated innate immune responses. When TLR4 is activated by infection or damaged tissues NF-kB and MAPK pathways start signaling and the release of pro-inflammatory cytokines including IL-6, IL-8 and TNF-α. In addition, tissue damage induces the synthesis and posttranslational modification of matrix molecules like MMP1 and MMP13. Molecules produced due to pathogens or tissue damage are highlighted in blue or red; respectively. IL; interleukin, MMP; matrix metalloprotease, P; phosphorylation, COL2; collagen type II. Taken from (Piccinini et al., 2016).

1.4.2 Tenascin C and microglia

As part of body innate immunity, microglia express endogenous TLR4 which is activated by TNC. Indeed, stimulation of the active inflammatory phenotype of microglia by TNC was detected in the twitcher mouse model of Krabbe disease. additionally, microglial response to psychosine was enhanced by TNC in terms of higher cytotoxicity to oligodendrocytes, globoid-like cell formation and MMP3 mRNA expression (Claycomb et al., 2014). As a reciprocal interaction between microglia and TNC, *in vitro* studies revealed that microglial-conditioned medium promotes astrocytic expression of tenascin by 2.5-fold (Smith and Hale, 1997). Taking in consideration as well that the expression level of TNC correlates with the active status of microglia in the developing brain and in pathology, TNC was chosen as a potential modulator of microglial functions in one of the present studies.

2 Aim of the study

Microglia are the first line innate sentinels in the brain parenchyma. Over the last few decades, it has become clear that microglia contribute significantly to the pathology of many CNS diseases as well. Recent genome-wide association studies (GWASs) revealed a large number of risk genes of many CNS diseases that are expressed in microglia. Such disorders include schizophrenia, Alzheimer's disease (AD), Parkinson's disease, autism, multiple sclerosis, as well as glioblastoma multiforme (GBM) (Gutmann and Kettenmann, 2019, Prinz et al., 2019). However, the development and progression of diseases often rely on the immunosuppressive or –promoting microenvironment rather than being cell-autonomous. Accordingly, microglia have emerged as a promising therapeutic target to treat CNS disorders.

I aimed in the present studies at finding extrinsic and intrinsic modulators of microglial functional phenotypes, in an attempt to highlight these targets for future clinical application. As extrinsic modulators, I focused on the effect of VGF or rather the VGF-derived peptide TLQP21, which is released by neurons, as well as the extracellular matrix protein TNC, which is generated by astrocytes, on microglia. As a microglia-intrinsic target, I investigated NF1, a known downstream effector of tyrosine kinase receptor. Additionally, I am still investigating the effect of the VGF-derived peptide TLQP21 on tumor-associated microglia in GBM and plaque-associated microglia in AD as follow-up studies that are currently in progress.

Specific scientific questions are:

1. VGF/TLQP21

1.1 Does TLQP21 have an impact on microglial membrane currents and Ca^{2+} release?

1.2 Are microglial migration, process movement and phagocytic activity affected by external TLQP21 application?

1.3 Which microglial receptors and intracellular pathways are involved in TLQP21 action on microglia?

1.4 Does knocking out VGF have any implications on microglial phenotypes in the brain diseases?

2. NF1

2.1 Does a heterozygous *Nf1* KO have an impact on microglial membrane currents? Are there sex-specific differences?

2.2 Are microglial migration, process movement and phagocytic activity affected by a heterozygous *Nf1* KO? Are there sex-specific differences?

2.3 Which intracellular pathways are involved in microglial alterations in *Nf1*+/-?

3. TNC

3.1 Are microglial phagocytic activity, HDAC1 expression, cytokines release and migration affected by TNC KO postnatally and during adulthood?

3.2 Which microglial receptors and intracellular pathways are involved in TNC action on microglia?

3 Project I: The VGF-derived peptide TLQP21 impairs purinergic control of chemotaxis and phagocytosis in mouse microglia

Contribution in detail:

1. Participation -with the corresponding authors- in the brainstorming meetings to evolve the story.

2. Performing the following experiments: whole-cell patch clamping, in vitro phagocytosis assay, in situ phagocytosis assay, and migration assay.

3. Analysis of all the corresponding data.

4. Figures preparation, and writing the manuscript with Dr. Marcus Semtner.

5. Doing follow up experiments after releasing the manuscript to further elucidate the role of VGF and TLQP21 in the disease context; see outlook session.

Signature of the doctoral candidate

Signature of the supervisor

3.1 Summary

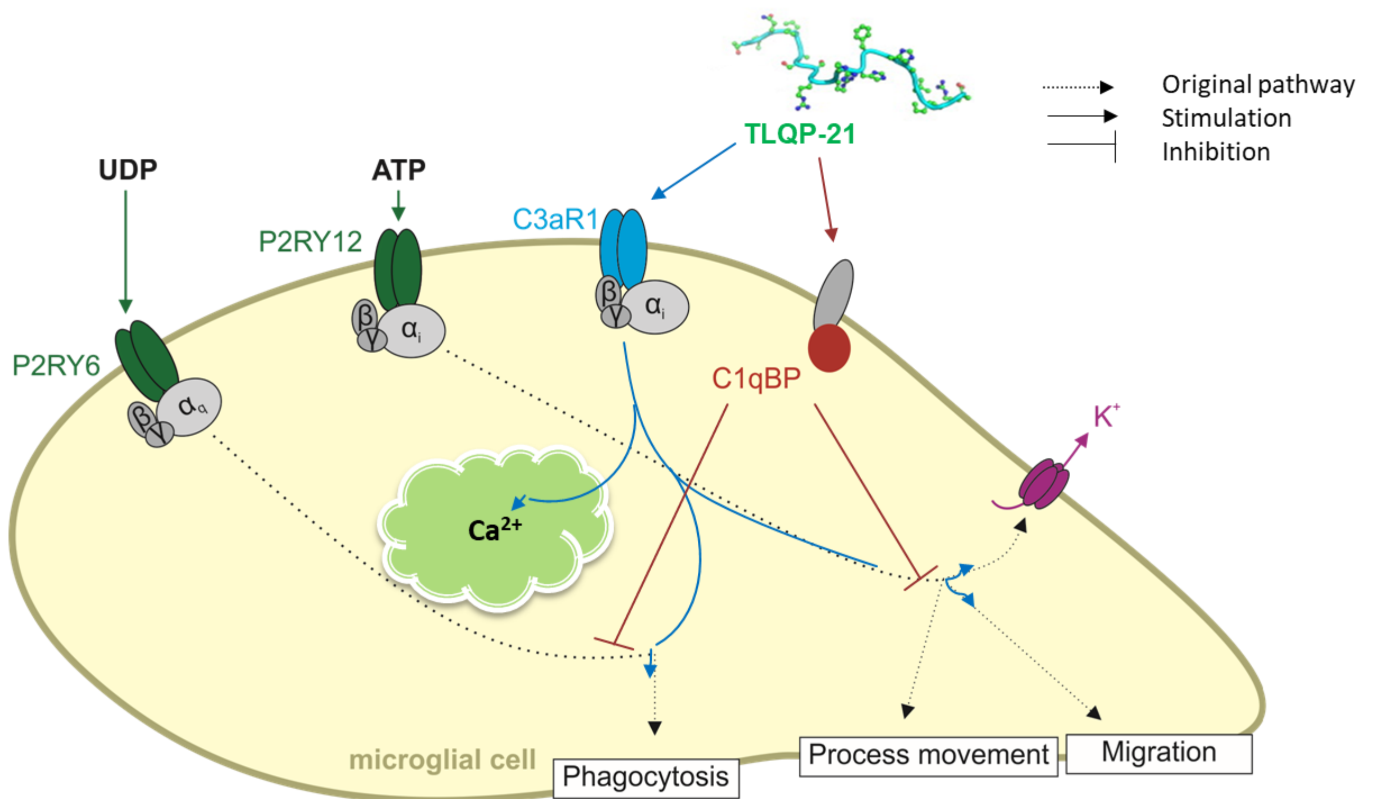

Figure 11. Graphical abstract summarizing the effect of TLQP21 on microglia. TLQP21 activates C3aR1 and consequently stimulates $[Ca^{2+}]_i$ release, phagocytosis, migration, and outwardly rectify K^+ currents. Moreover, when TLQP21 binds to C1qbP, it interferes with purinergic signaling and consequently suppress P2RY12-mediated K^+ currents, process movement and migration, as well as P2RY6-dependant phagocytosis.

In brief

Elmadany *et. al.* investigated the effect of the VGF-derived peptide TLQP21 on microglia as an emerging therapeutic target to manage many CNS disorders. TLQP21 activates microglial C3aR1 *in vitro* and consequently stimulate intracellular calcium release, outward rectifying potassium currents, phagocytosis, and migration. Furthermore, TLQP21 activates microglial C1qbp *in vitro* and *in situ,* and impairs metabotropic purinergic signaling in microglia; leading to suppression of P2Y6-mediated phagocytosis, and P2Y12-mediated outwardly rectifying potassium currents, process movement and migration. These data establish TLQP21 as a modulator of microglia.

3.2 Original publication

The Journal of Neuroscience, March 25, 2020 • 40(13):2606-2617
https://doi.org/10.1523/JNEUROSCI.1458-19.2020 (Please read this part online.)

3.3 Supplementary Figures

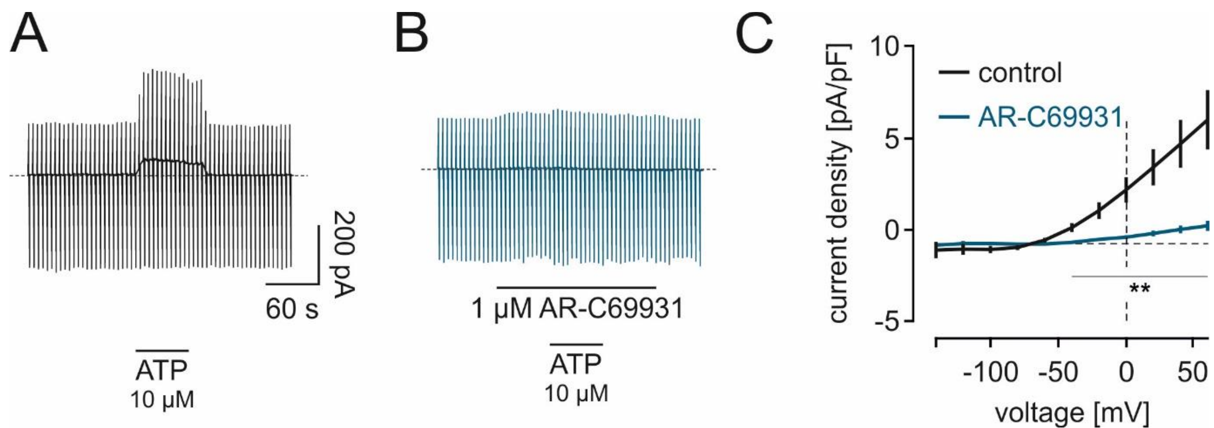

Figure 2-1. Blockade of ATP evokes membrane currents by a $P2Y_{12}$ receptor antagonist. (**A**) Representative traces of patch-clamp experiments on neonatal cultured microglia. Cells were voltage-clamped at 20 mV, and a series of pulses ranging from -170 mV to +60 mV was applied every 5 s. ATP (10 µM) was applied as indicated by bar. (**B**) Application of ATP (10 µM) in the presence of $P2Y_{12}$ receptor blocker AR-C69931 (1 µM). Applications indicated by bars. (**C**) Average current-voltage relationships of ATP (10 µM) - evoked currents in the absence (n = 9, control) or presence (n = 16) of 1 µM AR-C69931. Figure 2-1, TIF file

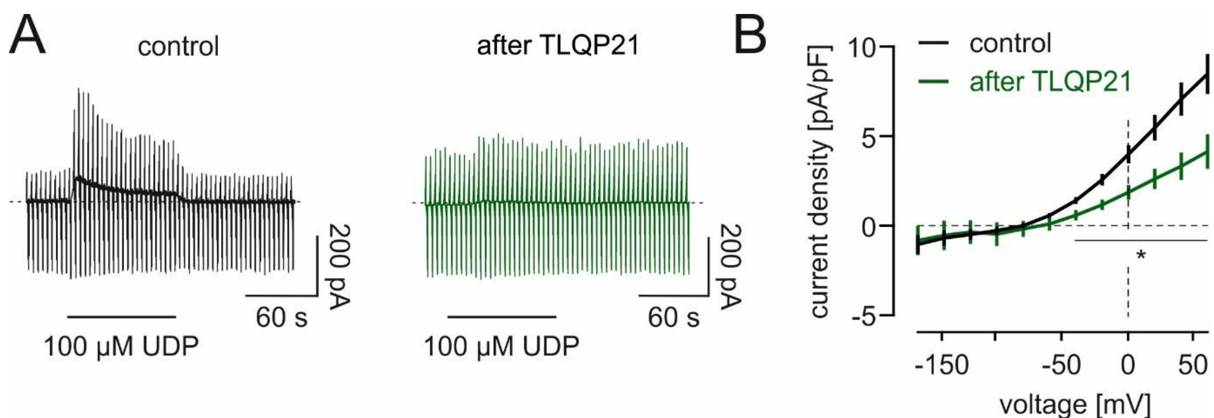

Figure 5-1. TLQP21 affects UDP-dependent signaling. (**A**) Representative patch-clamp recording on cultured neonatal microglia. Membrane currents recorded by the patch clamp technique of a microglial cell. UDP (100 µM) was applied as indicated by bars under control conditions (left) and 5 min after TLQP21 (100 nM for 60 s, right). (**B**) Average current density-voltage relationships of UDP (100 µM) - evoked currents without (black) or with (gray) prior application of 100 nM TLQP21 (60 s). Download Figure 5-1, TIF file

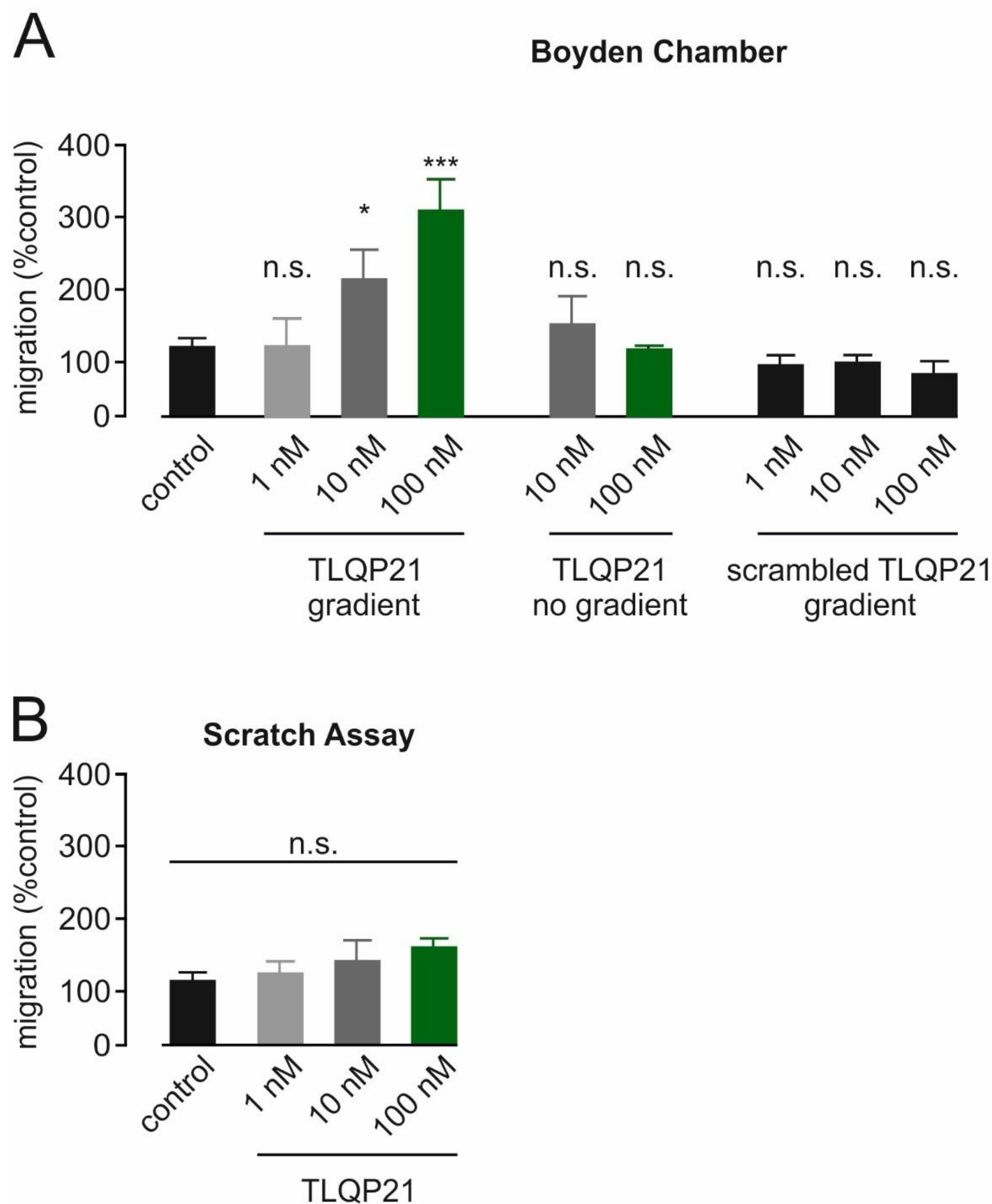

Figure 6-1. **Effect of TLQP21 on microglial migration.** (**A**) Boyden chamber assay: cells crossing the polycarbonate membrane (8 µm pore size) into the TLQP21-containing lower chamber were stained by Diff-quick and were displayed in light microscopic images, later processed with ImageJ for counting. TLQP21 was applied at the concentrations indicated, either with without (no) gradient as indicated. A scrambled TLQP21 peptide was also applied using a gradient. No gradient means that TLQP21 was present at the same concentration in the upper and lower chambers. (**B**) Scratch assay: the scratch was implemented on a layer of microglial cells, and cells migrating into the initial scratched area were analyzed after an incubation for 3 h (n = 9/group). TLQP21 was applied at the indicated concentrations. Download Figure 6-1, TIF file

3.4 Limitation

Producing the VGF knock out mouse model. The original plan of this project was to generate a VGF knock out (KO) mouse line and investigate the changes in microglial functional phenotype in comparison to WT mice, then to stimulate microglia of the VGF KO mice with the sole peptides. However, due to challenges in the viability of the mice I was unable to move forward with this plan. Consistent with findings from other labs, VGF knockout mice from the Salton lab were small, thin, hyperactive, hypermetabolic, and relatively infertile; the thing affected the viability of the pups and restricted the number of available mice for the experiments (Salton et al., 2000). Moreover, the brain of the *VGF +/-* pups was smaller than in WT at the same age. Therefore, I decided to go for conditional knocking out of VGF at the age of 4-6 weeks to permit physiological growth and maturation of the brain (see outlook section).

Age and gender consideration. All the *in situ* experiments in the present project were performed on male adult mice due to the availability of the mice. To our knowledge, no sexual dimorphism was demonstrated on TLQP21 effect in the CNS.

3.5 Follow up experiments and outlook

3.5.1 Elucidating the role of VGF in Alzheimer's disease through microglia

In 2017, our lab showed an impairment of microglial phagocytosis in a model of Alzheimer disease called 5xFAD mice*. This decrease in the phagocytosis index was linked to P2RY6 dysfunction, as well as alterations in microglial membrane currents in the vicinity of amyloid ß-plaques (Wendt et al., 2017). Knowing that the VGF-derived peptide TLQP21 interferes with P2RY6 signaling and modulates microglial membrane currents, I explored the influence of VGF and its peptide on plaque-associated microglia. As a pilot study, I stained 50 µm coronal slices from the cerebral cortex of 5xFAD mice (Figure 12). I could observe a clear pattern of VGF expression completely surrounding the plaques. Moreover, I found that VGF is colocalized with microglia in the vicinity of the plaques; suggesting that VGF might be involved in generating the unique phenotype of plaque-associated microglia.

