Microglia promote synaptic loss and depressive-like behavior under chronic-stress exposure

Inaugural-Dissertation

to obtain the Doctoral Degree

Faculty of Biology, Albert-Ludwigs-Universität Freiburg im Breisgau

presented by

Simone Brioschi

Born in Milan (Italy), 23 June 1986

Dekanin: Prof. Dr. Bettina Warscheid

Promotionsvorsitzender: Prof. Dr. Andreas Hiltbrunner

Betreuer der Arbeit: Prof. Dr. Knut Biber, Faculty of Medicine, Dept. of Psychiatry University of Freiburg Medical Center

Verantwortlicher Betreuer seitens der Fakultät: Prof. Dr. Dierk Reiff, Faculty of Biology, Dept. for Animal Physiology, Neurobiology and Behavior

Affidavit

I herewith declare that I have prepared the present work without any unallowed help from third parties and without the use of any aids beyond those given. All data and concepts taken either directly or indirectly from other sources are so indicated along with a notation of the source. In particular, I have not made use of any paid assistance from exchange or consulting services (doctoral degree advisors or other persons). No one has received remuneration from me either directly or indirectly for work which is related to the content of the present dissertation. Furthermore, I declare that this work has not been submitted in this country or abroad to any other examination board in this or similar form. Moreover, I declare that **the presented work is entirely original and none of the displayed data are published.**

St. Louis (Missouri, USA), December 2018

Simone Brioschi

Examination committee members:

Referent: Prof. Dr. Dierk Reiff

Koreferent: Prof. Dr. Marco Prinz

Drittprüferin: Prof. Dr. Melanie Meyer-Lühmann

Datum der Prüfung: 27.05.2019

Disclosure of personal and external contributions

I personally performed and analyzed all experiments presented in this thesis with the few exceptions listed below. Most of the work was done in my lab of affiliation: Laboratory of Translational Psychiatry, Molecular Psychiatry research group, Department of Psychiatry and Psychotherapy, University of Freiburg Medical Center, Faculty of Medicine, University of Freiburg, Hauptstraße 5, 79104 Freiburg (Germany).

Additional experiments were performed in the following laboratories:

- Figure 3 (panels A, B and C) and Figure 12 (panels D, E and F): The experimental work was performed at the Institute of Neuropathology, University of Freiburg Medical Center, 79106 Freiburg (Germany). The data were analyzed in the Laboratory of Translational Psychiatry, Department of Psychiatry and Psychotherapy, University of Freiburg Medical Center, 79104 Freiburg (Germany).
- Figure 5 (panels A, B, C and D): Both the experimental work and the data analysis were performed at the Department of Pathology and Immunology, Washington University School of Medicine, MO 63110 Saint Louis (USA).
- Figure 10: The experimental work was performed at the German Center for Neurodegenerative Diseases (DZNE), 53127 Bonn (Germany). The data were analysed at the Institute for Multiple Sclerosis Research (IMSF), 37075 Göttingen (Germany).

The following collaborative projects described in this dissertation contain data generated by people other than myself:

- Figure 1 (panel D): Experiments and data analysis performed by **Laura Kracht**, Department of Neuroscience, University Medical Center Groningen (UMCG), 9713AV Groningen (The Netherlands)
- Figure 5 (panels E, F, G and H): Experiments and data analysis performed by **David Wozniak**, Department of Pathology and Immunology, Washington University School of Medicine, MO 63110 Saint Louis (USA).
- Figure 6 (panel A): Experiments and data analysis performed by **Yochai Wolf,** Weizmann Institute, 76100 Rehovot (Israel)

Acknowledgements

First, I want to thank my supervisor *Knut Biber* (University of Freiburg Medical Center, Germany) for giving me the opportunity to carry out my PhD work in his lab, for all the time he dedicated to me and for the uncountable number of precious advises.

A special thank goes to all those people who helped or assisted me with my work, including *Laura Kracht and Bart Eggen* (University Medical Center of Groningen, The Netherlands), *Felix Nebeling and Martin Fuhrmann* (DZNE Bonn, Germany), *Yochai Wolf* (Weizmann Institute, Rehovot, Israel), *Marco Colonna* (Washington University of St. Louis, USA), and *Rosa Paolicelli* (University of Zürich, Switzerland).

I thank with all my heart my dear colleagues and friends *Annette Masuch, Peter Wieghofer, Lukas Amann, Marta Jordão and Paolo d'Errico* (University of Freiburg Medical Center, Germany) for sharing materials, mice and especially their knowledge and experience.

I also would like to thank *Francesca Odoardi and Alexander Flügel* (IMSF Göttingen, Germany) for giving me the chance to complete my data analysis and my thesis in their lab. I really enjoyed the time we spent together. I also thank *Franziska Scharkowski and Lea Sendler* (Max Plank Institute Göttingen, Germany) for helping with the translation of the abstract.

Moreover, I really would like to express my gratitude to the present *PhD committee* for reading this thesis and evaluating my work. I am sure I will make the best use of your comments and criticisms.

Last, I need to thank my amazing fiancé *Hana Janova* for all the support during the most difficult period of my PhD. Your commitment and motivation have always been my driving force!

Sincerely,

Simone Brioschi

Summary

Summary

Growing experimental evidences highlight microglial alterations in psychiatric diseases, including depression and stress-related disorders. The central goal of my thesis was to investigate microglia in a mouse model of chronic-stress consisting of 5 days of Repeated Forced Swim Stress (RFSS). Mice exposed to RFSS the paradigm exhibit behavioral changes resembling depressive-like behavior. I first analyzed microglia by confocal imaging in the hippocampus. My analysis showed that the number of microglial cells was unaltered in mice undergoing the RFSS paradigm. However, microglia exhibited an increased ramification of the processes (hyper-ramified morphology) in the hippocampal regions DG, CA1 and CA3. By contrast, I found no change in microglial morphology or cell number in brain regions that are presumably not affected by chronic-stress (somatosensory cortex, caudate-putamen striatal nucleus, cerebellum). Since the phenotypic and functional properties of hyper-ramified microglia are unknown, I analyzed the expression of cytokines and microglial activation markers. By qPCR, I showed that the expression of the classical pro-inflammatory cytokines CCL2, IL6 and IL1β, and of the macrophage-activation marker CD11b was increased in hippocampal microglia upon the RFSS paradigm. By confocal imaging, I showed increased CD11b immunoreactivity and phagosomes volume in hippocampal microglia from RFSS mice. These data suggested that RFSS promotes a pro-inflammatory phenotype in hippocampal microglia. Next, I assessed the motility of microglia in CA1 by *in vivo* two-photon microscopy. Time-lapse imaging in *Cx3cr1*$^{GFP/+}$ mice (with GFP-positive microglia) before and immediately after the RFSS paradigm showed that RFSS markedly reduced the motility of microglial processes. Together, these data showed that RFSS affects the homeostatic functions of microglia in the hippocampus, however, it was not yet clear whether microglia are important for the behavioral phenotype of the RFSS model. To answer this question, I investigated the *IL34* knock-out mouse line, exhibiting a 50% reduction in the number of microglial cells. The cytokine IL34 is a ligand for CSF1-receptor, expressed in microglia and required for their survival. My data showed that *IL34* knock-out mice did not develop RFSS-induced behavioral changes, whereas learning and memory skills at the Morris-Water Maze were unaffected. These data suggest that hyper-ramified microglia play a crucial role for the behavioral phenotype of the RFSS model. In line with this assumption, I could show that LPS injection in RFSS mice induced a rapid de-ramification of microglial processes, along with a partial "rescue" of the RFSS-induced behavioral changes. Further studies are needed to understand the biological underpinnings of this mechanism.