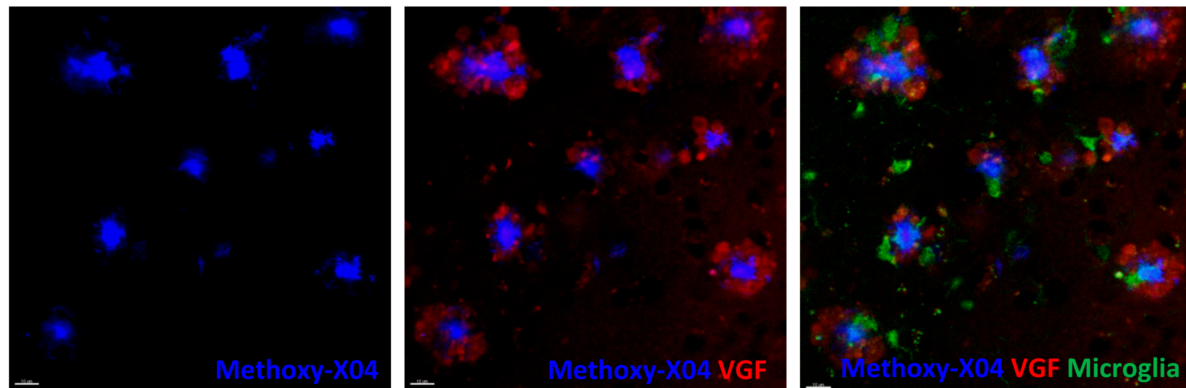

Figure 12. Accumulation of VGF peptides in the vicinity of amyloid ß-plaques. We equipped immunohistochemistry technique to visualize VGF, the plaques, and microglia. To stain the amyloid ß-plaques, we followed the same protocol used in our paper by (Wendt et al., 2017); using 100 µM Methoxy-X04-solution 40 % ethanol/ 60 % water solution adjusted to pH 10 with NaOH, in blue. C-terminus polyclonal antibodies were used to stain VGF; in red, whereas polyclonal antibodies against Iba1 were used to stain microglia; in green. Under 40X oil-immersion objective of LSM700 confocal microscope, VGF appeared surrounding the plaques and colocalized with microglia. Scale bar = 10 µm. Unpublished data.

Next, we designed a novel mouse model that permits conditional KO of VGF while preserving the 5xFAD mutations (Figure 13).

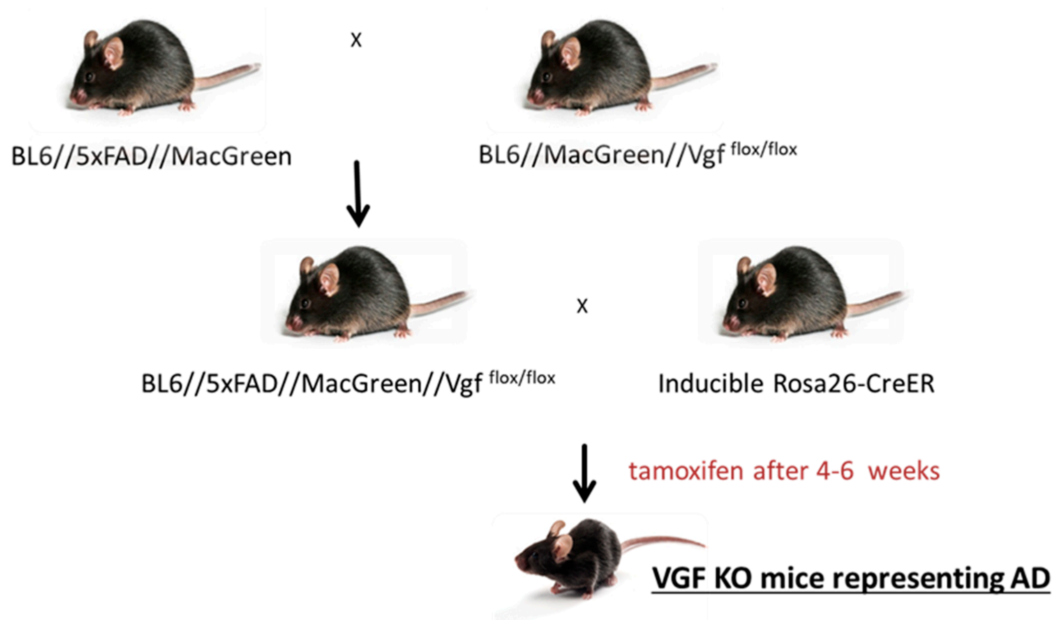

Figure 13. A novel mouse line of 5xFAD//VGF KO. The crossbreeding was started with 5xFAD mice from MacGreen BL6 background and VGF$^{flox/flox}$ mice from the same background. The resultant BL6//5xFAD//MacGreen//Vgf $^{flox/flox}$ mice were then crossbred with Rosa26-CreER[§] mice, and knocking out of VGF was induced by gavage administration of tamoxifene in an attempt to have VGF KO mice representing AD.

The crossbreeding was started with 5xFAD*//MacGreen mice and VGF$^{flox/flox¥}$ mice on a Bl6N background. Next, BL6//5xFAD//MacGreen//Vgf$^{flox/flox}$ mice were crossbred with the tamoxifen-inducible Rosa26-CreER§ mice. Tamoxifen was first administered by gavage, however, VGF was still detected as shown in Figure 14. Kocking out of VGF is currently achieved by i.p. administration of tamoxifen (100 mg/kg body weight). We will validate the KO using qPCR (F: CCCTCGACCATCGCTCATAC, R: GGTTTTCATGACCAACGGGC) and immunohistochemistry analysis after 4 weeks.

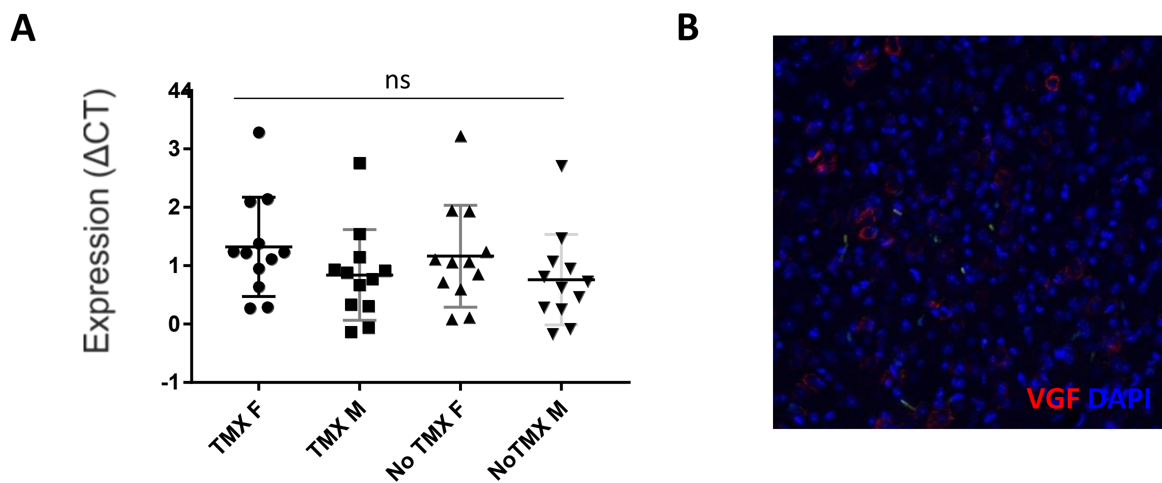

Figure 14. VGF expression after gavage administration of tamoxifen onto BL6//5xFAD//MacGreen//Vgf $^{flox/flox}$//Rosa26-CreER. (**A**) qPCR analysis of mRNA expression of VGF showing that VGF is still detected 4 weeks after tamoxifen administration, CT values were normalized to the house keeping gene hypoxanthine phosphoribosyl transferase (HPRT). (**B**) Immunohistochemistry staining confirmed the existence of VGF in the cortex, 1ry anti-VGF antibodies, 2ry antibodies (Cy3), 10X, LSM700 confocal microscope. TMX: Tamoxifen, F: female, M; male.

After confirming the KO of VGF in our novel 5xFAD//VGF KO mouse line the plan is:

- To investigate the memory, cognition and motor function of the mice at the age of 2, 5 and 8 months in comparison with 5XFAD and WT mice by behaviour tests.
- To explore microglial phagocytosis and process movement at the age of 2, 5 and 8 months in comparison with 5xFAD and WT mice.
- To study the single cell transcriptome of microglia at the age of 5 and 8 months in comparison with 5xFAD and WT mice.
- To study the electrophysiology of microglia at the age of 8 months in comparison with 5xFAD and WT mice.
- To explore the effect of exogenouse TLQP21 in absence of endogenous VGF and its peptides on microglia *in vitro* and *in situ*.

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* 5xFAD is a mouse model that represent AD. It expresses human APP and PSEN1 transgenes with a total of five AD-linked mutations: the Swedish (K670N/M671L), Florida (I716V), and London (V717I) mutations in APP, and the M146L and L286V mutations in PSEN1. www.alzforum.org/research-models

MacGreen is a reporter Cfs1r-EGFP transgenic mouse line, in which an expression of enhanced green fluorescent protein (EGFP) to cells expressing the macrophage colony-stimulating factor receptor gene (Cfs1r, c-fms, CSFlR, CD115); including microglia, is detectable (Sasmono and Williams, 2012).

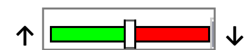
¥ VGF$^{flox/flox}$ is a mouse line in which vgf gene flanked by two knockin recognition sites of the Cre recombinase in order to delete (Kocking out) this gene after activation of the Cre recombinase by tamoxifen (Jackson and Abbott, 2000).

§ Rosa26-CreER is a mouse line in which a conditional Cre-ER cassette was introduced onto Gt(ROSA)26Sor promoter to generate tamoxifen-induced, Cre-mediated targeted deletions. www.jax.org/strain/004847

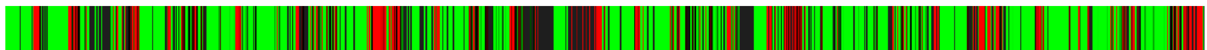
3.5.2 Elucidating the role of VGF/TLQP21 in glioblastoma multiforme

Wang *et al.* demonstrated that the neuropeptide VGF plays a critical role in maintaining the stemness and promoting the survival of glioblastoma stem cells (GSCs) (Wang et al., 2018). In addition, the authors found that VGF potentiates the survival of differentiated glioblastoma cells (DGCs) and triggers the release of BDNF that activates NTRK2 receptors on GSCs. This VGF-BDNF-NTRK2 paracrine signaling confer growth advantage for GBM. Therefore, I hypothesized that the VGF-derived peptide TLQP21 might itself contribute to GBM tumorigenesis and might affect TAMs as well and modulate their functions.

To research whether a correlation between TLQP21 and GBM exist, I checked the online RNA-seq database to see if GBM human samples express TLQP21 receptors (Figure 15). Indeed, GBM human samples were found to express TLQP21 receptors, however, it is not clear if these receptors are expressed in TAMs or the cancerous cells themselves.



C1qbp



C3aR1

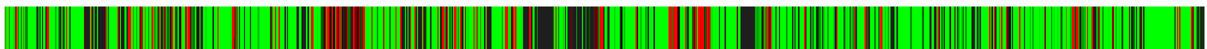


Figure 15. TlQP21 receptors are expressed in GBM at variant degrees. TLQP21 receptors, C1qbp and C3aR1 are expressed in human GBM samples. Data extracted from online Allen Institute RNAseq database https://glioblastoma.alleninstitute.org/rnaseq.

As a preliminary step to see if TLQP21 affects the tumor growth differently in tissues where microglia are present, I (with my master student Ms. Fatma Cherif) inoculated TLQP21-stimulated mCherry-labelled GL261 cells onto organotypic brain slices (OBS) from macGreen mice at P14 age according to the established protocol in our lab; see (Huang et al., 2020). Intrestingly, TLQP21 significantly increased the size of the tumor *in situ*, indicating that TLQP21-mediated tumor growth is through microglia. By depleting microglia from the OBS slices, the TLQP21 effect on tumor growth was suppressed (Figure 16).

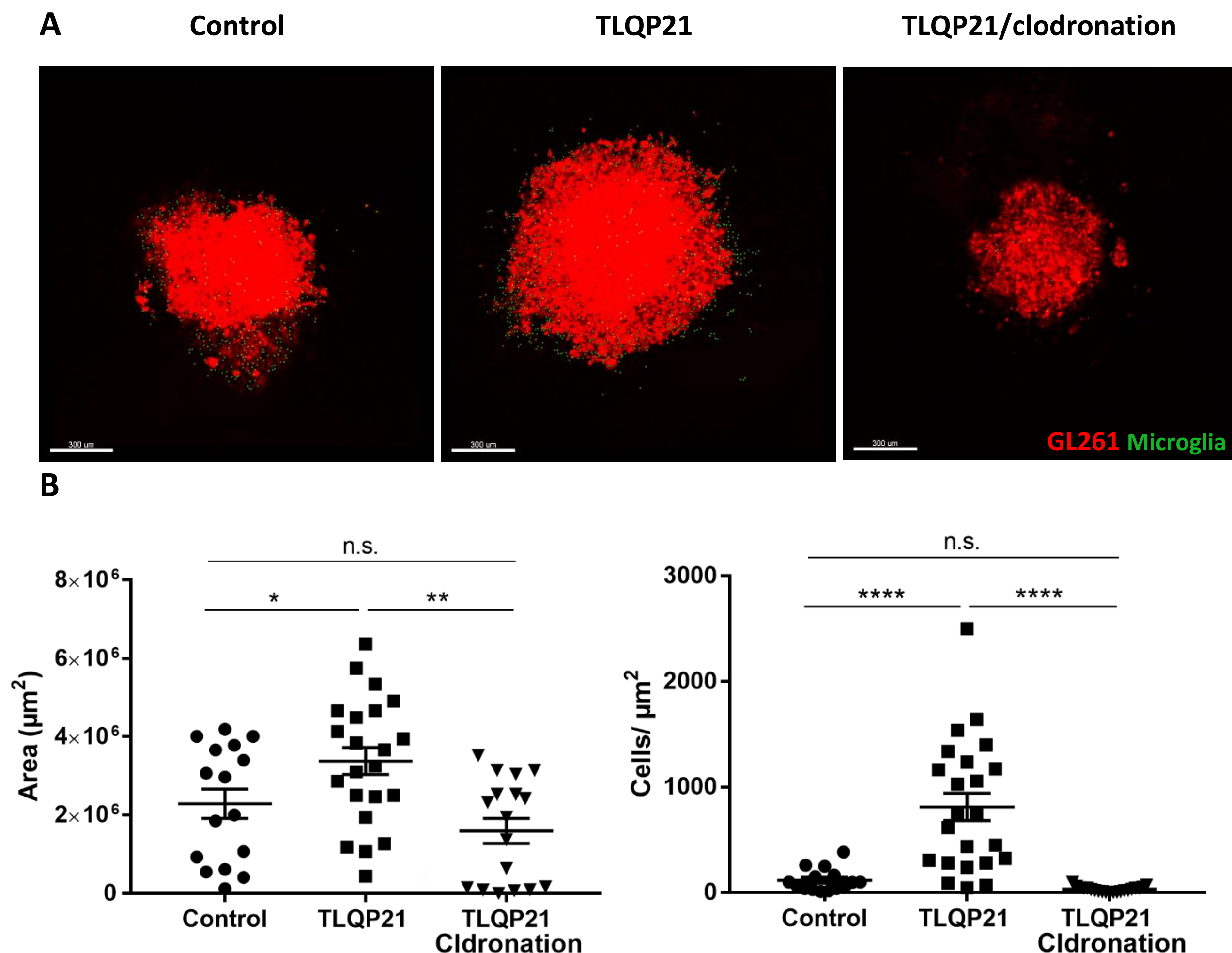


Figure 16. TLQP21 increases GL261 tumor growth *in situ*. (**A**) Inoculating TLQP21-stimulated mCherry-GL261 cells onto OBS from MacGreen mice produce larger tumors in 12 slices from 4 macGreen mice at the age of P14. (**B**) The tumor area in the OBS was increased by TLQP21 stimulation, and this effect was suppressed by depleting microglia from the slices. (**C**) TLQP21 increased the migration of microglia in the tumor. (**B**, **C**) The significance was measured by one-way annova followed by tukey's post hoc test. Scale bar = 10 µm. Unpublished data.

For this part of the project, the following points are to be investigated:

- Inoculating mCherryGL261 cells onto OBS from our VGF KO mice and compare the size to OBS from WT mice
- Inestigating the tumor and the tumor associated microglia response to TLQP21 in VGF KO and VGF WT OBS.
- Inestigating the tumor response of to temazolamide in VGF KO and VGF WT OBS with and without stimulation by TLQP21.

4 Project II: Neurofibromatosis type 1 - mutant microglia exhibit a sexually-dimorphic cAMP-dependent impairment of purinergic function

Contribution in detail:

1. Participation -with the corresponding authors- in the brainstorming meetings to discuss my data and evolve the story.

2. Performing the following experiments: whole-cell patch clamp and in situ phagocytosis assay.

3. Teaching the trainee and my colleague (R. P. and F. L.) the *in situ* phagocytosis assay, as they helped me; mainly in the scanning and Imaris rendering steps.

3. Analysis of the corresponding data.

4. Figures preparation, and writing the manuscript with Dr. Marcus Semtner.

5. Doing follow up experiments after releasing the manuscript to further characterize NF1 mutation in iPSCs-derived microglia; see outlook session.

Signature of the doctoral candidate

Signature of the supervisor

4.1 Summary

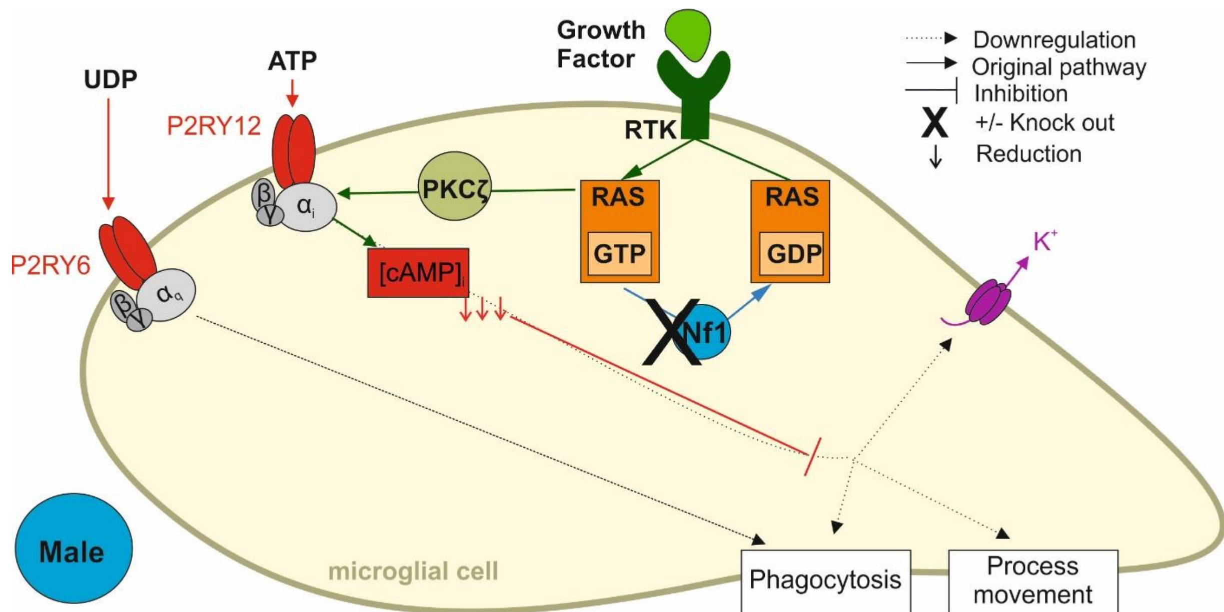


Figure 17. Graphical abstract illustrating the sexually dimorphic cAMP-dependant defects in purinergic signaling that was observed in male *Nf1 +/-* microglia. Knocking out NF1in male mice stops its GTP-inactivating signals, activates RAS, and promotes PKCζ-mediated stimulation of P2RY12 which is G_iPCR. Consequently, adenylyl cyclase is inhibited and produces lower level of cAMP. The low level of cAMP impairs P2RY12-mediated K^+ currents, process movement and phagocytosis, whereas no changes were observed on P2Y6-dependant phagocytosis.
RTK-RAS-PKCζ-GPCR-cAMP pathway is based on (Gutmann et al., 2017).