Summary

Because TNFα was found to be increased in depressed patients, and microglia are the main producer of TNFα in the bran, I hypothesized that microglia may elicit RFSS-induced behavioral changes in a TNFα-dependent manner. To test this, I analyzed mice harboring a conditional genetic deletion of the TNFα locus in microglia. My results showed that mice with TNFα-deficient microglia exhibit RFSS-induced behavioral changes indistinguishable from wild-type. These data suggested that microglia-derived TNFα does not play an important role at the RFSS paradigm. I then hypothesized that hyper-ramified microglia in the hippocampus may affect synaptic function during the RFSS paradigm. Hence, I analyzed the number of glutamatergic synapses in CA1 and somatosensory cortex in RFSS and control mice by confocal imaging. In CA1 of RFSS mice the number of glutamatergic synapses was significantly reduced whereas the somatosensory cortex was unaffected. Given that hyper-ramified microglia were found in the CA1, but not in somatosensory cortex, it is possible that microglia played a role in the synaptic loss.

It was shown that microglia can sense the activity of glutamatergic synapses via the purinergic receptors. Moreover, growing evidences suggest that the brain's purinergic signaling is involved in chronic-stress. I then set out to investigate the role of P2Y12R (a microglial purinergic receptor) in the RFSS model. To do so, I tested both P2Y12R knock-out and wild-type mice to the RFSS paradigm. Interestingly, P2Y12R knock-out mice did not develop RFSS-induced behavioral changes. Moreover, RFSS-induced microglia hyper-ramification and synaptic loss in CA1 were partially reduced in P2Y12R-deficient mice. With a following experiment, I showed that wild-type mice treated with a P2Y12R-inhibitor did not exhibit RFSS-induced behavioral changes, indicating that pharmacological blockage of the P2Y12R-signalling in wild-types recapitulates the RFSS-resilient phenotype of the P2Y12R knock-out mice. Together, these data emphasize the importance of microglia in this model of RFSS and reveal a previously unappreciated role for the P2Y12R signaling during chronic-stress.

Summary

Zusammenfassung

Zahlreiche neue Forschungsergebnisse zeigen Veränderungen von Mikroglia in psychiatrischen Erkrankungen, wie zum Beispiel Depressionen und stressassoziierte Störungen. In meiner Doktorarbeit, untersuchte ich Mikroglia in einem Mausmodell für chronischen Stress, ausgelöst durch wiederholten Schwimmstress an 5 aufeinander folgenden Tagen (repetitive forced swim stress; RFSS). Nach erfolgtem RFSS-Versuch, zeigen die Mäuse Verhaltensänderungen, die einem depressiven Verhalten ähneln und durch Antidepressiva Gabe reversibel sind

Zunächst analysierte ich Anzahl und Morphologie der Mikroglia im Hippocampus mittels konfokaler Mikroskopie. Meine Analyse zeigte, dass die Anzahl der Mikrogliazellen bei Mäusen, die dem RFSS-Paradigma unterzogen wurden, unverändert blieb. Jedoch zeigten die Mikroglia eine erhöhte Verzweigung der Fortsätze (hyperverzweigte Morphologie) in den Hippocampus Regionen DG, CA1 und CA3. Es waren keine morphologischen Veränderungen im somatosensorischer Kortex, Nukleus Caudate-Putamen Striatum und Cerebellum zu beobachten, Hirnregionen, die vermutlich nicht von chronischem Stress betroffen sind. Um mehr über die funktionellen Eigenschaften von hyperverzweigten Mikroglia zu erfahren wurden zunächst qPCR Analysen durchgeführt. Hiermit konnte ich zeigen, dass die Expression der pro-inflammatorischen Zytokine CCL2, IL6 und IL1β und des Makrophagen-Aktivierungsmarkers CD11b nach RFSS in Mikroglia im Maus Hippocampus erhöht waren. Durch konfokale Mikroskopie zeigte ich, dass Mikroglia im Hippocampus von RFSS-Mäusen eine verstärkte CD11b-Immunreaktivität haben und das Volumen von Phagosomen erhöht war. Darüber hinaus, habe ich die Motilität von CA1-Mikroglia mittels *in vivo* Zwei-Photonen Mikroskopie bewertet. Zeitraffer-Aufnahmen in *Cx3cr1*$^{GFP/+}$ Mäusen (green fluorescent protein (GFP)-positive Mikroglia) zeigten, dass RFSS die Motilität von Mikrogliazellen deutlich verringert. Diese Ergebnisse verdeutlichen, dass RFSS die homöostatischen Funktionen von Mikroglia im Hippocampus verändert. Es war jedoch noch nicht klar, ob diese Veränderungen der Mikroglia für den Verhaltensphänotyp im RFSS Maus-Modells wichtig sind.

Um diese Frage zu beantworten, untersuchte ich zunächst die IL34-Knock-Out-Mauslinie, welche bis zu 50% weniger Mikrogliazellen aufweist. In diesen Tieren waren keine RFSS-induzierten Verhaltensänderungen zu messen. Hingegen war Lernen und Gedächtnis in dieser Mauslinie unverändert. Diese Daten deuten darauf hin, dass Mikroglia für den Verhaltensphänotyp des RFSS

Summary

Maus-Modells eine entscheidende Rolle spielen. Da die Injektion von LPS einen schnellen Rückgang der hyperverzweigten Morphologie von Mikroglia induzierte und einen teilweisen Rückgang der RFSS-induzierten Verhaltensänderungen zur Folge hatte, legen meine Versuche den Schluss nahe, dass hyperverzweigte Mikroglia für die Verhaltensänderungen im RFSS Model eine Rolle spielen.

TNFα, welches im Gehirn hauptsächlich von Mikroglia produziert wird, ist häufig bei Patienten mit Depressionen erhöht. Daher stellte ich die Hypothese auf, dass Mikroglia durch TNFα-Freisetzung RFSS-induzierte Verhaltensänderungen auslösen könnten. Um diese Hypothese zu testen, analysierte ich Mäuse, mit einer Deletion des TNFα-Gens spezifisch in Mikroglia. Da ich keine Unterschiede im RSS-Versuch zwischen Mäusen mit TNFα-defizienten Mikroglia und Wildtyp Mäuse gefunden habe, wird eine Rolle von TNFα aus Mikroglia- in diesem Maus-Modell ausgeschlossen.

Im Moment wird diskutiert ob Mikroglia Synapsen aufnehmen können und so neuronale Kommunikation beeinflussen können. Um zu untersuchen ob dies im RFSS Model wichtig ist analysierte ich zunächst die Anzahl der glutamatergen Synapsen in der CA1-Region und des somatosensorischen Cortex in RFSS- und Kontrollmäusen mittels konfokaler Mikroskopie. Nach dem RFSS-Paradigma war die Anzahl glutamaterger Synapsen in der CA1-Region signifikant reduziert. Im Gegensatz dazu, war die Zahl glutamaterger Synapsen im somatosensorischen Cortex unverändert. Da hyperverzweigte Mikroglia in der CA1-Region, nicht aber im somatosensorischen Cortex gefunden wurden, ist es möglich, dass Mikroglia beim synaptischen Verlust eine Rolle gespielt.

Mikroglia interagieren mit glutamatergen Synapsen mit Hilfe von purinergen Rezeptoren zB. P2Y12R. Aus diesem Grund habe ich eine mögliche Rolle von P2Y12R im RFSS-Modell untersuchen. Interessanterweise entwickelten P2Y12R-Knock-out-Mäuse keine RFSS-induzierten Verhaltensänderungen und sowohl die RFSS-induzierte Mikroglia-Hyperverzweigung als auch der synaptische Verlust in der CA1-Region unter P2Y12R-defizienten Bedingungen waren signifikant reduziert. Übereinstimmend mit den Knock-out Daten zeigte ich, dass Wildtyp-Mäuse, die mit einem P2Y12R-Inhibitor behandelt wurden, keine RFSS-induzierten Verhaltensänderungen zeigten. Zusammenfassend unterstreichen diese Daten die Bedeutung der Mikroglia in diesem RFSS-Modell. Meine Daten legen den Schluss nahe, dass im RFSS-Modell Mikroglia über P2Y12 Aktivität hyperverzweigen und diese Zellen für den Verlust von glutamatergen Synapsen verantwortlich sind. Ob dies jedoch mechanistisch zu den RFSS-induzierten Verhaltensänderungen führt ist noch nicht klar und sollte in weiteren Studien untersucht werden.