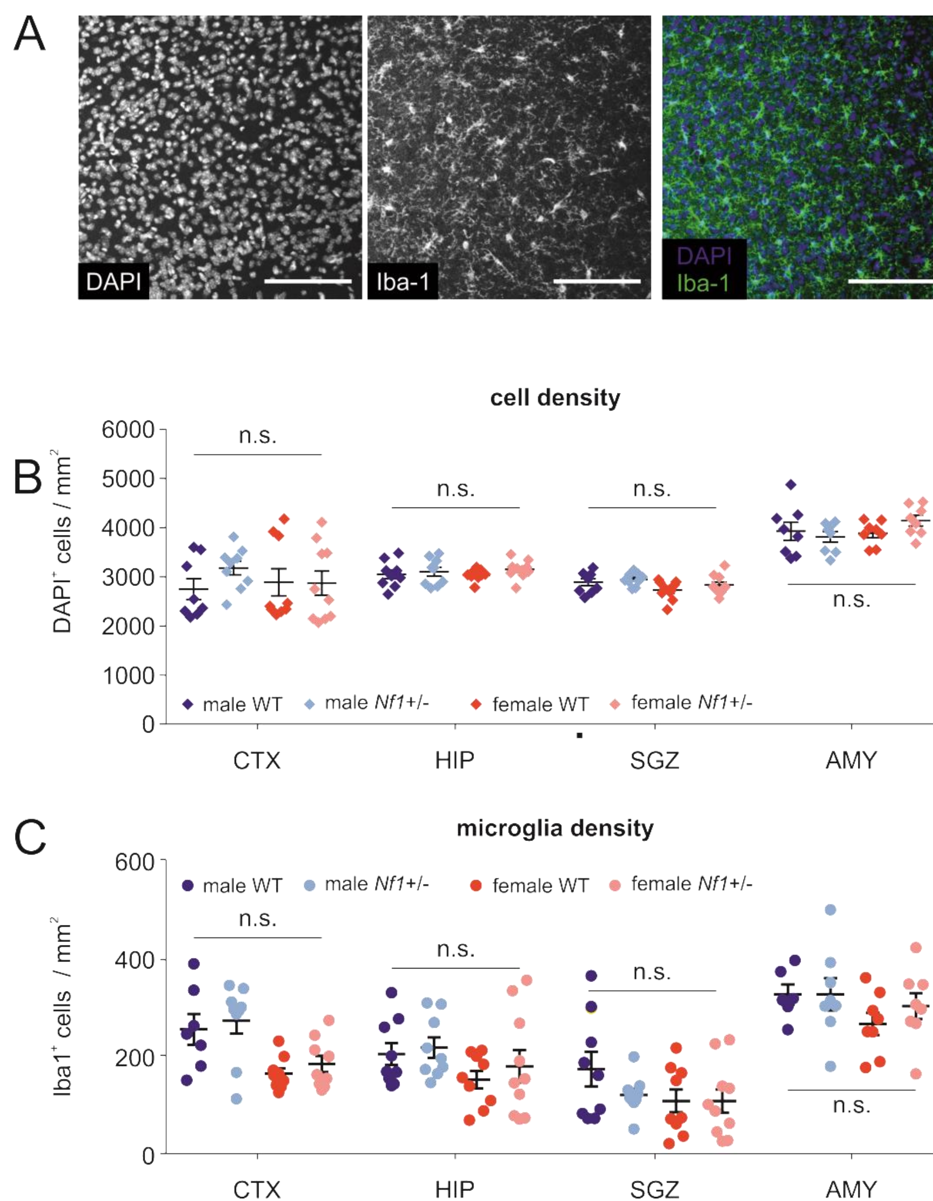
In brief

Elmadany *et. al.* investigated the influence of sex and NF1 knock out, on microglial activities. *Nf1 +/-* microglia exhibit reduced basal phagocytosis regardless the sex, whereas P2RY12-dependent stimulation of phagocytosis was diminished only in male *Nf1 +/-* microglia. Consistently, ATP-induced P2RY12-mediated membrane currents and laser lesion-induced process movement were suppressed only in male *Nf1 +/-* mutant microglia. Elmadany *et. al.* further elaborated the mechanism behind this purinergic signaling impairment and detected a reduction in the intracellular cAMP levels in male but not female *Nf1 +/-* microglia which could be resolved by pharmacological blockade of phosphodiesterase 3 to increase cAMP level.

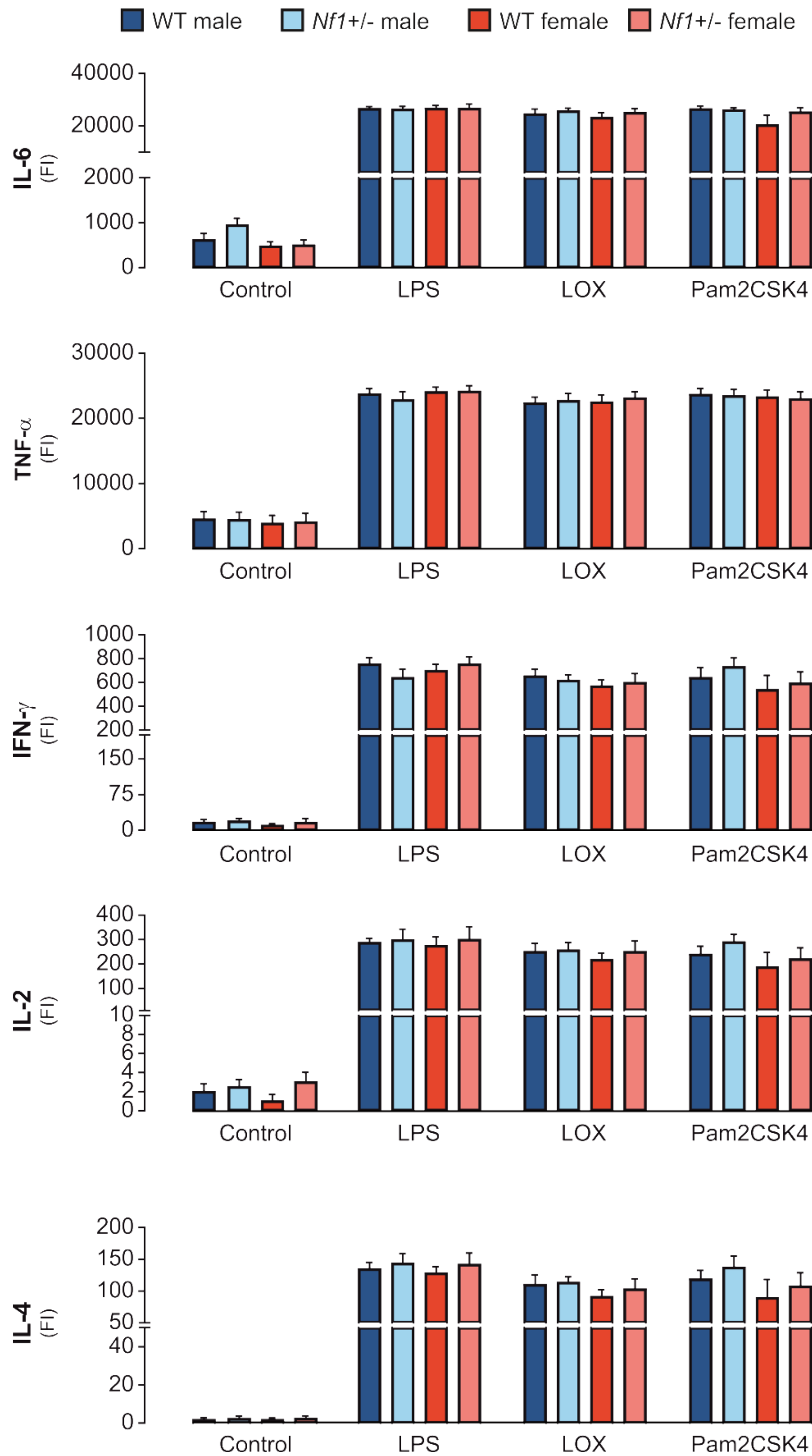
4.2 Original publication

Neurobiology of Disease 144 (2020) 105030
https://doi.org/10.1016/j.nbd.2020.105030 (Please read this part online.)

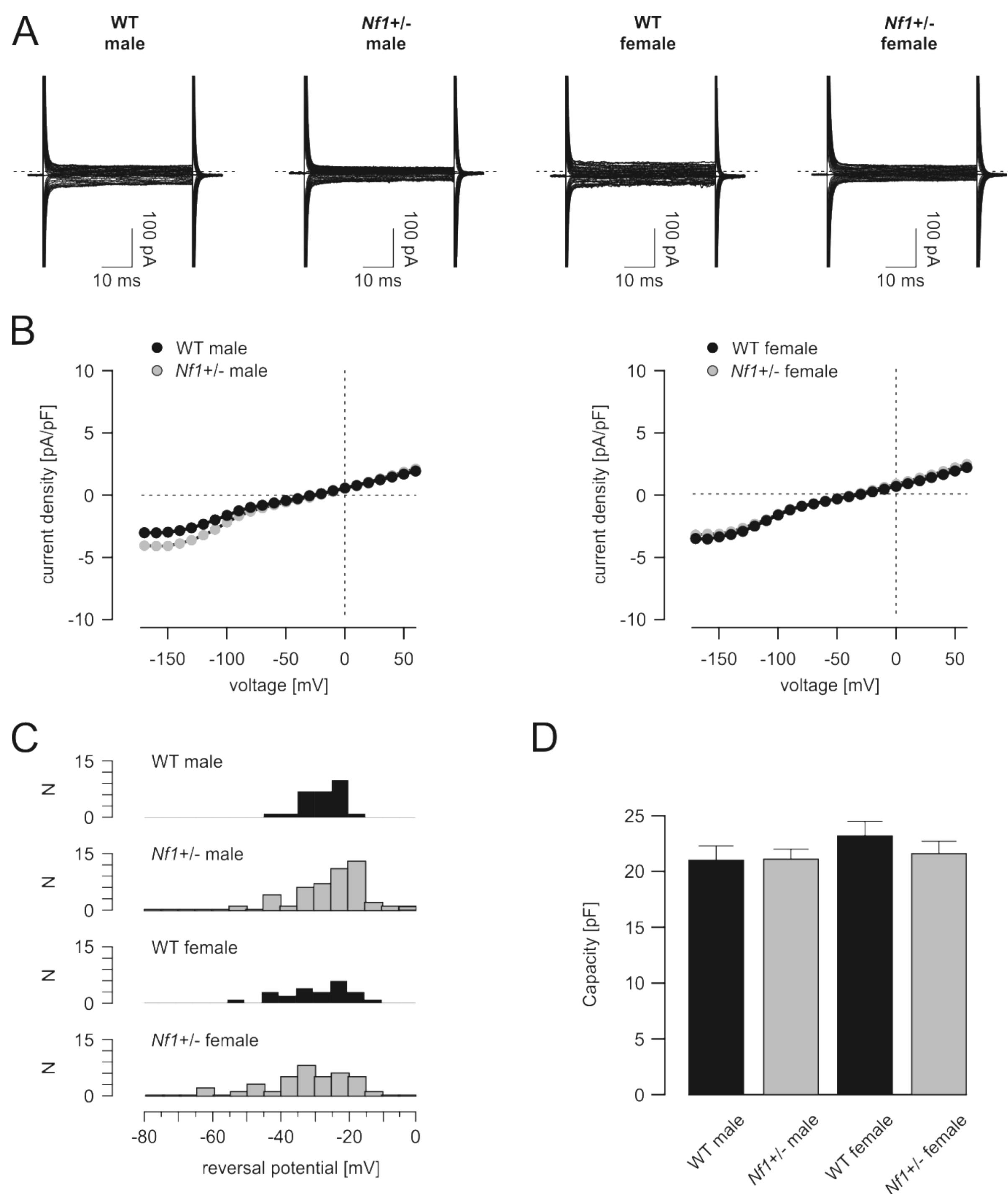
4.3 Supplementary Figures



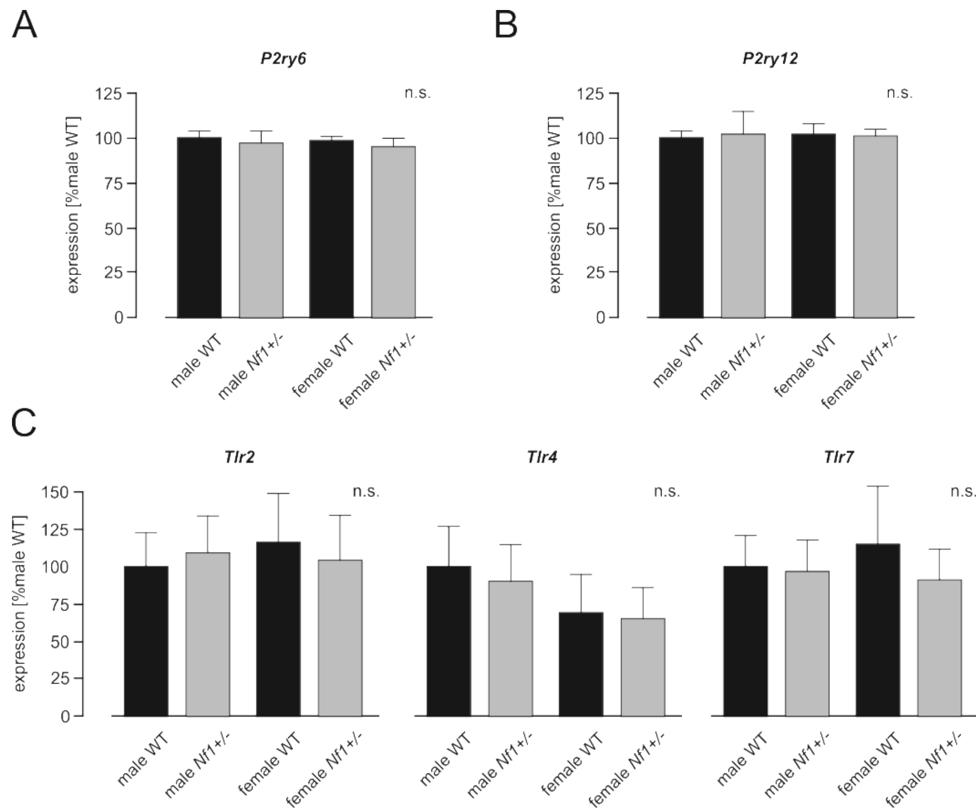
Supplementary Figure 1. Cellular and microglial densities in different brain regions of male and female WT and *Nf1 +/-* mice. (**A**) Cortical DAPI and Iba1 staining from a representative male WT mouse brain (Scale bar: 100 µM). (**B** and **C**) Total cellular (**B**) and microglial (**C**) density values obtained from brain slices of 12-16-week-old male and female WT and *Nf1+/-* mice. The following brain regions were analyzed: medial prefrontal cortex (CTX), hippocampal CA2-CA3 regions (HIP), subgranular zone (SGZ), and amygdala (AMY). N=9-10 animals per condition were analyzed. Two way ANOVA followed by Bonferroni post hoc tests were performed, which revealed no significant differences in cell numbers between the four groups (male and female WT and *Nf1+/-* mice).



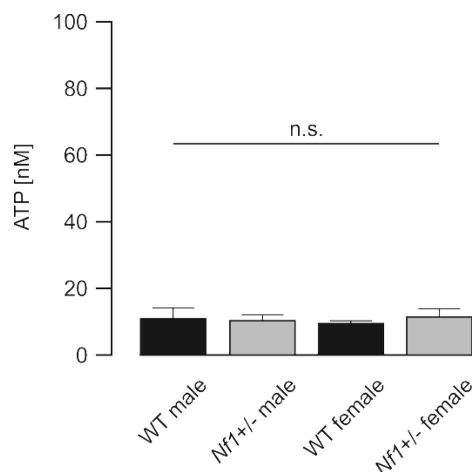
Supplementary Figure 2. Adult cultured microglia, derived from *Nf1+/-* and WT male and female mice, do not differ in terms of cytokine release following TLR activation. Adult microglia cultures were stimulated with LPS (100ng/ml), LOX (1mM) or Pam2CSK4 (100ng/ml) for 24 hours. Supernatants were collected, and Multiplex ELISA was performed to analyze microglial release of IL-6, TNF-α, IFN-γ, IL-2 and IL-4. Data are shown as the mean Fluorescent Intensity (FI). Unstimulated cells were used as controls. N=5 independent microglia cultures were used per condition. One way ANOVA followed by Bonferroni post hoc tests revealed no significant differences in cytokine release between male and female WT and *Nf1*+/- microglia.



Supplementary Figure 3. Membrane properties of *Nf1+/-* microglia are similar to WT microglia. (**A**) Sample currents of microglia from 12-16 week old WT and *Nf1+/-* mice, which were obtained during a series of voltage pulses ranging from -170 to +60 mV, with 10 mV increments from a holding potential of -70 mV. (**B**) Summarized and averaged current-voltage relationships from 14-16 week old WT and *Nf1+/-* microglia. Currents were normalized to the membrane capacitance before averaging. See panel A for sample traces. There were no significant sex- or *Nf1*-dependent differences (ANOVA/Tukey). (**C**) Distribution of the reversal potentials (indicative of the membrane potential), shown as averaged histograms of all recorded microglial cells from male and female WT and *Nf1+/-* mice. (**D**) Summary of the membrane capacitances of microglia from WT and *Nf1+/-* mice. There were no significant sex- or *Nf1*-dependent differences (ANOVA/Tukey). Number of recorded cells (mice): male WT, 32 (13); male *Nf1+/-*, 42 (18); female WT, 23 (13); female *Nf1*+/-, 37 (17).



Supplementary Figure 4. Expression of purinergic or Toll-like receptors is not sexually dimorphic or influenced by *Nf1* mutation. (**A** – **C**) qPCR on cDNA from male and female WT and *Nf1+/-* microglia revealed no changes in *P2ry6* (**A**), *P2ry6* (**B**) or *Tlr2*, *Tlr4*, and *Tlr7* (**C**) mRNA expression. N = 4-5 mice were used per condition. One way ANOVA followed by Tukey post hoc tests revealed no significant differences in mRNA expression between male and female WT and *Nf1*+/- microglia (**A**-**C**).



Supplementary Figure 5. Viability of MACS-isolated microglia is not sexually dimorphic or influenced by *Nf1* mutation. Viability was tested using a commercial assay (see Materials and Methods section) based on determining the intracellular ATP content as a measure of cell metabolism. Male and female WT and *Nf1*+/- microglia displayed similar intracellular ATP levels indicative of similar viability following the MACS isolation procedure. N = 3 mice were used per condition. One-way ANOVA followed by Tukey post hoc tests revealed no significant differences in the cell viability of MACS-isolated microglia from male and female WT and *Nf1*+/- mice.

4.4 Follow up experiments and outlook

Characterization of *Nf1 +/-* mutant microglia derived from human iPSCs

By using three isogenic series of human iPSC and the same control human iPSC line, a follow up project in our lab by Leonard D. Kuhrt -I am part of the team working on this study- is running to confirm my murine NF1 findings on iPSCs-derived microglia (iMG) model. Each of the three lines containing one different NF1 patient germline NF1 gene mutations engineered by CRISPR/Cas9 technology. In this follow up project, I have started studying the electrophysiology of *Nf1 +/-* iMG and compare it to WT iMG. Basal membrane currents of mutant and WT iMG were similar, whereas purinergic signaling is altered in *Nf1 +/-* iMG (Figure 18). Intriguingly and unlike the findings on murine microglia, P2X was absent in mutant microglia but not in WT. Moreover, the P2Y-mediated K^+ currents were lower in mutant iMG consistent with the findings in murine *Nf1 +/-* microglia. Further experiments are performed currently for a detailed characterization of *Nf1 +/-* iMG.