Table of content

Table of content (index) Page

Table of content

Table of content

Table of content

Table of content (list of figures) Page

Abbreviation

Abbreviations

Amy	_	Amygdala
ANOVA	_	Analysis of Variance
BBB	_	Blood Brain Barrier
BDNF	_	Brain-Derived Neurotrophic Factor
CA1	_	Cornu Ammonis regon 1
CA3	_	Cornu Ammonis regon 3
CCL2	_	C-C chemokine ligand-2
CER	_	Cerebellum
CNS	_	Central Nervous System
CR3	_	Complement Receptor-3
CSD	_	Chronic Social Defeat
CSF1	_	Colony Stimulating Factor-1
CSF1R	_	Colony Stimulating Factor-1 Receptor
CX3CL1	_	CX3 Chemokine Ligand-1 (fractalkine)
CX3CR1	_	CX3 Chemokine Receptor-1 (fractalkine receptor)
DAPI	_	4′,6-Aiamidino-2-Phenylindole’
DG	_	Dentate Gyrus
FACS	_	Fluorescence-Activated Cell Sorting
FST	_	Forced Swim Test
GCL	_	Granule Cell Layer
GFAP	_	Glial Fibrillary Acidic Protein
GFP	_	Green Fluorescent Protein
HPA	_	Hypothalamic Pituitary Adrenal axis
Iba1	_	Ionized calcium Binding Adapter molecule-1
ip.	_	intraperitoneal

Abbreviation

IT	_	Immobility Time
LPS	_	Lipopolysaccharide
MDD	_	Major Depressive Disorder
ML	_	Molecular Layer
mPFC	_	medial Prefrontal Cortex
MWM	_	Morris Water Maze
NAc	_	Nucleus Accumbens
NPC	_	Neuronal Progenitor Cells
P2Y12R	_	Purinergic 2Y Receptor-12
PCL	_	Pyramidal Cell Layer
qPCR	_	Quantitative Polymerase Chain Reaction
RFSS	_	Repeated Forced Swim Stress
SGZ	_	Sub-Granular Zone
SO	_	Stratum Oriens
SR	_	Stratum Radiatum
ssCTX	_	Somatosensory Cortex
STR	_	Striatum Caudate Putamen
SVZ	_	Sub-Ventricular Zone
TST	_	Tail Suspension Test
VGLUT1	_	Vesicular Glutamate Transporter-1

Introduction

1. Definition of “microglia”: the brain-resident macrophages

Every organ in the body hosts a population of professional phagocytic cells named “macrophages” which are generated through the hematopoietic lineage. Macrophages are essentially myeloid-derived leukocytes and, together with other types of myeloid cells, represent the innate immune system of the organism. In case of lesions or infections macrophages can promptly proliferate and migrate towards a site of inflammation, thus producing inflammatory mediators and apoptotic corpses are scavenged by phagocytosis. Moreover, tissue-resident macrophages are important for development and homeostasis of the tissue itself, as well as for the immunological surveillance of the neighboring cells. Like other organs, also the central nervous system (CNS) (brain and spinal cord) is colonized by its own type of resident macrophages, known as "Microglia". This name relies on the traditional concept according to which all brain’s cells are classified into two main categories: namely neurons and glia. Glia encompasses heterogeneous cells types, mostly astrocytes, oligodendrocytes and microglia. The first two share with neurons the same neuroectodermal ontogeny and are known to exert a variety physiological properties essential for brain’s function (Barres, 2008). By contrast, microglia are myeloid cells of mesodermal origin, immigrated in the CNS during the embryonic development. The whole CNS is anatomically separated from the peripheral circulation by the blood-brain barrier (BBB), a particularly tight structure of the neuro-vascular unit which prevents leakage of plasmatic proteins and infiltration of circulating immune cells that might harm the fine architecture of the brain. Misleadingly, the brain was often described as an immune-privileged organ, in which leukocytes’ reactions could be observed only under pathological conditions. However, microglia fully populate the brain’s parenchyma and exhibit a variety of immunological functions even under homeostasis. Several studies reported that microglia are very important for maintenance of brain’s physiology through a continuous exchange of biological information with neurons and other glial cells. Moreover, microglia are present in the CNS of several organisms including humans, monkeys, rodents, birds, amphibians and fishes. Interestingly, archaic macrophages were also found in organisms endowed of a primitive nerve system, such as leeches, snails and insects. This indicates that the evolutionary origin of microglia is very ancient and potentially started with the appearance of the first neuronal ganglia in the invertebrates. Given the intimate relationship (across different species) between the CNS and this unique kind of macrophages, it is tempting to speculate how the brain would develop and perform if microglia were dysfunctional or just absent. After decades of research we certainly gained some clues, but we still have no clear picture of what microglia really do in the brain.