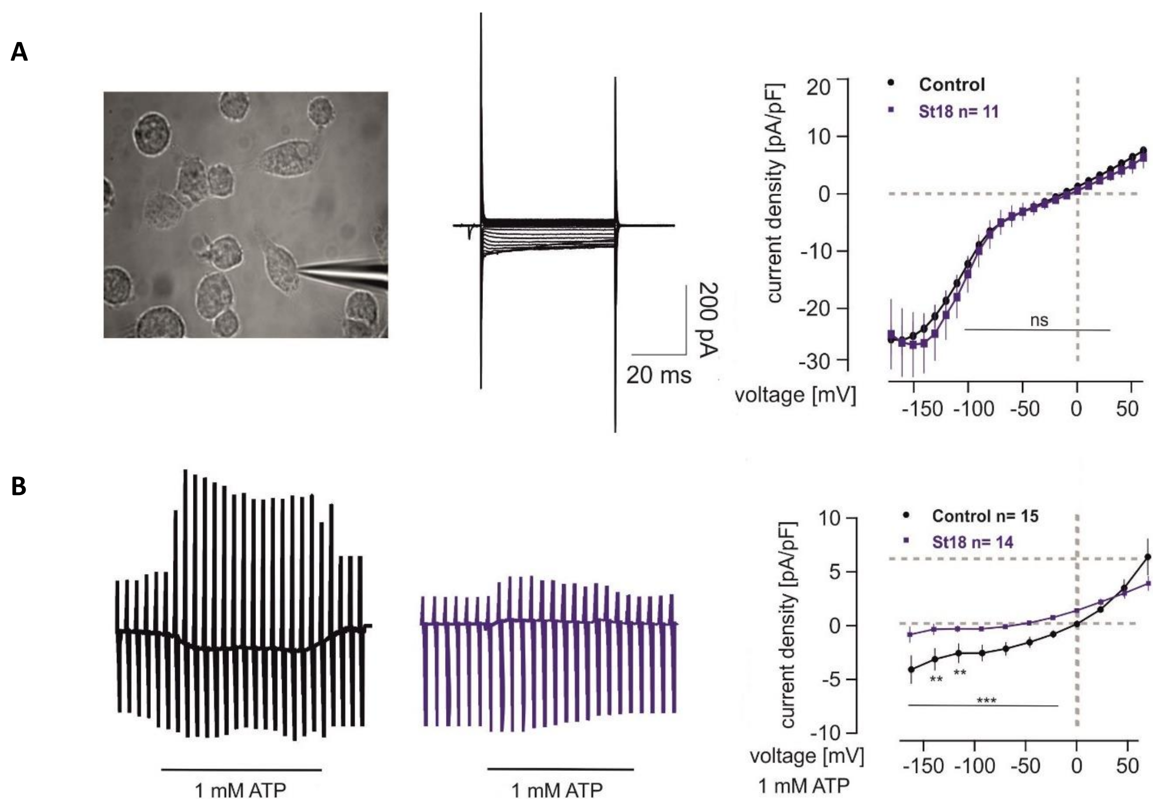


Figure 18. Purinoreceptors-mediated Potassium currents but not basal currents are altered in *Nf1 +/-* iMG. (**A**) Transmission light image showing iMG that was clamped for basal currents recording, no differences were reported among *Nf1* +/- and WT iMG. (**B**) When 1mM ATP was applied, P2X-mediated inward potassium currents were detected only in WT but not *Nf1* +/- iMG cells. St18: *Nf1* +/- iMG line. Unpublished data.

5 Project III: Tenascin C regulates multiple microglial functions involving TLR4 signaling and HDAC1

Contribution in detail:

1. Performing the *in situ* phagocytosis assay in WT, TNC KO and Tlr4 KO mice

2. Optimizing the rendering and analysis protocol to measure the intensity of TNC surrounding microglia in correlation to HDAC1 expression, using Imaris software.

3. Analysing data resulted from the *in situ* phagocytosis assay, preparing the corresponding figures, and writing a description of the assay in the method section of the manuscript.

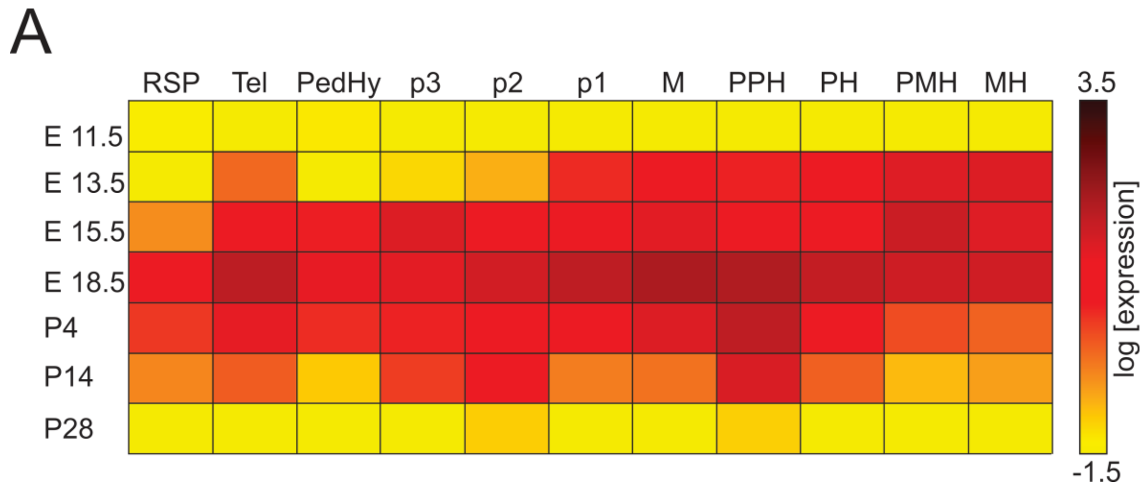
Signature of the doctoral candidate

Signature of the supervisor

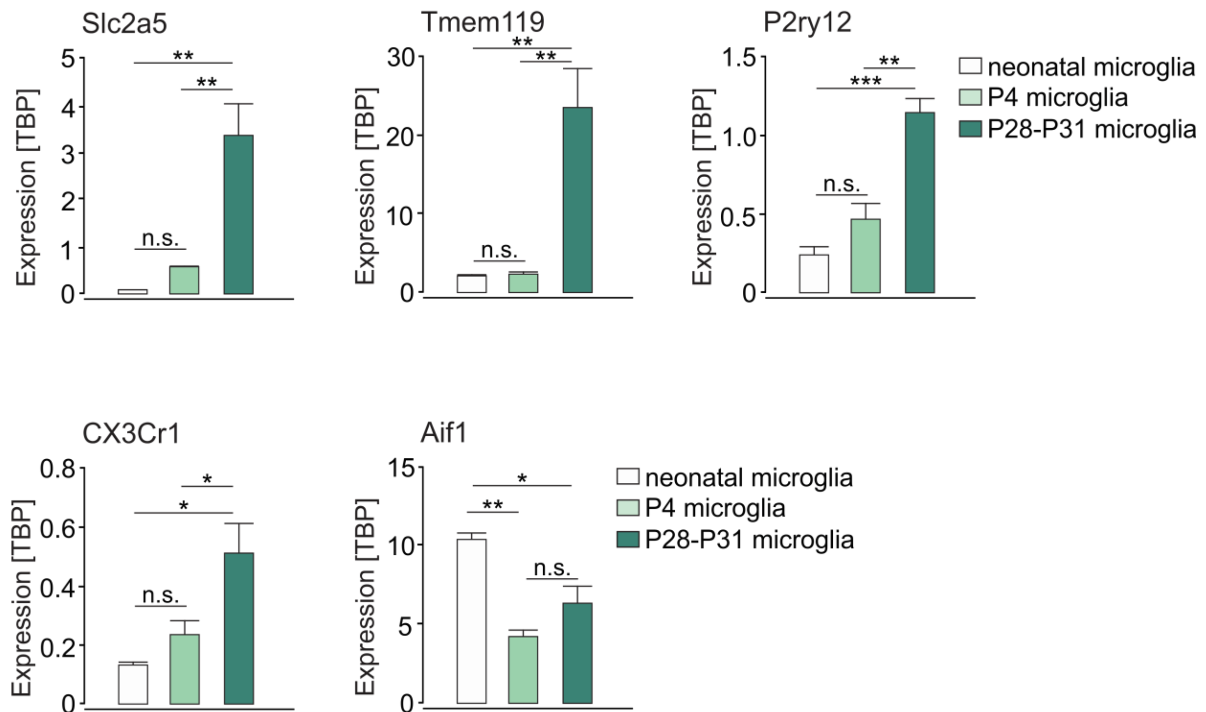
5.1 Original publication

Brain, Behavior, and Immunity 81 (2019) 470-483
https://doi.org/10.1016/j.bbi.2019.06.047 (Please read this part online.)

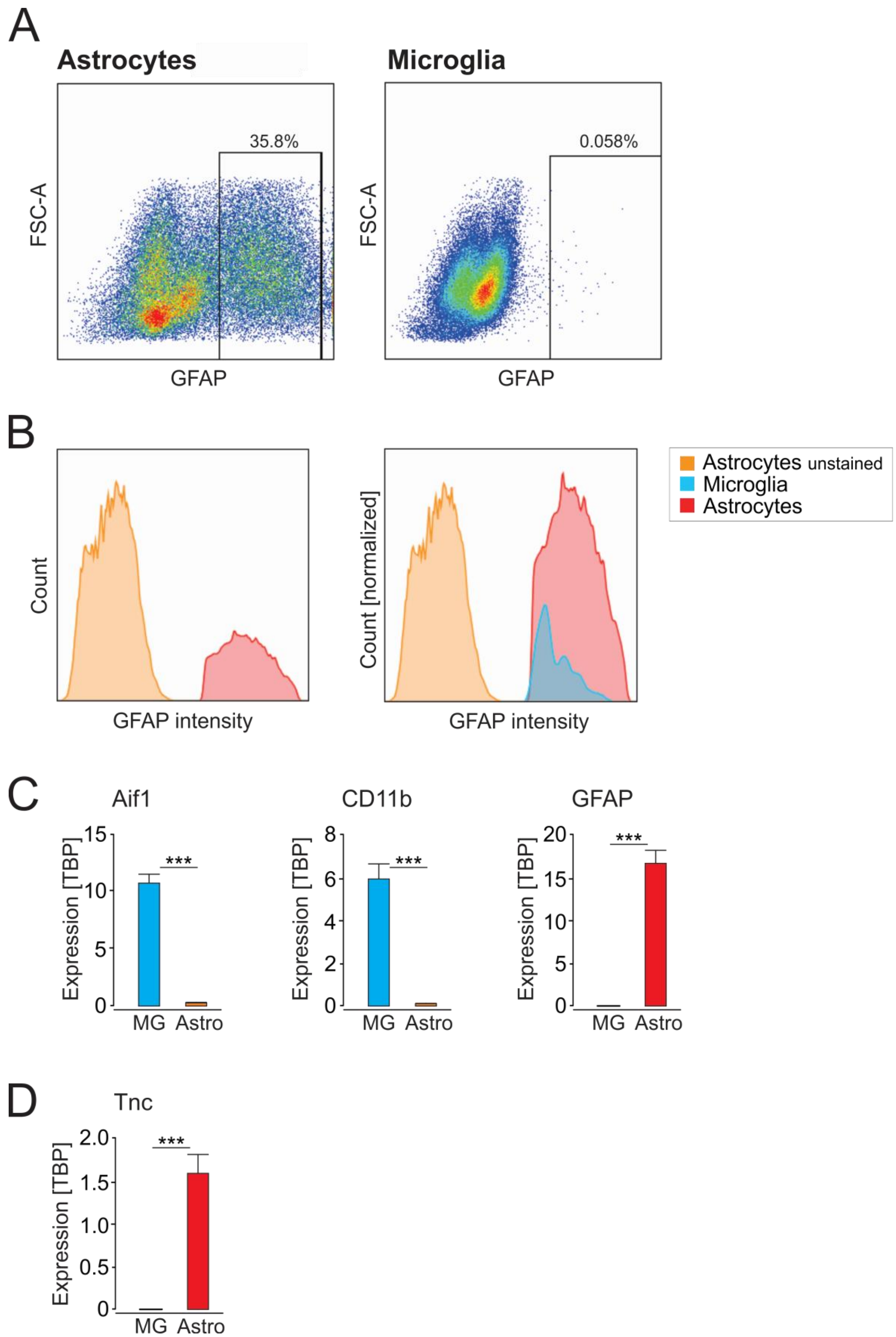
5.2 Supplementary Figures



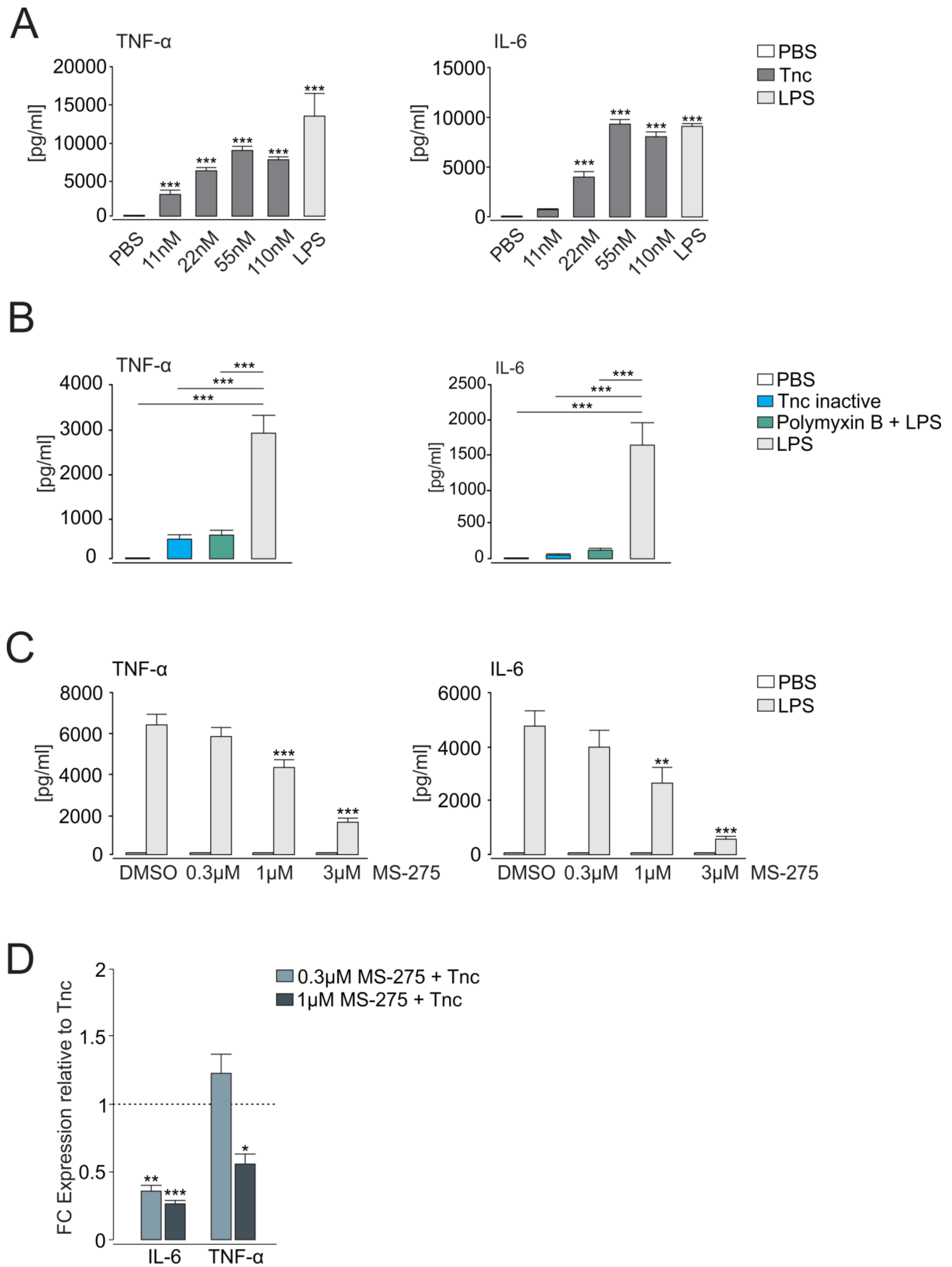
Supplementary Figure 1. Tenascin C expression at selected time points during mouse CNS development. A. Heatmap showing the expression of Tnc at different developmental stages (E11.5; E.13.5; E15.5; E18.5; P4; P14; P28) in different brain regions, modified from the Allen Developing Mouse Brain Atlas (RSP = rostral secondary prosencephalon; Tel = telencephalic vesicle; PedHy = peduncular hypothalamus; p3 = prosomere 3; p2 = prosomere 2; p1 = prosomere 1; M = midbrain; PPH = prepontine hindbrain; PH = pontine hindbrain; PMH = pontomedullary hindbrain; MH = medullary hindbrain).



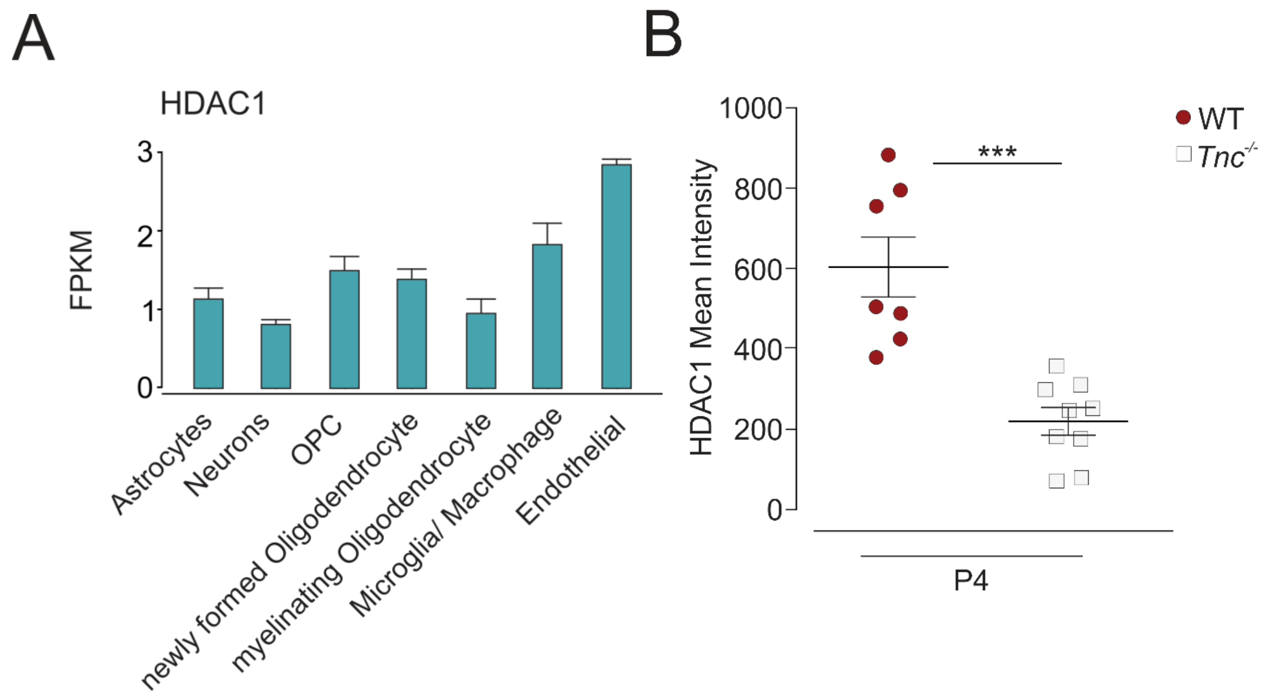
Supplementary Figure 2. Expression of microglia markers in primary neonatal microglia and freshly isolated microglia from P4 and P28-P31 WT mice. Primary neonatal microglia, prepared as previously described (Prinz et al., 1999), possess a range of microglia-specific features including electrophysiological properties (Boucsein et al., 2000), spontaneous calcium elevations (Korvers et al., 2016), the ability to migrate (Ifuku et al., 2016), to phagocyte and to produce cytokines. Recent studies challenged the nature of primary microglia cultures and their comparability with freshly isolated microglia (Butovsky et al., 2014; Gosselin et al., 2017). We assessed the expression of several microglia marker genes (Haage et al., 2019) in our cultures and compared them to freshly isolated microglia from P4 and P28-P31 mice. **A**. Expression levels of the microglia markers *Slc2a5*, *Tmem119*, *P2ry12*, *Cx3Cr1* and *Aif1* in primary neonatal microglia and microglia freshly isolated from P4 and P28-P31 mice measured by qRT-PCR (n = 3). CT values were normalized to the housekeeping gene *Tbp* (TATA-box binding protein). Values are given as mean ± SEM. For statistical analysis, one-way ANOVA followed by Tukey´s multiple comparisons test was performed. n.s. = $p > 0.05$; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. In conclusion, marker expression of primary neonatal microglia reflects P4 microglia more than P28-P31 microglia and they therefore render early postnatal microglia *in vitro*. Differences in marker expression between neonatal and P4 vs. P28-P31 microglia are due to an increase of some of the markers in the course of postnatal development (*Slc2a5*, *Tmem119, P2ry12*, *Cx3Cr1*) (Bennett et al., 2016). Differences in *Aif1* expression between neonatal microglia vs. P4 and P28-P31 microglia might be explained by cell culture conditions which rather mimick a pathologic environment (He et al., 2018).


A
Astrocytes
Microglia
35.8%
0.058%
FSC-A
GFAP
B
Count
Count [normalized]
GFAP intensity
Astrocytes unstained
Microglia
Astrocytes
C
Aif1
CD11b
GFAP
Expression [TBP]
***
MG Astro
D
Tnc

Supplementary Figure 3. Quality control of primary neonatal microglia cultures. Since primary neonatal microglia are harvested from an astrocytic feeding layer, where astrocytes are the main source of Tnc (Faissner and Steindler, 1995; Gates et al., 1995), we additionally tested the purity of our primary neonatal microglia cultures by fluorescence activated cell sorting, as well as by qRT-PCR for microglial and astrocytic marker expression. We here confirmed the purity of our primary microglia cultures excluding the possibility that Tnc derived from primary astrocytes might perturb our *in vitro* platform. **A.** Flow cytometry analysis of primary neonatal microglia. Graphs show forward scatter area (FSC-A) vs. GFAP (glial fibrillary acidic protein) intensity in astrocytes (left graph) and microglia (right graph). Astrocytes were obtained directly after shaking off the microglia. **B.** Left graph represents histogram of GFAP intensity in unstained astrocytes (orange), GFAP-stained astrocytes (red) and GFAP-stained microglia (blue) without normalization to counts; right graph shows histogram of GFAP intensity in unstained astrocytes (orange), GFAP-stained astrocytes (red) and GFAP-stained microglia (blue) with normalization to counts. **C.** Expression of microglia markers *Aif*, *CD11b* and astrocyte marker *GFAP* in primary neonatal microglia and astrocytes measured by qRT-PCR (microglia $n = 3$; astrocytes $n = 4$). CT values were normalized to the housekeeping gene *Tbp* (TATA-box binding protein). Values are given as mean ± SEM. For statistical analysis, Student´s t- test was performed. *** = $p < 0.001$. **D.** Expression of Tnc in astrocytes compared to primary neonatal microglia, as measured by qRT-PCR (microglia $n = 3$; astrocytes $n = 4$). CT values were normalized to the housekeeping gene *Tbp* (TATA-box binding protein). Values are given as mean ± SEM. For statistical analysis, Student´s t- test was performed. *** = $p < 0.001$.


A
TNF-α
IL-6
[pg/ml]
20000
15000
10000
5000
0
PBS
11nM
22nM
55nM
110nM
LPS
***
PBS
Tnc
LPS
B
TNF-α
IL-6
4000
3000
2000
1000
2500
1500
500
PBS
Tnc inactive
Polymyxin B + LPS
LPS
C
TNF-α
IL-6
8000
6000
4000
2000
DMSO
0.3µM
1µM
3µM
MS-275
**
D
FC Expression relative to Tnc
2
1.5
1
0.5
0
0.3µM MS-275 + Tnc
1µM MS-275 + Tnc
IL-6
TNF-α
*

Supplementary Figure 4. A. Dose-response curve of IL-6 and TNF-α secretion from primary neonatal microglia treated with PBS as control or different concentrations of Tnc (11 nM, 22 nM, 55 nM, 110 nM) or LPS (100 ng/ml) as positive control for 24hrs. Harvested supernatant was measured by ELISA for secreted IL-6 or TNF-α (n = 5-7). **B. ELISA control experiments with heat-inactivated Tnc and LPS-inhibitor Polymyxin B.** Cytokine release of TNF-α and IL-6 from WT primary microglia measured by ELISA 24hrs after treatment (n = 3). Microglia were treated with heat-inactivated Tnc (55 nM; Tnc inactive), pretreated with LPS-inhibitor Polymyxin B (25µg/ml) for 30min prior to LPS stimulation (100ng/ml) or treated with LPS (100ng/ml) as a positive control. For statistical analysis, one-way ANOVA followed by Tukey´s multiple comparisons test was performed. Values are given as mean ± SEM. *** = $p < 0.001$. **C and D. Effects of MS-275 on microglial cytokine/chemokine expression and synthesis. C.** ELISA showing dose-dependent inhibition of IL-6 and TNF-α secretion in LPS-stimulated microglia pretreated with MS-275. Primary neonatal microglia were pretreated with MS-275 (300 nM, 1 µM, 3 µM) or DMSO as control for 24hrs, then stimulated with LPS for 24hrs. Subsequently, the supernatants were collected, and cytokine secretion was measured by ELISA (n = 5). For statistical analysis, one-way ANOVA followed by Tukey´s multiple comparisons test was performed. ** = $p < 0.01$; *** = $p < 0.001$. Significance was tested versus LPS stimulation without inhibitor. **D**. Expression levels of the cytokines, *Il6* and *Tnf-α*, in primary microglia pretreated with 300 nM or 1 µM MS-275 or DMSO for 24hrs before stimulation with Tnc (55 nM) for 6hrs measured by qRT-PCR (n = 3). CT values were normalized to the housekeeping gene *Tbp* (TATA-box binding protein). Bars represent log fold change in expression of each cytokine in relative to Tnc-treated DMSO control. Values are given as mean ± SEM. For statistical analysis, Student´s t-test was performed. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.



Supplementary Figure 5. A. HDAC1 expression in different CNS cell types at age P7. Data were extracted from published RNA-sequencing datasets (Zhang et al., 2014) and show HDAC1 expression (FPKM) in astrocytes, neurons, oligodendrocyte precursor cells (OPCs), newly formed oligodendrocytes, myelinating oligodendrocytes, microglia/macrophages and endothelial cells. **B. Quantification of HDAC1 intensity in nuclei of cortical slices of P4 WT and** ***Tnc$^{-/-}$*** **mice.** P4 cortical slices (WT or *Tnc$^{-/-}$*) were stained for HDAC1 (red) and DAPI (blue). Following confocal imaging, nuclei were rendered and the HDAC1 mean intensity was quantified (n = 3 animals per group; analysis of 2-4 fields of view per slice). For statistical analysis, Student´s t- test was performed.

References

Bennett, M.L., Bennett, F.C., Liddelow, S.A., Ajami, B., Zamanian, J.L., Fernhoff, N.B., Mulinyawe, S.B., Bohlen, C.J., Adil, A., Tucker, A., Weissman, I.L., Chang, E.F., Li, G., Grant, G.A., Hayden Gephart, M.G., Barres, B.A., 2016. New tools for studying microglia in the mouse and human CNS. Proc. Natl. Acad. Sci. 113, E1738–E1746. https://doi.org/10.1073/pnas.1525528113

Boucsein, C., Kettenmann, H., Nolte, C., 2000. Electrophysiological properties of microglial cells in normal and pathologic rat brain slices. Eur. J. Neurosci. 12, 2049–2058. https://doi.org/10.1046/j.1460-9568.2000.00100.x

Butovsky, O., Jedrychowski, M.P., Moore, C.S., Cialic, R., Lanser, A.J., Gabriely, G., Koeglsperger, T., Dake, B., Wu, P.M., Doykan, C.E., Fanek, Z., Liu, L., Chen, Z., Rothstein, J.D., Ransohoff, R.M., Gygi, S.P., Antel, J.P., Weiner, H.L., 2014. Identification of a unique TGF-β–dependent molecular and functional signature in microglia. Nat. Neurosci. 17, 131–143. https://doi.org/10.1038/nn.3599

Faissner, A., Steindler, D., 1995. Boundaries and inhibitory molecules in developing neural tissues. Glia 13, 233–254. https://doi.org/10.1002/glia.440130402

Gates, M.A., Thomas, L.B., Howard, E.M., Laywell, E.D., Sajin, B., Faissner, A., Götz, B., Silver, J., Steindler, D.A., 1995. Cell and molecular analysis of the developing and adult mouse subventricular zone of the cerebral hemispheres. J. Comp. Neurol. 361, 249–266. https://doi.org/10.1002/cne.903610205

Gosselin, D., Skola, D., Coufal, N.G., Holtman, I.R., Schlachetzki, J.C.M., Sajti, E., Jaeger, B.N., O'Connor, C., Fitzpatrick, C., Pasillas, M.P., Pena, M., Adair, A., Gonda, D.D., Levy, M.L., Ransohoff, R.M., Gage, F.H., Glass, C.K., 2017. An environment-dependent transcriptional network specifies human microglia identity. Science (80-.). 356, eaal3222. https://doi.org/10.1126/science.aal3222

Haage, V., Semtner, M., Vidal, R.O., Hernandez, D.P., Pong, W.W., Chen, Z., Hambardzumyan, D., Magrini, V., Ly, A., Walker, J., Mardis, E., Mertins, P., Sauer, S., Kettenmann, H., Gutmann, D.H., 2019. Comprehensive gene expression meta-analysis identifies signature genes that distinguish microglia from peripheral monocytes/macrophages in health and glioma. Acta Neuropathol. Commun. 7, 20. https://doi.org/10.1186/s40478-019-0665-y

He, Y., Yao, X., Taylor, N., Bai, Y., Lovenberg, T., Bhattacharya, A., 2018. RNA sequencing analysis reveals quiescent microglia isolation methods from postnatal mouse brains and limitations of BV2 cells. J. Neuroinflammation 15, 153. https://doi.org/10.1186/s12974-018-1195-4

Ifuku, M., Buonfiglioli, A., Jordan, P., Lehnardt, S., Kettenmann, H., 2016. TLR2 controls random motility, while TLR7 regulates chemotaxis of microglial cells via distinct pathways. Brain. Behav. Immun. 58, 338–347. https://doi.org/10.1016/J.BBI.2016.08.003

Korvers, L., de Andrade Costa, A., Mersch, M., Matyash, V., Kettenmann, H., Semtner, M., 2016. Spontaneous Ca2+ transients in mouse microglia. Cell Calcium 60, 396–406. https://doi.org/10.1016/J.CECA.2016.09.004

Prinz, M., Kann, O., Draheim, H.J., Schumann, R.R., Kettenmann, H., Weber, J.R., Hanisch, U.K., 1999. Microglial activation by components of gram-positive and -negative bacteria: distinct and common routes to the induction of ion channels and cytokines. J. Neuropathol. Exp. Neurol. 58, 1078–89.

6 Discussion

It has been more than a hundred years following microglia discovery in 1919 by Pío del Río-Hortega (Prinz et al., 2019). The modern era of microglia research reached the conclusion that is microglia are no longer full-time brain defenders, rather their contribution to neurological diseases is demonstrated (Wolf et al., 2017, Prinz et al., 2019). Therefore, microglia-targeted therapy has emerged as a promising strategy to combat CNS diseases, however, better understanding of microglial biology, functions, and fate is needed. Recently, Garaschuk and Verkhratsky summarized the receptors expressed in microglia in their chapter “Physiology of Microglia” (Garaschuk and Verkhratsky, 2019) (Figure 19). This multitude of receptors expressed in microglia might be potential bases for microglia-targeted therapy. Out of these receptors, complement receptors (C3aR1, C1qbp), purinoreceptors (P2RY6, P2RY12) and toll like receptors (Tlr4) were involved in my projects. Additionally, I focused on NF1 as an intracellular effector in Trk downstream. I demonstrate in the present dissertation that activation of microglial C3aR1 and C1qbp by non-microglial TLQP21, as well as knocking out Nf1, TNC, or Tlr4 could successfully modulate microglial functional phenotypes. In the following subsections, each of these modulators and their action on microglia are discussed.

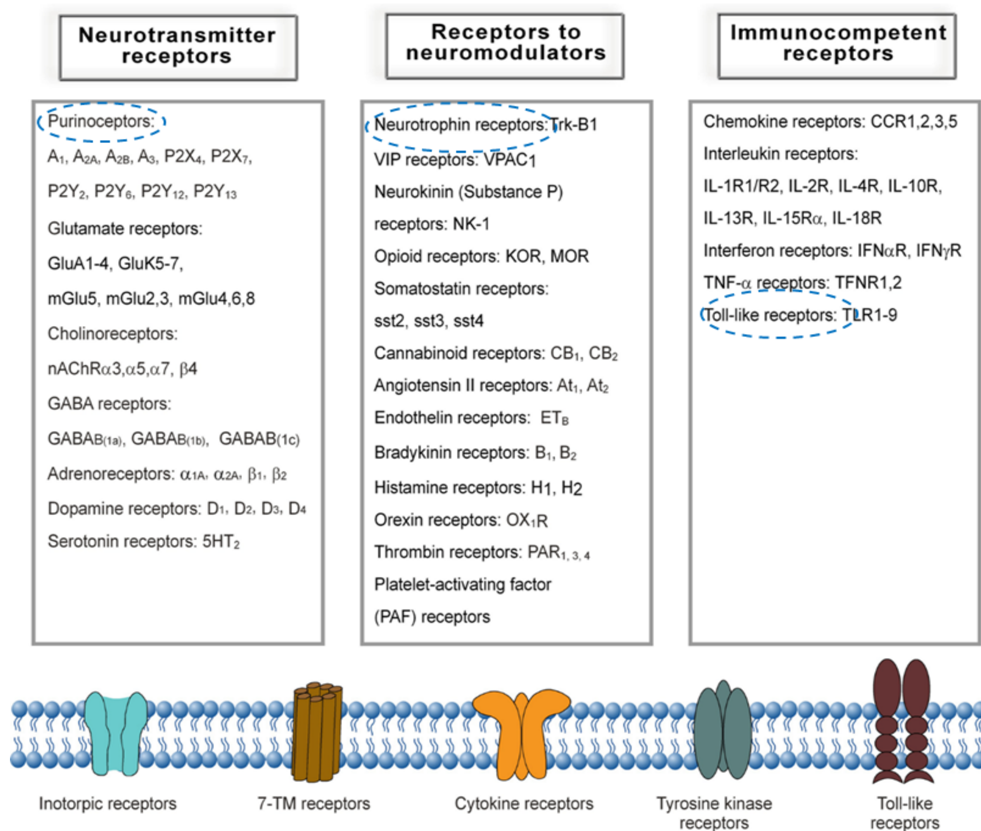


Figure 19. A summary of the receptors expressed **in microglia.** Highlighted in blue the receptors that were involved in my studies. Adapted from (Garaschuk and Verkhratsky, 2019).

6.1 Effect of the VGF-derived peptide TLQP21 on microglia

VGF and its peptides are the subject of extensive research related to the CNS diseases. Several studies reported a decrease in VGF expression in the brains of patients with depression and neurodegenerative disease, including Parkinson's disease, Alzheimer's disease (AD), and amyotrophic lateral sclerosis (Llano et al., 2017, Li et al., 2020) . Knowing that the VGF-derived peptide TLQP21 itself is involved in many CNS disorders (Fairbanks et al., 2014, Brancia et al., 2018), and that its receptors are expressed in microglia encouraged me and the involved co-authors to investigate the effect of TLQP21 on microglia. First, I explored the electrophysiological properties of microglia in brain slices *ex vivo* while applying 100 nM of TLQP21 and found that there was no alteration on microglial ion currents. However, the ATP-induced rectifying K^+ current was abolished after pre-treatment of the slices with TLQP21. Interestingly, purinergic responses were affected even minutes after the washout of TLQP21. To confirm whether this impairment of purinergic signaling is due to direct effect of TLQP21 on microglia or due to non-microglial interaction in the brain slices, the same steps were repeated on primary neonatal microglia culture. Intriguingly, we could detect *in vitro* TLQP21-induced rectifying K^+ currents, in addition to the expected TLQP21 interference with the P2RY12-mediated rectifying K^+ currents. These finding revealed for the first time that there are two distinct effects of TLQP21 on microglia. Next, I used blockers of TLQP21 receptors to further elucidate the involved pathways, and incorporate further microglial activities; including Ca^{2+} release, process movement, migration, and phagocytosis. I demonstrate that the VGF-derived peptide TLQP21 influences microglial functional phenotypes via two distinct pathways; linked to the differential expression of TLQP21 receptors in microglia: (1) via a C3aR1-dependent pathway that triggers membrane currents, Ca^{2+} responses, and stimulates microglial migration and phagocytic activity; and (2) via a C1qBP-dependent pathway that interferes with purinergic signaling through metabotropic P2Y receptors. The C1qBP-dependent impairment of purinergic responses leads to a suppression of membrane currents evoked by metabotropic P2Y, chemo-attractancy and process movement by ATP, and the induction of microglial phagocytosis by UDP.

6.1.1 The C3aR1-dependant effect of TLQP21 on microglia

In the past, C3aR1 was only considered as part of the complement cascade that is involved in innate immunity. However, recent studies highlighted further participation of C3aR1 in neurogenesis, cancer, and neuroendocrine and endocrine hormones release (Bresciani et al., 2020, Hannedouche et al., 2013). Early in 2013, C3aR1 has been identified as a receptor for TLQP21 (Hannedouche et al., 2013), in addition to being activated by the complement protein 3 (C3). C3 is generated by astrocytes (Lian et al., 2016), whereas VGF is mainly released by neurons (Figure 8a) and subsequently generate TLQP21. The TLQP21 receptor C3aR1 is expressed in microglia (Figure 8c). Taken together, there could be a contribution of neuronal VGF, astrocytic C3, and microglial C3aR1 in the neurons-astrocytes-microglia crosstalk. Therefore, studying the association between VGF, its peptides, and microglia becomes an important topic to be addressed. In depth understanding of TLQP21-microglial C3aR1 interaction might explain microglial behavior in health and disease.

Consistent with our data, studies on TLQP21 and its receptors described enhancement of Ca^{2+} signals by TLQP21 in microglia through C3aR1 (Chen et al., 2013, Hannedouche et al., 2013). A recent study by El Gaamouch *et. al.* that was released at the same time as our paper confirmed our findings (El Gaamouch et al., 2020). The authors explored the effect of TLQP21, or alternatively C3a super agonist (C3aSA), on microglial migration and phagocytosis in BV2 microglial cells and primary microglia isolated from *C3aR1 -/-* KO or WT mice. Indeed, they found that TLQP21 promotes microglial migration and phagocytosis only in BV2 microglial cells and primary microglia isolated from WT mice; indicating that TLQP21 effect is C3aR1 dependent.

In the present project, C3aR1 activation by TLQP21 triggered outwardly rectifying K^+ currents. Intriguingly, these TLQP21-C3aR1 mediated stimulatory effects were only found in primary cultured microglia but not *in situ*. Sharma *et. al.* in 2015, consistent with our findings, showed that the C3aR1 protein is detected in primary cultures but lacking in freshly isolated microglia (Sharma et al., 2015). In contrast, on the mRNA level, Tabula Muris Consortium *et. al.*, reported a robust transcription of the C3ar1 gene in acutely isolated microglia. This contradiction might be due to post-translational modifications on the C3aR1 protein.

Under CNS pathological conditions such as dementia, AD, epilepsy, and traumatic brain injury, the expression of microglial C3ar1 was found to be elevated (Zhao et al., 2018, Bodea et al., 2014). This elevation might be linked to microglia activation under pathological condition. Therefore, it could be that the stimulatory effect of TLQP21 on microglia becomes significant *in vivo* in CNS diseases.

VGF was reported to increase GBM tumorigenesis (Wang et al., 2018). Moreover, C3aRs are thought to act as immune checkpoint receptors by suppressing the activity of effector T cells and inhibiting the release of IL-10, which is important for T cell-mediated antitumor immunity (Wang et al., 2019). Blocking C3aRs restores T cells activity and enhance the response to immunotherapy (Wang et al., 2019). On the other hand, since complement cascade steer microglial phagocytosis, mice deficient in C3 are characterized by abnormalities in synapses elimination and consequently neuronal function (Illes et al., 2020). In such cases, stimulation of the existent C3aR1 by TLQP21 might help to restore microglial phagocytosis and reconstitute homeostasis.