2. Historical overview

2.1 Golgi and Cajal

In the end of the 19th century, neuropathologists were aware of at least two populations of cells in the brain, namely neurons and glial cells. The invention of microscope together with new staining techniques of the nerve tissue (especially the silver-nitrate staining), paved the way to the first pioneering observations of *Camillo Golgi* and *Santiago Ramón y Cajal*. Between the early 1870s and the late 1890s, the two scientists described an arborized structure of neuronal cells, which were identified as the basic functional unit of the nervous system. Both *Golgi* and *Cajal* were awarded the Nobel Prize in 1906 for their revolutionary discovery. Since neurons were the first brain cells to be discovered and characterized at that time, neuropathologists refer them as "first element". During the following years, *Cajal* identified a second population of brain cells other than neurons, which were accordingly indicated as "second element" or "glial cells". These cells exhibited a peculiar starry shape and were much more abundant than neurons themselves. The term "glia" or "neuroglia" (from Greek "Glue") was already introduced in the 19th century by *Rudolf Virchow*, indicating a sort of biological matter providing structural consistency to the nerve tissue, filling the gaps between neighboring cells. However, before Cajal's observations it was still unclear whether "glia" was an independent cellular entity or merely a connective matrix devoid of own nuclei and cell bodies. Only in the 1895 the Hungarian neuroanatomist *Michael von Lenhossék* introduced the term "Astrocyte", referring to the typical morphology of these cells (from Greek "Star Cell") (Wolf *et al.*, 2017).

2.2 Del Rio-Hortega

The first theories about microglia date back to the beginning of 20th century, when *Cajal* postulated the existence of a third cellular element in the brain, distinct from both neurons and astrocytes. Other neuropathologists, such as *Alois Alzheimer* and *Franz Nissl*, witnessed abundant amoeboid-like cells in the human brain under pathological conditions. Nevertheless, a careful characterization of these cells was not attempted before the beginning of the 1920s. The name "microglia" was originally introduced by *Pio Del Rio-Hortega*, an alumnus from the Cajal school, to distinguish them from astrocytes (or macroglia because of their bigger size) (Wolf *et al.*, 2017).

Introduction

Del Rio-Hortega developed a modified version of a silver carbonate staining that allowed to observe microglia in more detail (**Figure I).** With this technique, the Spanish neuroscientist described two new kinds of glial cells, namely microglia and oligodendrocytes. His observations on these so called "microgliocytes" (term that *Hortega* used to indicate single microglial cells) were collected in a series of publications in the period between 1919 and 1927 (Perez-Cerda *et al.,* 2015; Kettenmann *et al.,* 2011; Sierra *et al.,* 2016; Sousa *et al.,* 2017), and eventually outlined in a book chapter entitled "Microglia" written for the issue "Cytology and Cellular Pathology of the Nervous System". The silver-carbonate staining allowed *Del Rio-Hortega* to establish a series of key points on microglial biology. 1) Microglia were described as glial cells of mesodermal origin (unlike astrocytes and neurons which are neuroectodermal-derived), which probably enter the brain parenchyma migrating through the blood vessels during embryonic development. 2) Initially, microglia accumulate in the white matter, but then spread throughout the gray matter, eventually colonizing all the CNS. 3) In the adult brain, microglia appear as apolar cells, endowed of ramified processes radially distributed around the cell body. 4) Microglial cells are evenly dispersed throughout the CNS, occupying not-overlapping territories. 5) Upon brain injury, microglia become activated and undergo a dramatic morphological change, acquiring a sort of amoeboid shape. 6) In this state, microglia migrate towards the injury site where they proliferate and help remove tissue debris by phagocytosis (Kettenmann *et al.,* 2011). It is interesting to note how these basic concepts are still valid nowadays (Prinz and Priller 2014).