6.1.2 TLQP21 impairs purinergic signaling in microglia in a C1qbp-dependent manner

I introduced in the present project TLQP21, and via C1qbp interaction, as a first biological modulator of microglial purinergic signaling. Purinergic signalling is critical for microglial activities. Thus, by modulating these signal pathways scientists might be able to modify microglial phenotypes in a favor to the therapeutic purposes.

In the present study, I demonstrated that TLQP21-microglial C1qbp interactions interfere with P2Y-mediated phagocytic activity and responses to injury. TLQP21 suppressed P2RY12-mediated outwardly rectifying K^+ current, process movement and migration, and P2Y6-dependent phagocytosis *in situ* and *in vitro*. While several studies reported the elevation of intracellular Ca^{2+} as a response to TLQP21 (Chen et al., 2013, Hannedouche et al., 2013, Norgauer et al., 1993), none of these studies, neither ours, described an attenuation of the subsequent ATP-induced Ca^{2+} responses after a pre-treatment with TLQP21. Blocking C1qbp by monoclonal antibodies restored the ATP-induced rectifying K^+ current, process movement and migration, as well as the UDP-induced phagocytosis.

Finding the appropriate agonists and antagonists for purinergic receptors might profoundly change microglial functional phenotypes to restore the homeostasis in CNS disorders and neuroinflammation. As an example, the expression of P2RY12 was recognized as an essential component of the post-natal synaptic pruning process in the visual cortex (Prinz et al., 2019). Excessive synaptic pruning was reported in schizophrenia (Sellgren et al., 2019b), which was mainly linked to C4 complement protein. Since TLQP21 interferes with P2RY12 signaling, studying the effect of TLQP21 in schizophrenia cases might improve the course of the disease. Other disorders that imply upregulation of P2RY12 are facial nerve axotomy (Sasaki et al., 2003) and neuropathic pain (Tozaki-Saitoh et al., 2008).

Taken together, exogenous administration of TLQP21 peptide or using selective inhibitors for TLQP21 receptors might be an emerging immunotherapeutic tool to manage CNS diseases through microglia.

6.2 Male neurofibromatosis 1 +/- microglia exhibit sexually dimorphic cyclic AMP-associated defect

The discovery of NF1 protein, its role and mutations in NF1 disease facilitates designing several genetically engineered mouse models with differential Nf1 mutations. However, none of these models exhibit all symptoms and complications of NF1 disease together. Previous work from the Gutmann lab attempted to study NF1-linked OPG by homozygous knocking out of *Nf1 -/-* in astrocytes. Nevertheless, the resultant increase in the astrocytic cells' proliferation *in vitro* and *in vivo* was insufficient to produce glioma. Additional heterozygous *Nf1 +/-* knockout of microglia leads to the formation of OPG by 2–3 months of age (Daginakatte and Gutmann, 2007). This finding may suggest the involvement of microglia in NF1 disease.

To better understand the mechanism behind NF1 disease, I investigated the influence of variants like sex and genotype on microglia. Indeed, I found that *Nf1 +/-* in microglia leads to male-specific defects in purinergic signalling and suppression of the associated microglial functions like P2RY12-mediated phagocytosis, process movement, and membrane currents. These findings support prior observations that male microglia participate differentially to brain diseases. In one of the studies, Sorge *et. al.* reported that male microglia upregulate

P2RX4 after injury and trigger pain hypersensitivity (Sorge et al., 2015). Similarly, male microglia were found to differentially exhibit ATP-dependent p38-MAPK phosphorylation and the consequent brain-derived neurotrophic factor (BDNF) release (Mapplebeck et al., 2018). Moreover, a previous study from my lab showed that male microglia have differential expression of purinoreceptors (e.g., P2RY12, P2RX4, P2RX7) (Guneykaya et al., 2018). However, no differences in the expression of purinoreceptors among mutant and WT microglia regardless of sex was observed in the present project. Rather, a differential expression of cAMP in male *Nf1 +/-* microglia was shown to underlie the sexually dimorphic phenotype in murine *Nf1 +/-* microglia. The role of microglial cAMP in controlling ramification and process movement has previously been described using phosphodiesterase (PDE) inhibitors and GPCRs activation (Bernier et al., 2019). In the context of NF1 disease, NF1 protein was found to induce cAMP homeostasis in mouse and human neurons via RAS activation and the consequent stimulation of PKCζ that regulates cAMP levels (Gutmann et al., 2017). However, I could not confirm a clear mechanism to explain why cAMP defect was found only in males and whether gonad or sex hormones underlie this sexual dimorphism.

Gender medicine has become a hot topic in the time of precision medicine, it has been considered the first move towards personalized regimens (Gemmati et al., 2019). Therefore, the sexually dimorphic cAMP-defects in my study were assumed as an important finding that could help in achieving better management for NF1 disease. Accordingly, using a blocker of PDE as a strategy to counteract this defect would work only for males but not females NF1 patients.

Consistent with the present data, sexual dimorphism was previously reported in the context of NF1 disease. For example boys exhibit impulsivity and hyperactivity more than girls (Cohen et al., 2018). In addition, there was a male bias in the prevalence of autism spectrum disorders (ASD) among NF1 children (Garg et al., 2016). On the other hand, female bias was observed in NF1-linked OPGs, in which vision loss is 3- to 5-times higher in females compared to males (Diggs-Andrews et al., 2014). Further studies are needed to find out if *Nf1 +/-* microglial phenotype contribute to the sexual dimorphism in NF1-linked complications.

Understanding the mechanism behind these sexual dimorphisms might provide new insights regarding the interplay between disease risk factors and cellular function in brain pathology, as well as provide better therapeutic strategies.

6.3 Tenascin C regulates multiple functions of microglia

In the present study, I demonstrate that TNC plays an important role in regulating of microglial function during postnatal development. TNC was shown to trigger the production of pro inflammatory chemokines and cytokines, as well as to stimulate phagocytosis and chemotaxis. Interestingly, TNC effects on microglia in the developing brain were partially dependent on Tlr4 and were not completely abolished in *Tlr4 −/−* microglia, rather reduced. This finding implies that TNC activates not only the Tlr4 pathway in microglia, as demonstrated for human macrophages (Midwood et al., 2009), but other pathways as well.

For the first time, me and my colleagues showed that TNC induces the expression of microglial histone deacetylase 1 (HDAC1) partially in a Tlr4-dependent manner, indicating that the TNC-Tlr4 pathway is involved in the epigenetics modification of microglial proteins and associated functional phenotypes. Furthermore, the TNC-mediated release of IL-6 and TNF-α cytokines involved HDAC1 and could be inhibited by using the HDAC inhibitor MS-275. A study by Das Gupta *et. al.* in 2016 demonstrated that HDAC1 has several tasks in regulating monocytes/macrophages activation and metabolism (Das Gupta et al., 2016). In microglia, LPS, another Tlr4 ligand, stimulation of microglial cells was diminished by the inhibition of HDAC (Kannan et al., 2013). However, to our knowledge the TNC-HDAC1 interaction in microglia has not been described before. Consistent with our first findings, we reported that TNC-induced HDAC1 expression was reduced, but not completely abolished in *Tlr4 -/-* mice, indicating that Tlr4 is not the only receptor by which TNC modulates microglial function.

Further studies are needed to elucidate other potential pathways involved in TNC control of microglial functions. Indeed, TNC has several binding sites and interact with several integrin receptors (Tucker and Chiquet-Ehrismann, 2015, Chiquet-Ehrismann and Tucker, 2011). In another study, integrin-dependent phagocytosis in microglia was reported (Koenigsknecht and Landreth, 2004). TNC was found to activate the epidermal growth factor (EGF) receptor as well (Fujimoto et al., 2016). In the mentioned study, Fujimoto *et. al.*, found that EGF-like repeats in the TNC activate EGFR and ERK1/2 in rat cerebral arteries. Interestingly, the EGF receptor is highly expressed during development at E18.5 (The Allen Developing Mouse Brain Atlas), and associated with migration and phagocytosis of polymorphonuclear neutrophil (Li et al., 2014). Several other receptors are linked to microglial phagocytosis during

development; including C3 (Schafer et al., 2012) and IL1 receptor like-1 (IL1RL1) (Vainchtein et al., 2018).

Our findings on TNC were mainly in early postnatal microglia, consistent with the fact that TNC is minimally expressed in healthy adult within the neurogenic niches. TNC expression is upregulated in brain pathology and upon injury by reactive astrocytes regardless of age (Yalcin et al., 2020, Claycomb et al., 2014, Smith and Hale, 1997). Considering the multifactorial nature of diseases, studying TNC under physiological-like could provide more precise data on the sole effect of TNC on microglia, and help in expecting what changes on microglial functions under pathological conditions in which TNC is upregulated.

Taking a close look at our reported TNC effects on microglia, microglial phagocytosis was significantly reduced in P4, but not in P28, in *TNC -/-* mice. Microglial phagocytosis play an important role in synaptic pruning during development (Diaz-Aparicio et al., 2020). However, excessive synaptic pruning might lead to CNS disorders and was reported in schizophrenic patients (Sellgren et al., 2019a). Inhibition of TNC might be a promising therapeutic strategy in such cases. *In vivo* experiments examining microglial synaptic pruning capacity in *TNC -/-* mice might be necessary to further clarify TNC impact on microglial pruning. Microglial phagocytosis at P4 was suppressed as well in *Tlr4 -/-* mice; suggesting that Tlr4 is involved in microglial phagocytosis. However, at P4 *Tlr4 -/-* mice showed higher levels of phagocytosis than WT mice at P28-P31; indicating the involvement of another TNC-dependent, Tlr4-independent pathway in regulating microglial phagocytosis postnatally.

In our study, we found that cultured *Tlr4 -/-* microglia exhibit significant reduction in chemotaxis towards TNS and LPS as chemoattractants. Earlier studies described the influence of TNC on microglial migration. However, these reports were contradicting. TNC was reported to stimulate, as well as inhibit, cell migration (Faissner, 1997, Sánchez-López et al., 2004). It seems that the effects of TNC on cell migration might be dependent on the temporal and spatial contexts.

We further found a reciprocal interaction between TNC and HDAC1 in microglia during early postnatal development, that is TNC induces HDAC1 expression, while HDAC1 is critical for the TNC-mediated cytokines release. Pharmacological blockade of HDAC1 by the HDAC inhibitor MS-275 suppresses the TNC-induced IL-6 and TNF-α secretion. TAMs contribute to glioma

growth by IL-6 (Dzaye et al., 2016), thus TNC-HDAC-TNC-IL6 correlation might partially explain the role of HDAC inhibitors in reducing glioblastoma growth (Lee et al., 2015).

Taken together, TNC regulates microglial activities on different levels; epigenetically and post-translationally, and could be considered as a modulator of microglial functional phenotypes.

7 Conclusion

Microglial cells are the first line of the CNS immune defence, thus, any changes in the brain environment or any neuropathological events activate microglia to orchestrate the immunological response and restore the homeostasis (Wolf et al., 2017). On the other hand, several studies described the contribution of microglia to the progression of neurological diseases (Hammond et al., 2018). Therefore, microglia have emerged as a promising therapeutic target with a potential broad therapeutic landscape. Finding modulators of microglial activities is one strategy to favor the beneficial functional phenotype in accordance to a disease.

I demonstrate that besides intrinsic targets, proteins derived from non-microglial cells can also strongly affect the functional profile of microglia, and can therefore be considered as potential targets for future clinical applications. Within the course of this thesis, TLQP21, NF1 and TNC were identified as regulators of microglial functions. Our findings highlight a mechanistic association between TLQP21 and the stimulation (via C3aR1) or attenuation (via C1qBP) of microglial functions in health and disease. The ECM protein TNC was shown to control key functions in microglia partially via Tlr4, and to reciprocally interact with microglial HDAC1. These finding unveil a novel role of TNC as a modulator of microglia on the epigenetical and posttranslational levels. Moreover, by shedding a light on microglia in the context of NF1 disease, we could report sexual dimorphism in microglia harboring a germline *Nf1 +/-* mutation. A cAMP-defect was only detected in male NF1 KO microglia but not in female and could be restored by pharmacological blockade of PDE3 enzyme. Understanding the mechanisms that underlie sex differences would provide new insights into understanding the disease course and in selecting the optimal therapeutic strategy based on precision medicine. Further studies are needed to investigate whether the addressed actions on microglia are preserved in the disease contexts, and affect the disease progression.

8 Bibliography

ABBRACCHIO, M. P., BURNSTOCK, G., BOEYNAEMS, J. M., BARNARD, E. A., BOYER, J. L., KENNEDY, C., KNIGHT, G. E., FUMAGALLI, M., GACHET, C., JACOBSON, K. A. & WEISMAN, G. A. 2006. International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacol Rev,* 58**,** 281-341.

ALDER, J., THAKKER-VARIA, S., BANGASSER, D. A., KUROIWA, M., PLUMMER, M. R., SHORS, T. J. & BLACK, I. B. 2003. Brain-derived neurotrophic factor-induced gene expression reveals novel actions of VGF in hippocampal synaptic plasticity. *J Neurosci,* 23**,** 10800-8.

ANDERSON, J. L. & GUTMANN, D. H. 2015. Neurofibromatosis type 1. *Handb Clin Neurol,* 132**,** 75-86.

BACHILLER, S., JIMÉNEZ-FERRER, I., PAULUS, A., YANG, Y., SWANBERG, M., DEIERBORG, T. & BOZA-SERRANO, A. 2018. Microglia in Neurological Diseases: A Road Map to Brain-Disease Dependent-Inflammatory Response. *Frontiers in Cellular Neuroscience,* 12.

BARTOLOMUCCI, A., POSSENTI, R., LEVI, A., PAVONE, F. & MOLES, A. 2007. The role of the vgf gene and VGF-derived peptides in nutrition and metabolism. *Genes & nutrition,* 2**,** 169-180.

BARTOLOMUCCI, A., POSSENTI, R., MAHATA, S. K., FISCHER-COLBRIE, R., LOH, Y. P. & SALTON, S. R. 2011. The extended granin family: structure, function, and biomedical implications. *Endocr Rev,* 32**,** 755-97.

BECKMANN, N. D., LIN, W.-J., WANG, M., COHAIN, A. T., CHARNEY, A. W., WANG, P., MA, W., WANG, Y.-C., JIANG, C. & AUDRAIN, M. 2020. Multiscale causal networks identify VGF as a key regulator of Alzheimer’s disease. *Nature Communications,* 11**,** 1-19.

BERNIER, L.-P., BOHLEN, C. J., YORK, E. M., CHOI, H. B., KAMYABI, A., DISSING-OLESEN, L., HEFENDEHL, J. K., COLLINS, H. Y., STEVENS, B. & BARRES, B. A. 2019. Nanoscale surveillance of the brain by microglia via cAMP-regulated filopodia. *Cell reports,* 27**,** 2895-2908. e4.

BIANCHI, M. E. 2007. DAMPs, PAMPs and alarmins: all we need to know about danger. *Journal of leukocyte biology,* 81**,** 1-5.

BODEA, L. G., WANG, Y., LINNARTZ-GERLACH, B., KOPATZ, J., SINKKONEN, L., MUSGROVE, R., KAOMA, T., MULLER, A., VALLAR, L., DI MONTE, D. A., BALLING, R. & NEUMANN, H. 2014. Neurodegeneration by activation of the microglial complement-phagosome pathway. *J Neurosci,* 34**,** 8546-56.

BOUCSEIN, C., KETTENMANN, H. & NOLTE, C. 2000. Electrophysiological properties of microglial cells in normal and pathologic rat brain slices. *Eur J Neurosci,* 12**,** 2049-58.

BRANCIA, C., NOLI, B., BOIDO, M., PILLERI, R., BOI, A., PUDDU, R., MARROSU, F., VERCELLI, A., BONGIOANNI, P., FERRI, G. L. & COCCO, C. 2018. TLQP Peptides in Amyotrophic Lateral Sclerosis: Possible Blood Biomarkers with a Neuroprotective Role. *Neuroscience,* 380**,** 152-163.

BRAWEK, B. & GARASCHUK, O. 2014. Network-wide dysregulation of calcium homeostasis in Alzheimer's disease. *Cell Tissue Res,* 357**,** 427-38.

BRESCIANI, E., POSSENTI, R., COCO, S., RIZZI, L., MEANTI, R., MOLTENI, L., LOCATELLI, V. & TORSELLO, A. 2020. TLQP-21, A VGF-Derived Peptide Endowed of Endocrine and Extraendocrine Properties: Focus on In Vitro Calcium Signaling. *International Journal of Molecular Sciences,* 21**,** 130.

BROSSIER, N. M. & GUTMANN, D. H. 2015. Improving outcomes for neurofibromatosis 1-associated brain tumors. *Expert Rev Anticancer Ther,* 15**,** 415-23.

BURNSTOCK, G. & VERKHRATSKY, A. 2010. Long-term (trophic) purinergic signalling: purinoceptors control cell proliferation, differentiation and death. *Cell Death Dis,* 1**,** e9.

CALOVI, S., MUT-ARBONA, P. & SPERLÁGH, B. 2019. Microglia and the Purinergic Signaling System. *Neuroscience,* 405**,** 137-147.

CATTANEO, A., SESTA, A., CALABRESE, F., NIELSEN, G., RIVA, M. A. & GENNARELLI, M. 2010a. The expression of VGF is reduced in leukocytes of depressed patients and it is restored by effective antidepressant treatment. *Neuropsychopharmacology,* 35**,** 1423-1428.

CATTANEO, A., SESTA, A., CALABRESE, F., NIELSEN, G., RIVA, M. A. & GENNARELLI, M. 2010b. The expression of VGF is reduced in leukocytes of depressed patients and it is restored by effective antidepressant treatment. *Neuropsychopharmacology,* 35**,** 1423-8.

CERO, C., VOSTRIKOV, V. V., VERARDI, R., SEVERINI, C., GOPINATH, T., BRAUN, P. D., SASSANO, M. F., GURNEY, A., ROTH, B. L., VULCHANOVA, L., POSSENTI, R., VEGLIA, G. & BARTOLOMUCCI, A. 2014. The TLQP-21 peptide activates the G-protein-coupled receptor C3aR1 via a folding-upon-binding mechanism. *Structure,* 22**,** 1744-1753.

CHEN, S., BENNET, L. & MCGREGOR, A. L. 2015. MacGreen mice: a novel tool to investigate inflammation following experimental stroke. *Journal of Experimental Stroke & Translational Medicine,* 8**,** 1-10.

CHEN, Y. C., PRISTERÁ, A., AYUB, M., SWANWICK, R. S., KARU, K., HAMADA, Y., RICE, A. S. & OKUSE, K. 2013. Identification of a receptor for neuropeptide VGF and its role in neuropathic pain. *J Biol Chem,* 288**,** 34638-46.

CHEN, Z. & HAMBARDZUMYAN, D. 2018. Immune microenvironment in glioblastoma subtypes. *Frontiers in immunology,* 9**,** 1004.

CHIQUET-EHRISMANN, R., MACKIE, E. J., PEARSON, C. A. & SAKAKURA, T. 1986. Tenascin: an extracellular matrix protein involved in tissue interactions during fetal development and oncogenesis. *Cell,* 47**,** 131-9.

CHIQUET-EHRISMANN, R. & TUCKER, R. P. 2011. Tenascins and the importance of adhesion modulation. *Cold Spring Harb Perspect Biol,* 3.

CIMINO, P. J. & GUTMANN, D. H. 2018. Neurofibromatosis type 1. *Handb Clin Neurol,* 148**,** 799-811.

CLAYCOMB, K. I., WINOKUR, P. N., JOHNSON, K. M., NICAISE, A. M., GIAMPETRUZZI, A. W., SACINO, A. V., SNYDER, E. Y., BARBARESE, E., BONGARZONE, E. R. & CROCKER, S. J. 2014. Aberrant production of tenascin-C in globoid cell leukodystrophy alters psychosine-induced microglial functions. *J Neuropathol Exp Neurol,* 73**,** 964-74.

COHEN, R., HALEVY, A., AHARON, S. & SHUPER, A. 2018. Attention deficit hyperactivity disorder in neurofibromatosis type 1: Evaluation with a continuous performance test. *Journal of Clinical Neurology,* 14**,** 153-157.

COLOMBO, F., BASTONI, M., NIGRO, A., PODINI, P., FINARDI, A., CASELLA, G., RAMESH, M., FARINA, C., VERDERIO, C. & FURLAN, R. 2018. Cytokines Stimulate the Release of Microvesicles from Myeloid Cells Independently from the P2X7 Receptor/Acid Sphingomyelinase Pathway. *Front Immunol,* 9**,** 204.

CRAPSER, J. D., SPANGENBERG, E. E., BARAHONA, R. A., ARREOLA, M. A., HOHSFIELD, L. A. & GREEN, K. N. 2020. Microglia facilitate loss of perineuronal nets in the Alzheimer's disease brain. *EBioMedicine,* 58.

DAGINAKATTE, G. C., GIANINO, S. M., ZHAO, N. W., PARSADANIAN, A. S. & GUTMANN, D. H. 2008. Increased c-Jun-NH2-kinase signaling in neurofibromatosis-1 heterozygous microglia drives microglia activation and promotes optic glioma proliferation. *Cancer Res,* 68**,** 10358-66.

DAGINAKATTE, G. C. & GUTMANN, D. H. 2007. Neurofibromatosis-1 (Nf1) heterozygous brain microglia elaborate paracrine factors that promote Nf1-deficient astrocyte and glioma growth. *Hum Mol Genet,* 16**,** 1098-112.

DAS GUPTA, K., SHAKESPEAR, M. R., IYER, A., FAIRLIE, D. P. & SWEET, M. J. 2016. Histone deacetylases in monocyte/macrophage development, activation and metabolism: refining HDAC targets for inflammatory and infectious diseases. *Clinical & Translational Immunology,* 5**,** e62.

DEL RIO-HORTEGA, P. 1932. *Cytology & [and] Cellular Pathology of the Nervous System*, Hoeber.

DEMBITZER, F. R., KINOSHITA, Y., BURSTEIN, D., PHELPS, R. G., BEASLEY, M. B., GARCIA, R., HARPAZ, N., JAFFER, S., THUNG, S. N., UNGER, P. D., GHEBREHIWET, B. & PEERSCHKE, E. I. 2012. gC1qR expression in normal and pathologic human tissues: differential expression in tissues of epithelial and mesenchymal origin. *J Histochem Cytochem,* 60**,** 467-74.

DI LUCENTE, J., NGUYEN, H. M., WULFF, H., JIN, L.-W. & MAEZAWA, I. 2018. The voltage-gated potassium channel Kv1.3 is required for microglial pro-inflammatory activation in vivo. *Glia,* 66**,** 1881-1895.

DIAZ-APARICIO, I., PARIS, I., SIERRA-TORRE, V., PLAZA-ZABALA, A., RODRÍGUEZ-IGLESIAS, N., MÁRQUEZ-ROPERO, M., BECCARI, S., HUGUET, P., ABIEGA, O. & ALBERDI, E. 2020a. Microglia actively remodel adult hippocampal neurogenesis through the phagocytosis secretome. *Journal of Neuroscience,* 40**,** 1453-1482.

DIAZ-APARICIO, I., PARIS, I., SIERRA-TORRE, V., PLAZA-ZABALA, A., RODRÍGUEZ-IGLESIAS, N., MÁRQUEZ-ROPERO, M., BECCARI, S., HUGUET, P., ABIEGA, O., ALBERDI, E. & MATUTE, C. 2020b. Microglia Actively Remodel Adult Hippocampal Neurogenesis through the Phagocytosis Secretome. 40**,** 1453-1482.

DIGGS-ANDREWS, K. A., BROWN, J. A., GIANINO, S. M., RUBIN, J. B., WOZNIAK, D. F. & GUTMANN, D. H. 2014. Sex Is a major determinant of neuronal dysfunction in neurofibromatosis type 1. *Annals of Neurology,* 75**,** 309-316.

DRAGO, F., LOMBARDI, M., PRADA, I., GABRIELLI, M., JOSHI, P., COJOC, D., FRANCK, J., FOURNIER, I., VIZIOLI, J. & VERDERIO, C. 2017. ATP modifies the proteome of extracellular vesicles released by microglia and influences their action on astrocytes. *Frontiers in pharmacology,* 8**,** 910.

DZAYE, O., HU, F., DERKOW, K., HAAGE, V., EUSKIRCHEN, P., HARMS, C., LEHNARDT, S., SYNOWITZ, M., WOLF, S. A. & KETTENMANN, H. 2016. Glioma Stem Cells but Not Bulk Glioma Cells Upregulate IL-

6 Secretion in Microglia/Brain Macrophages via Toll-like Receptor 4 Signaling. *J Neuropathol Exp Neurol,* 75**,** 429-40.

EGAN, T. M., SAMWAYS, D. S. & LI, Z. 2006. Biophysics of P2X receptors. *Pflugers Arch,* 452**,** 501-12.

EICHHOFF, G., BRAWEK, B. & GARASCHUK, O. 2011. Microglial calcium signal acts as a rapid sensor of single neuron damage in vivo. *Biochim Biophys Acta,* 1813**,** 1014-24.

EL GAAMOUCH, F., AUDRAIN, M., LIN, W.-J., BECKMANN, N., JIANG, C., HARIHARAN, S., HEEGER, P. S., SCHADT, E. E., GANDY, S. & EHRLICH, M. E. 2020. VGF-derived peptide TLQP-21 modulates microglial function through C3aR1 signaling pathways and reduces neuropathology in 5xFAD mice. *Molecular neurodegeneration,* 15**,** 1-19.

EYO, U. B. & WU, L.-J. 2019. Microglia: lifelong patrolling immune cells of the brain. *Progress in neurobiology,* 179**,** 101614.

FAIRBANKS, C. A., PETERSON, C. D., SPELTZ, R. H., RIEDL, M. S., KITTO, K. F., DYKSTRA, J. A., BRAUN, P. D., SADAHIRO, M., SALTON, S. R. & VULCHANOVA, L. 2014. The VGF-derived peptide TLQP-21 contributes to inflammatory and nerve injury-induced hypersensitivity. *Pain,* 155**,** 1229-37.

FAISSNER, A. 1997. The tenascin gene family in axon growth and guidance. *Cell Tissue Res,* 290**,** 331-41.

FERRI, G. L., LEVI, A. & POSSENTI, R. 1992. A novel neuroendocrine gene product: selective VGF8a gene expression and immuno-localisation of the VGF protein in endocrine and neuronal populations. *Brain Res Mol Brain Res,* 13**,** 139-43.

FRANCHINI, L., LEVI, G. & VISENTIN, S. 2004. Inwardly rectifying K+ channels influence Ca2+ entry due to nucleotide receptor activation in microglia. *Cell Calcium,* 35**,** 449-59.

FUJIMOTO, M., SHIBA, M., KAWAKITA, F., LIU, L., NAKASAKI, A., SHIMOJO, N., IMANAKA-YOSHIDA, K., YOSHIDA, T. & SUZUKI, H. 2016. Epidermal growth factor-like repeats of tenascin-C-induced constriction of cerebral arteries via activation of epidermal growth factor receptors in rats. *Brain research,* 1642**,** 436-444.

GARASCHUK, O. & VERKHRATSKY, A. 2019. Physiology of Microglia. *Methods Mol Biol,* 2034**,** 27-40.

GARCION, E., HALILAGIC, A. & FAISSNER, A. 2004. Generation of an environmental niche for neural stem cell development by the extracellular matrix molecule tenascin C. *Development,* 131**,** 3423-3432.

GARG, S., HEUVELMAN, H., HUSON, S., TOBIN, H., GREEN, J. & NORTHERN, U. 2016. Sex bias in autism spectrum disorder in neurofibromatosis type 1. *Journal of neurodevelopmental disorders,* 8**,** 26.

GEMMATI, D., VARANI, K., BRAMANTI, B., PIVA, R., BONACCORSI, G., TRENTINI, A., MANFRINATO, M. C., TISATO, V., CARÈ, A. & BELLINI, T. 2019. "Bridging the Gap" Everything that Could Have Been Avoided If We Had Applied Gender Medicine, Pharmacogenetics and Personalized Medicine in the Gender-Omics and Sex-Omics Era. *Int J Mol Sci,* 21.

GONG, Q. J., LI, Y. Y., XIN, W. J., ZANG, Y., REN, W. J., WEI, X. H., LI, Y. Y., ZHANG, T. & LIU, X. G. 2009. ATP induces long-term potentiation of C-fiber-evoked field potentials in spinal dorsal horn: the roles of P2X4 receptors and p38 MAPK in microglia. *Glia,* 57**,** 583-91.

GUNEYKAYA, D., IVANOV, A., HERNANDEZ, D. P., HAAGE, V., WOJTAS, B., MEYER, N., MARICOS, M., JORDAN, P., BUONFIGLIOLI, A. & GIELNIEWSKI, B. 2018. Transcriptional and translational differences of microglia from male and female brains. *Cell reports,* 24**,** 2773-2783. e6.

GUTMANN, D. H., FERNER, R. E., LISTERNICK, R. H., KORF, B. R., WOLTERS, P. L. & JOHNSON, K. J. 2017. Neurofibromatosis type 1. *Nat Rev Dis Primers,* 3**,** 17004.

GUTMANN, D. H. & KETTENMANN, H. 2019. Microglia/Brain Macrophages as Central Drivers of Brain Tumor Pathobiology. *Neuron,* 104**,** 442-449.

HAMMOND, T. R., ROBINTON, D. & STEVENS, B. 2018. Microglia and the Brain: Complementary Partners in Development and Disease. *Annu Rev Cell Dev Biol,* 34**,** 523-544.

HANNEDOUCHE, S., BECK, V., LEIGHTON-DAVIES, J., BEIBEL, M., ROMA, G., OAKELEY, E. J., LANNOY, V., BERNARD, J., HAMON, J., BARBIERI, S., PREUSS, I., LASBENNES, M. C., SAILER, A. W., SUPLY, T., SEUWEN, K., PARKER, C. N. & BASSILANA, F. 2013. Identification of the C3a receptor (C3AR1) as the target of the VGF-derived peptide TLQP-21 in rodent cells. *J Biol Chem,* 288**,** 27434-43.

HICKMAN, S., IZZY, S., SEN, P., MORSETT, L. & EL KHOURY, J. 2018. Microglia in neurodegeneration. *Nature neuroscience,* 21**,** 1359-1369.

HIDETOSHI, T. S., MAKOTO, T. & INOUE, K. 2012. P2Y receptors in microglia and neuroinflammation. *Wiley Interdisciplinary Reviews: Membrane Transport and Signaling,* 1**,** 493-501.

HUANG, Y., ZHANG, Q., LUBAS, M., YUAN, Y., YALCIN, F., EFE, I. E., XIA, P., MOTTA, E., BUONFIGLIOLI, A. & LEHNARDT, S. 2020. Synergistic Toll-like receptor 3/9 signaling affects properties and impairs glioma-promoting activity of microglia. *Journal of Neuroscience,* 40**,** 6428-6443.

ILLES, P., RUBINI, P., ULRICH, H., ZHAO, Y. & TANG, Y. 2020. Regulation of Microglial Functions by Purinergic Mechanisms in the Healthy and Diseased CNS. *Cells,* 9**,** 1108.

INOUE, K., KOIZUMI, S., KATAOKA, A., TOZAKI-SAITOH, H. & TSUDA, M. 2009. P2Y(6)-Evoked Microglial Phagocytosis. *Int Rev Neurobiol,* 85**,** 159-63.

IZQUIERDO, P., ATTWELL, D. & MADRY, C. 2019. Ion Channels and Receptors as Determinants of Microglial Function. *Trends Neurosci,* 42**,** 278-292.

JACKSON, I. J. & ABBOTT, C. M. 2000. *Mouse genetics and transgenics*, Oxford University Press.

JETHWA, P. H. & EBLING, F. J. 2008. Role of VGF-derived peptides in the control of food intake, body weight and reproduction. *Neuroendocrinology,* 88**,** 80-7.

KANNAN, V., BROUWER, N., HANISCH, U.-K., REGEN, T., EGGEN, B. J. L. & BODDEKE, H. W. G. M. 2013. Histone deacetylase inhibitors suppress immune activation in primary mouse microglia. *Journal of Neuroscience Research,* 91**,** 1133-1142.

KARASAWA, A. & KAWATE, T. 2016. Structural basis for subtype-specific inhibition of the P2X7 receptor. 5.

KETTENMANN, H., HANISCH, U. K., NODA, M. & VERKHRATSKY, A. 2011. Physiology of microglia. *Physiol Rev,* 91**,** 461-553.

KETTENMANN, H., HOPPE, D., GOTTMANN, K., BANATI, R. & KREUTZBERG, G. 1990. Cultured microglial cells have a distinct pattern of membrane channels different from peritoneal macrophages. *J Neurosci Res,* 26**,** 278-87.

KOENIGSKNECHT, J. & LANDRETH, G. 2004. Microglial phagocytosis of fibrillar β-amyloid through a β1 integrin-dependent mechanism. *Journal of Neuroscience,* 24**,** 9838-9846.

KÖNNECKE, H. & BECHMANN, I. 2013. The Role of Microglia and Matrix Metalloproteinases Involvement in Neuroinflammation and Gliomas. *Clinical and Developmental Immunology,* 2013**,** 914104.

LEE, P., MURPHY, B., MILLER, R., MENON, V., BANIK, N. L., GIGLIO, P., LINDHORST, S. M., VARMA, A. K., VANDERGRIFT, W. A., 3RD, PATEL, S. J. & DAS, A. 2015. Mechanisms and clinical significance of histone deacetylase inhibitors: epigenetic glioblastoma therapy. *Anticancer Res,* 35**,** 615-25.

LEVI, A., ELDRIDGE, J. & PATERSON, B. 1985. Molecular cloning of a gene sequence regulated by nerve growth factor. *Science,* 229**,** 393-395.

LEVI, A., FERRI, G.-L., WATSON, E., POSSENTI, R. & SALTON, S. R. 2004. Processing, distribution, and function of VGF, a neuronal and endocrine peptide precursor. *Cellular and molecular neurobiology,* 24**,** 517-533.

LEWIS, J. E., BRAMELD, J. M. & JETHWA, P. H. 2015. Neuroendocrine Role for VGF. *Frontiers in Endocrinology,* 6.

LI, K. J., SIAO, S. C., WU, C. H., SHEN, C. Y., WU, T. H., TSAI, C. Y., HSIEH, S. C. & YU, C. L. 2014. EGF receptor-dependent mechanism may be involved in the Tamm-Horsfall glycoprotein-enhanced PMN phagocytosis via activating Rho family and MAPK signaling pathway. *Molecules,* 19**,** 1328-43.

LI, X., GE, H., ZHOU, D., WU, X., QI, G., CHEN, Z., YU, C., ZHANG, Y., YU, H. & WANG, C. 2020. Reduced serum VGF levels are linked with suicide risk in Chinese Han patients with major depressive disorder. 20**,** 225.

LIAN, H., LITVINCHUK, A., CHIANG, A. C.-A., AITHMITTI, N., JANKOWSKY, J. L. & ZHENG, H. 2016. Astrocyte-microglia cross talk through complement activation modulates amyloid pathology in mouse models of Alzheimer's disease. *Journal of Neuroscience,* 36**,** 577-589.

LIDDELOW, S. A., GUTTENPLAN, K. A., CLARKE, L. E., BENNETT, F. C., BOHLEN, C. J., SCHIRMER, L., BENNETT, M. L., MÜNCH, A. E., CHUNG, W.-S. & PETERSON, T. C. 2017. Neurotoxic reactive astrocytes are induced by activated microglia. *Nature,* 541**,** 481-487.

LIU, P. W., YUE, M. X., ZHOU, R., NIU, J., HUANG, D. J., XU, T., LUO, P., LIU, X. H. & ZENG, J. W. 2017. P2Y(12) and P2Y(13) receptors involved in ADPβs induced the release of IL-1β, IL-6 and TNF-α from cultured dorsal horn microglia. *J Pain Res,* 10**,** 1755-1767.

LLANO, D. A., BUNDELA, S., MUDAR, R. A. & DEVANARAYAN, V. 2017. A multivariate predictive modeling approach reveals a novel CSF peptide signature for both Alzheimer's Disease state classification and for predicting future disease progression. *PLoS One,* 12**,** e0182098.

LOU, N., TAKANO, T., PEI, Y., XAVIER, A. L., GOLDMAN, S. A. & NEDERGAARD, M. 2016. Purinergic receptor P2RY12-dependent microglial closure of the injured blood-brain barrier. *Proc Natl Acad Sci U S A,* 113**,** 1074-9.

LOW, D. & GINHOUX, F. 2018. Recent advances in the understanding of microglial development and homeostasis. *Cellular immunology,* 330**,** 68-78.

LUND, S., CHRISTENSEN, K. V., HEDTJÄRN, M., MORTENSEN, A. L., HAGBERG, H., FALSIG, J., HASSELDAM, H., SCHRATTENHOLZ, A., PÖRZGEN, P. & LEIST, M. 2006. The dynamics of the LPS triggered inflammatory response of murine microglia under different culture and in vivo conditions. *J Neuroimmunol,* 180**,** 71-87.

MADRY, C. & ATTWELL, D. 2015. Receptors, ion channels, and signaling mechanisms underlying microglial dynamics. *J Biol Chem,* 290**,** 12443-50.

MADRY, C., KYRARGYRI, V., ARANCIBIA-CÁRCAMO, I. L., JOLIVET, R., KOHSAKA, S., BRYAN, R. M. & ATTWELL, D. 2018. Microglial Ramification, Surveillance, and Interleukin-1β Release Are Regulated by the Two-Pore Domain K(+) Channel THIK-1. *Neuron,* 97**,** 299-312.e6.

MAPPLEBECK, J. C., DALGARNO, R., TU, Y., MORIARTY, O., BEGGS, S., KWOK, C. H., HALIEVSKI, K., ASSI, S., MOGIL, J. S. & TRANG, T. 2018. Microglial P2X4R-evoked pain hypersensitivity is sexually dimorphic in rats. *Pain,* 159**,** 1752-1763.

MIDWOOD, K., SACRE, S., PICCININI, A. M., INGLIS, J., TREBAUL, A., CHAN, E., DREXLER, S., SOFAT, N., KASHIWAGI, M., OREND, G., BRENNAN, F. & FOXWELL, B. 2009. Tenascin-C is an endogenous activator of Toll-like receptor 4 that is essential for maintaining inflammation in arthritic joint disease. *Nat Med,* 15**,** 774-80.

MILNER, R. & CAMPBELL, I. L. 2003. The extracellular matrix and cytokines regulate microglial integrin expression and activation. *J Immunol,* 170**,** 3850-8.

MIRZAEI, R., SARKAR, S., DZIKOWSKI, L., RAWJI, K. S., KHAN, L., FAISSNER, A., BOSE, P. & YONG, V. W. 2018. Brain tumor-initiating cells export tenascin-C associated with exosomes to suppress T cell activity. *Oncoimmunology,* 7**,** e1478647-e1478647.

MONIF, M., REID, C. A., POWELL, K. L., SMART, M. L. & WILLIAMS, D. A. 2009. The P2X7 receptor drives microglial activation and proliferation: a trophic role for P2X7R pore. *J Neurosci,* 29**,** 3781-91.

NEHER, J. J., NENISKYTE, U., HORNIK, T. & BROWN, G. C. 2014. Inhibition of UDP/P2Y6 purinergic signaling prevents phagocytosis of viable neurons by activated microglia in vitro and in vivo. *Glia,* 62**,** 1463-75.

NEWELL, E. W. & SCHLICHTER, L. C. 2005. Integration of K+ and Cl- currents regulate steady-state and dynamic membrane potentials in cultured rat microglia. *J Physiol,* 567**,** 869-90.

NIMMERJAHN, A., KIRCHHOFF, F. & HELMCHEN, F. 2005. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science,* 308**,** 1314-8.

NIU, J., HUANG, D., ZHOU, R., YUE, M., XU, T., YANG, J., HE, L., TIAN, H., LIU, X. & ZENG, J. 2017. Activation of dorsal horn cannabinoid CB2 receptor suppresses the expression of P2Y(12) and P2Y(13) receptors in neuropathic pain rats. *J Neuroinflammation,* 14**,** 185.

NÖRENBERG, W., GEBICKE-HAERTER, P. J. & ILLES, P. 1992. Inflammatory stimuli induce a new K+ outward current in cultured rat microglia. *Neurosci Lett,* 147**,** 171-4.