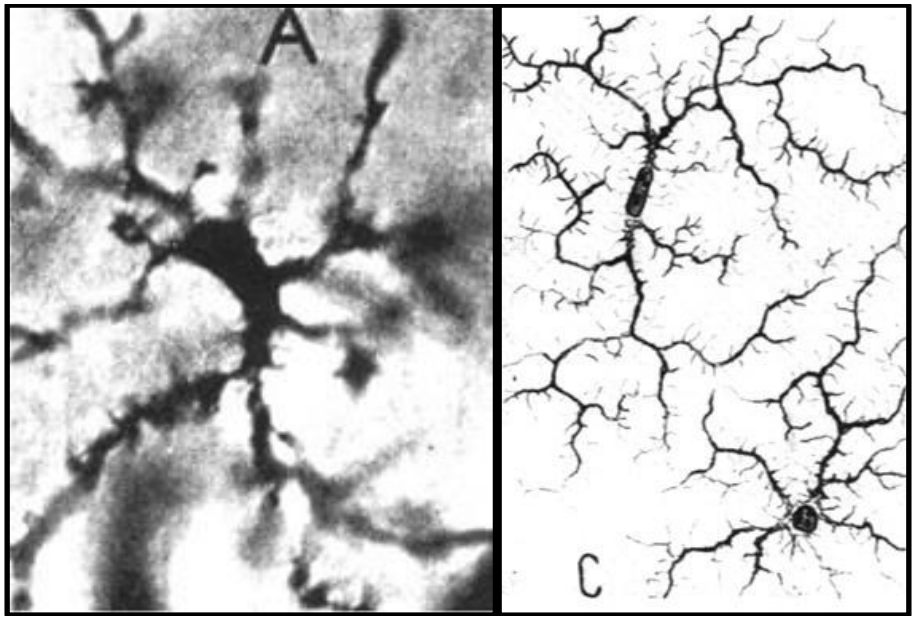

Figure I: Original photomicrographs and hand drawn microglial cells (by Del Rio-Hortega). (Left) Original photomicrographs by del Rio-Hortega showing a microglial cell in Silver-Carbonate staining. (**Right)** Two microglial cells hand drawn by Del Rio-Hortega. Adapted from (Kettenmann *et al.,* 2011).

2.3 Georg Kreutzberg

At the end of the 1960s, the German neuropathologist *Georg W. Kreutzberg* investigated behavior of microglia in a rodent model of facial nerve axotomy. Transection of the facial nerve causes a rapid degeneration of neurons located in facial motor nucleus of the brainstem. This event is accompanied by a massive proliferation of the local microglia, followed by a slow recovery (Moran and Graeber, 2004). It appeared clear that neuronal damage can be sensed by neighboring microglia, which in turn promote a long-lasting inflammatory reaction within the injured area. *Kreutzberg* monitored the ongoing pathological process by electron microscopy. The ultrastructural resolution allowed to highlight the physical interaction between microglial cells and neuronal cell bodies, indicating the existence of some sort of a cell-mediated communication. Importantly, these images showed that upon inflammatory activation, microglia were able to engulf synapses, which were then removed from the neuronal surface (Blinzinger and Kreutzberg, 1968). With these studies, *Kreutzberg and colleagues* described a mechanism of synaptic displacement/removal mediated by microglia (**Figure II**), which was termed as "synaptic stripping" (Kettenmann *et al.*, 2013). In summary, microglia are relatively quiescent phagocytes under homeostasis, however, upon injury microglia become "active" and efficiently scavenge debris of apoptotic/necrotic cells. Moreover, microglia may help shape synaptic connections under neuro-inflammation, possibly exerting a neuroprotective function.