NORGAUER, J., DOBOS, G., KOWNATZKI, E., DAHINDEN, C., BURGER, R., KUPPER, R. & GIERSCHIK, P. 1993. Complement fragment C3a stimulates Ca2+ influx in neutrophils via a pertussis-toxin-sensitive G protein. *Eur J Biochem,* 217**,** 289-94.

OLMEDILLAS DEL MORAL, M., ASAVAPANUMAS, N., UZCÁTEGUI, N. L. & GARASCHUK, O. 2019. Healthy Brain Aging Modifies Microglial Calcium Signaling In Vivo. *Int J Mol Sci,* 20.

PAN, Y., XIONG, M., CHEN, R., MA, Y., CORMAN, C., MARICOS, M., KINDLER, U., SEMTNER, M., CHEN, Y. H., DAHIYA, S. & GUTMANN, D. H. 2018. Athymic mice reveal a requirement for T-cell-microglia interactions in establishing a microenvironment supportive of Nf1 low-grade glioma growth. 32**,** 491-496.

PANNASCH, U., FÄRBER, K., NOLTE, C., BLONSKI, M., CHIU, S. Y., MESSING, A. & KETTENMANN, H. 2006. The potassium channels Kv1. 5 and Kv1. 3 modulate distinct functions of microglia. *Molecular and Cellular Neuroscience,* 33**,** 401-411.

PELTONEN, S., KALLIONPÄÄ, R. A. & PELTONEN, J. 2017. Neurofibromatosis type 1 (NF1) gene: Beyond café au lait spots and dermal neurofibromas. *Experimental Dermatology,* 26**,** 645-648.

PETRELLA, C., BROCCARDO, M., POSSENTI, R., SEVERINI, C. & IMPROTA, G. 2012. TLQP-21, a VGF-derived peptide, stimulates exocrine pancreatic secretion in the rat. *Peptides,* 36**,** 133-6.

PICCININI, A. M., ZULIANI-ALVAREZ, L., LIM, J. M. & MIDWOOD, K. S. 2016. Distinct microenvironmental cues stimulate divergent TLR4-mediated signaling pathways in macrophages. *Science signaling,* 9**,** ra86-ra86.

POZNER, A., XU, B., PALUMBOS, S., GEE, J. M., TVRDIK, P. & CAPECCHI, M. R. 2015. Intracellular calcium dynamics in cortical microglia responding to focal laser injury in the PC:: G5-tdT reporter mouse. *Frontiers in Molecular Neuroscience,* 8**,** 12.

PRINZ, M., JUNG, S. & PRILLER, J. 2019. Microglia Biology: One Century of Evolving Concepts. *Cell,* 179**,** 292-311.

QUINTAS, C., PINHO, D., PEREIRA, C., SARAIVA, L., GONÇALVES, J. & QUEIROZ, G. 2014. Microglia $P2Y_6$ receptors mediate nitric oxide release and astrocyte apoptosis. *J Neuroinflammation,* 11**,** 141.

RANSOHOFF, R. M. 2016. A polarizing question: do M1 and M2 microglia exist? *Nat Neurosci,* 19**,** 987-91.

REILLY, K. M., LOISEL, D. A., BRONSON, R. T., MCLAUGHLIN, M. E. & JACKS, T. 2000. Nf1;Trp53 mutant mice develop glioblastoma with evidence of strain-specific effects. *Nat Genet,* 26**,** 109-13.

RIEDL, M. S., BRAUN, P. D., KITTO, K. F., ROIKO, S. A., ANDERSON, L. B., HONDA, C. N., FAIRBANKS, C. A. & VULCHANOVA, L. 2009. Proteomic analysis uncovers novel actions of the neurosecretory protein VGF in nociceptive processing. *J Neurosci,* 29**,** 13377-88.

RIVOLTA, I., BINDA, A., MOLTENI, L., RIZZI, L., BRESCIANI, E., POSSENTI, R., FEHRENTZ, J.-A., VERDIÉ, P., MARTINEZ, J., OMELJANIUK, R. J., LOCATELLI, V. & TORSELLO, A. 2017. JMV5656, A Novel Derivative of TLQP-21, Triggers the Activation of a Calcium-Dependent Potassium Outward Current in Microglial Cells. *Frontiers in Cellular Neuroscience,* 11.

ROESCH, S., RAPP, C., DETTLING, S. & HEROLD-MENDE, C. 2018. When Immune Cells Turn Bad-Tumor-Associated Microglia/Macrophages in Glioma. *Int J Mol Sci,* 19.

ROTH, T. L., NAYAK, D., ATANASIJEVIC, T., KORETSKY, A. P., LATOUR, L. L. & MCGAVERN, D. B. 2014. Transcranial amelioration of inflammation and cell death after brain injury. *Nature,* 505**,** 223-8.

SAKAMOTO, M., MIYAZAKI, Y., KITAJO, K. & YAMAGUCHI, A. 2015. VGF, Which Is Induced Transcriptionally in Stroke Brain, Enhances Neurite Extension and Confers Protection Against Ischemia In Vitro. *Transl Stroke Res,* 6**,** 301-8.

SALTON, S. R., FERRI, G. L., HAHM, S., SNYDER, S. E., WILSON, A. J., POSSENTI, R. & LEVI, A. 2000. VGF: a novel role for this neuronal and neuroendocrine polypeptide in the regulation of energy balance. *Front Neuroendocrinol,* 21**,** 199-219.

SÁNCHEZ-LÓPEZ, A., CUADROS, M. A., CALVENTE, R., TASSI, M., MARÍN-TEVA, J. L. & NAVASCUÉS, J. 2004. Radial migration of developing microglial cells in quail retina: a confocal microscopy study. *Glia,* 46**,** 261-273.

SASAKI, Y., HOSHI, M., AKAZAWA, C., NAKAMURA, Y., TSUZUKI, H., INOUE, K. & KOHSAKA, S. 2003. Selective expression of Gi/o-coupled ATP receptor P2Y12 in microglia in rat brain. *Glia,* 44**,** 242-250.

SASMONO, R. T. & WILLIAMS, E. 2012. Generation and characterization of MacGreen mice, the Cfs1r-EGFP transgenic mice. *Methods Mol Biol,* 844**,** 157-76.

SAUPE, F., SCHWENZER, A., JIA, Y., GASSER, I., SPENLÉ, C., LANGLOIS, B., KAMMERER, M., LEFEBVRE, O., HLUSHCHUK, R. & RUPP, T. 2013. Tenascin-C downregulates wnt inhibitor dickkopf-1, promoting tumorigenesis in a neuroendocrine tumor model. *Cell reports,* 5**,** 482-492.

SAVAGE, J. C., CARRIER, M. & TREMBLAY, M. 2019. Morphology of Microglia Across Contexts of Health and Disease. *Methods Mol Biol,* 2034**,** 13-26.

SAVIO, L. E. B., DE ANDRADE MELLO, P., DA SILVA, C. G. & COUTINHO-SILVA, R. 2018. The P2X7 Receptor in Inflammatory Diseases: Angel or Demon? *Front Pharmacol,* 9**,** 52.

SCHAFER, D. P., LEHRMAN, E. K., KAUTZMAN, A. G., KOYAMA, R., MARDINLY, A. R., YAMASAKI, R., RANSOHOFF, R. M., GREENBERG, M. E., BARRES, B. A. & STEVENS, B. 2012. Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. *Neuron,* 74**,** 691-705.

SCHILLING, T. & EDER, C. 2013. Patch clamp protocols to study ion channel activity in microglia. *Methods Mol Biol,* 1041**,** 163-82.

SCHILLING, T. & EDER, C. 2015. Microglial K(+) channel expression in young adult and aged mice. *Glia,* 63**,** 664-72.

SCHILLING, T., QUANDT, F. N., CHERNY, V. V., ZHOU, W., HEINEMANN, U., DECOURSEY, T. E. & EDER, C. 2000. Upregulation of Kv1.3 K(+) channels in microglia deactivated by TGF-beta. *Am J Physiol Cell Physiol,* 279**,** C1123-34.

SEELAND, S., KETTIGER, H., MURPHY, M., TREIBER, A., GILLER, J., KISS, A., SUBE, R., KRÄHENBÜHL, S., HAFNER, M. & HUWYLER, J. 2015. ATP-induced cellular stress and mitochondrial toxicity in cells expressing purinergic P2X7 receptor. *Pharmacol Res Perspect,* 3**,** e00123.

SELLGREN, C. M., GRACIAS, J., WATMUFF, B. & BIAG, J. D. 2019a. Increased synapse elimination by microglia in schizophrenia patient-derived models of synaptic pruning. 22**,** 374-385.

SELLGREN, C. M., GRACIAS, J., WATMUFF, B., BIAG, J. D., THANOS, J. M., WHITTREDGE, P. B., FU, T., WORRINGER, K., BROWN, H. E. & WANG, J. 2019b. Increased synapse elimination by microglia in schizophrenia patient-derived models of synaptic pruning. *Nature neuroscience,* 22**,** 374-385.

SHARMA, K., SCHMITT, S., BERGNER, C. G., TYANOVA, S., KANNAIYAN, N., MANRIQUE-HOYOS, N., KONGI, K., CANTUTI, L., HANISCH, U. K., PHILIPS, M. A., ROSSNER, M. J., MANN, M. & SIMONS, M. 2015. Cell type- and brain region-resolved mouse brain proteome. *Nat Neurosci,* 18**,** 1819-31.

SHIMAZAWA, M., TANAKA, H., ITO, Y., MORIMOTO, N., TSURUMA, K., KADOKURA, M., TAMURA, S., INOUE, T., YAMADA, M., TAKAHASHI, H., WARITA, H., AOKI, M. & HARA, H. 2010. An inducer of VGF protects cells against ER stress-induced cell death and prolongs survival in the mutant SOD1 animal models of familial ALS. *PLoS One,* 5**,** e15307.

SIBILIA, V., PAGANI, F., BULGARELLI, I., TULIPANO, G., POSSENTI, R. & GUIDOBONO, F. 2012. Characterization of the mechanisms involved in the gastric antisecretory effect of TLQP-21, a vgf-derived peptide, in rats. *Amino Acids,* 42**,** 1261-8.

SMITH, G. M. & HALE, J. H. 1997. Macrophage/Microglia regulation of astrocytic tenascin: synergistic action of transforming growth factor-beta and basic fibroblast growth factor. *J Neurosci,* 17**,** 9624-33.

SMOLDERS, S. M., KESSELS, S., VANGANSEWINKEL, T., RIGO, J. M., LEGENDRE, P. & BRÔNE, B. 2019. Microglia: Brain cells on the move. *Prog Neurobiol,* 178**,** 101612.

SORGE, R. E., MAPPLEBECK, J. C., ROSEN, S., BEGGS, S., TAVES, S., ALEXANDER, J. K., MARTIN, L. J., AUSTIN, J.-S., SOTOCINAL, S. G. & CHEN, D. 2015. Different immune cells mediate mechanical pain hypersensitivity in male and female mice. *Nature neuroscience,* 18**,** 1081-1083.

TATSUMI, E., YAMANAKA, H., KOBAYASHI, K., YAGI, H., SAKAGAMI, M. & NOGUCHI, K. 2015. RhoA/ROCK pathway mediates p38 MAPK activation and morphological changes downstream of P2Y12/13 receptors in spinal microglia in neuropathic pain. *Glia,* 63**,** 216-28.

THAKKER-VARIA, S. & ALDER, J. 2009. Neuropeptides in depression: Role of VGF. *Behavioural Brain Research,* 197**,** 262-278.

THION, M. S., GINHOUX, F. & GAREL, S. 2018. Microglia and early brain development: An intimate journey. *Science,* 362**,** 185-189.

TOZAKI-SAITOH, H., TSUDA, M., MIYATA, H., UEDA, K., KOHSAKA, S. & INOUE, K. 2008. P2Y12 receptors in spinal microglia are required for neuropathic pain after peripheral nerve injury. *Journal of Neuroscience,* 28**,** 4949-4956.

TRANI, E., GIORGI, A., CANU, N., AMADORO, G., RINALDI, A. M., HALBAN, P. A., FERRI, G. L., POSSENTI, R., SCHININÀ, M. E. & LEVI, A. 2002. Isolation and characterization of VGF peptides in rat brain. Role of PC1/3 and PC2 in the maturation of VGF precursor. *J Neurochem,* 81**,** 565-74.

TROVÓ-MARQUI, A. & TAJARA, E. 2006. Neurofibromin: a general outlook. *Clinical Genetics,* 70**,** 1-13.

TUCKER, R. P. & CHIQUET-EHRISMANN, R. 2015. Tenascin-C: Its functions as an integrin ligand. *The International Journal of Biochemistry & Cell Biology,* 65**,** 165-168.

TUROLA, E., FURLAN, R., BIANCO, F., MATTEOLI, M. & VERDERIO, C. 2012. Microglial microvesicle secretion and intercellular signaling. *Front Physiol,* 3**,** 149.

TVRDIK, P. & KALANI, M. Y. S. 2017. In Vivo Imaging of Microglial Calcium Signaling in Brain Inflammation and Injury. *Int J Mol Sci,* 18.

VAINCHTEIN, I. D., CHIN, G., CHO, F. S., KELLEY, K. W., MILLER, J. G., CHIEN, E. C., LIDDELOW, S. A., NGUYEN, P. T., NAKAO-INOUE, H. & DORMAN, L. C. 2018. Astrocyte-derived interleukin-33 promotes microglial synapse engulfment and neural circuit development. *Science,* 359**,** 1269-1273.

WALZ, W. & BEKAR, L. K. 2001. Ion channels in cultured microglia. *Microsc Res Tech,* 54**,** 26-33.

WANG, X., PRAGER, B. C., WU, Q., KIM, L. J. Y., GIMPLE, R. C., SHI, Y., YANG, K., MORTON, A. R., ZHOU, W., ZHU, Z., OBARA, E. A. A., MILLER, T. E., SONG, A., LAI, S., HUBERT, C. G., JIN, X., HUANG, Z., FANG, X., DIXIT, D., TAO, W., ZHAI, K., CHEN, C., DONG, Z., ZHANG, G., DOMBROWSKI, S. M., HAMERLIK, P., MACK, S. C., BAO, S. & RICH, J. N. 2018. Reciprocal Signaling between Glioblastoma Stem Cells and Differentiated Tumor Cells Promotes Malignant Progression. *Cell Stem Cell,* 22**,** 514-528.e5.

WANG, Y., ZHANG, H. & HE, Y.-W. 2019. The Complement Receptors C3aR and C5aR Are a New Class of Immune Checkpoint Receptor in Cancer Immunotherapy. *Frontiers in Immunology,* 10.

WENDT, S., MARICOS, M., VANA, N., MEYER, N., GUNEYKAYA, D., SEMTNER, M. & KETTENMANN, H. 2017. Changes in phagocytosis and potassium channel activity in microglia of 5xFAD mice indicate alterations in purinergic signaling in a mouse model of Alzheimer's disease. *Neurobiol Aging,* 58**,** 41-53.

WHITLON, D. S., ZHANG, X. & KUSAKABE, M. 1999. Tenascin-C in the cochlea of the developing mouse. *J Comp Neurol,* 406**,** 361-74.

WOLF, S. A., BODDEKE, H. W. & KETTENMANN, H. 2017. Microglia in Physiology and Disease. *Annu Rev Physiol,* 79**,** 619-643.

WU, C. Y., KAUR, C., SIVAKUMAR, V., LU, J. & LING, E. A. 2009. Kv1.1 expression in microglia regulates production and release of proinflammatory cytokines, endothelins and nitric oxide. *Neuroscience,* 158**,** 1500-8.

XIE, K., LIU, Y., HAO, W., WALTER, S., PENKE, B., HARTMANN, T., SCHACHNER, M. & FASSBENDER, K. 2013. Tenascin-C deficiency ameliorates Alzheimer's disease-related pathology in mice. *Neurobiol Aging,* 34**,** 2389-98.

YALCIN, F., DZAYE, O. & XIA, S. 2020. Tenascin-C Function in Glioma: Immunomodulation and Beyond. *Adv Exp Med Biol,* 1272**,** 149-172.

ZARRINMAYEH, H. & TERRITO, P. R. 2020. Purinergic Receptors of the Central Nervous System: Biology, PET Ligands, and Their Applications. *Mol Imaging,* 19**,** 1536012120927609.

ZHANG, X., XU, P., LI, C., ZHU, W., WU, S., YU, A., DING, Y., WANG, Q. & ZHANG, Z. 2017. Spinal microglial P2X4 receptor-brain-derived neurotrophic factor signaling regulates nicotine withdrawal-induced hyperalgesia. *Neuroreport,* 28**,** 339-347.

ZHAO, X., LIAO, Y., MORGAN, S., MATHUR, R., FEUSTEL, P., MAZURKIEWICZ, J., QIAN, J., CHANG, J., MATHERN, G. W., ADAMO, M. A., RITACCIO, A. L., GRUENTHAL, M., ZHU, X. & HUANG, Y. 2018. Noninflammatory Changes of Microglia Are Sufficient to Cause Epilepsy. *Cell Rep,* 22**,** 2080-2093.

9 List of Figures

Figure 1. Discovery of microglia.. 12

Figure 2. Microglia in mouse brain development... 13

Figure 3. Membrane ion channels and receptors shape the functions of microglia............. 15

Figure 4. Summary of microglial functional phenotypes.. 19

Figure 5. Role of purinergic receptors in modulating microglial phenotype at the different activation states... 22

Figure 6. RNA expression of VGF in the human body.. 23

Figure 7. Schematic representation of VGF translation and processing into the VGF-derived peptides.. 24

Figure 8. Expression of VGF, C3aR1 and C1qbp in microglia... 28

Figure 9. Activation of neurofibromin and associated signaling... 29

Figure 10. TLR4-mediated innate immune responses... 33

Figure 11. Graphical abstract summarizing the effect of TLQP21 on microglia...................... 37

Figure 12. Accumulation of VGF peptides in the vicinity of amyloid ß-plaques....................... 53

Figure 13. A novel mouse line of 5XFAD//VGF KO.. 53

Figure 14. VGF expression after gavage administration of tamoxifen onto BL6//5xFAD//MacGreen//Vgf flox/flox//Rosa26-CreER... 54

Figure 15. TlQP21 receptors are expressed in GBM at variant degrees.................................. 56

Figure 16. TLQP21 increase GL261 tumor size in situ... 57

Figure 17. Graphical abstract illustrating the sexually dimorphic cAMP-dependant defects in purinergic signaling observed in male NF1 +/- microglia.. 60

Figure 18. Purinoreceptors-mediated Potassium currents but not basal currents are altered in NF1 +/- iMG.. 78

Figure 19. A summary of the receptors expressed in microglia.. 102

10 List of Abbreviations

AD Alzheimer's disease

ADP Adenosine diphosphate

Akt Protein kinase B

ATP Adenosine triphosphate

cAMP Cyclic adenosine monophosphate

CNS Central nervous system

CSF1 Colony stimulating factor-1

DAMP Damage-associated molecular pattern

E Embryonic day

EGFP Enhanced green fluorescent protein

GBM Glioblastoma multiforme

GPCR G protein-coupled receptor

HDAC Histone-deacetylase

IL Interleukin

INF-δ Interferon gamma

IP3 Inositol triphosphate

Kir Inward rectifier K^+ channels

KO Knockout

Kv voltage-gated K^+ channels

LPS Lipopolysaccharide

MAPK Mitogen-activated protein kinases

MMP Matrix metalloproteinase

NERP Neuroendocrine regulatory peptides

NF1 Neurofibromin 1, Neurofibromatosis type 1

Nf1 +/- Heterozygous neurofibromin 1 knockout

NGF Nerve growth factor

P Postnatal day

PAMP Pathogen-associated molecular pattern

PC Prohormone convertase

PDE Phosphodiesterase

PI3K Phosphatidylinositol 3´-kinase

PKCζ Protein kinase C zeta

PLC Phospholipase C

PRR Pattern recognition receptors

P2Y Metabotropic purinergic signaling

RUNX Runt-related transcription factor 1

siRNA Short interfering ribonucleic acid

TAM Tumor associated microglia

THIK-1 Two-pore domain halothane-inhibited K^+ channel type 1

Tlr Toll like receptor

TNC Tenascin C

TNF-α Tumor necrosis factor alpha

Vm Membrane potential

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.