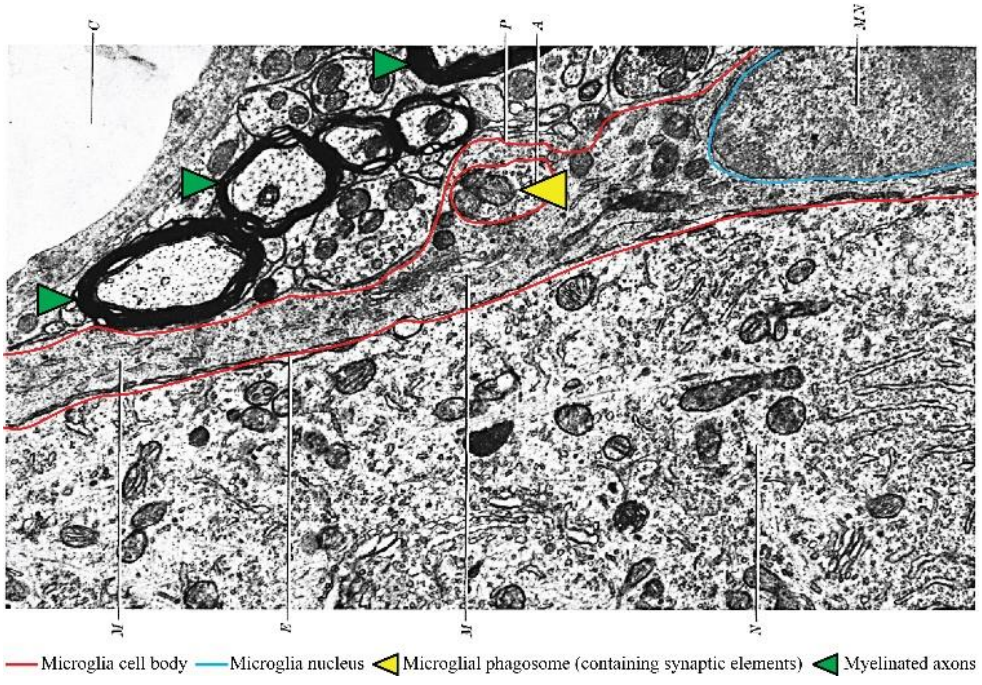

Figure II: Electron micrograph of a microglial cell (by Georg Kreutzberg). Electron micrograph of a microglial cell in close contact with a neuronal cell body. As visual aid, a red and a blue line have been drawn along the microglial cell body and the nucleus respectively. The yellow triangle highlights the presence of synaptic structures within a microglial phagosome. Green triangles point to the neighboring myelinated axons. Adapted from (Blinzinger and Kreutzberg, 1968).

2.4 The modern microglia research

2.4.1 Electrophysiological properties of microglia

The decade between early 1990s and beginning of 2000 provided an important background of knowledge for the modern microglia research. The work of *Helmut Kettenmann* uncovered electrophysiological properties of microglia either cultured *in vitro* or on acute brain slices. Application of patch clamp techniques revealed expression of a unique pattern of K^+ channels, which seemingly conferred to microglia a distinct electrophysiological profile compared to other macrophages (Kettenmann *et al.*, 1990; Brockhaus and Iischner, 1993; Ilschner *et al.*, 1995). Moreover, *Kettenmann* focused his research on the neurochemical signals governing mechanisms of microglia activation. In particular, microglia displayed a robust responsiveness to ATP and its metabolites, which were shown to activate microglia through an array of purinergic receptors (Walz *et al.*, 1993). The role of the purinergic receptors in microglia biology will be addressed more in detail in a dedicated chapter. Microglia was found to express different neurotransmitter receptors (at least *in vitro*), which may enable microglial to sense neuronal activity (Pocock and Kettenmann, 2007). Moreover, microglia challenged with different sort of pro-inflammatory stimuli (i.e. LPS, ATP, nerve injury) (**Figure III**) displayed a substantial modification of their electrophysiological properties, indicating that ion channels play an important role in microglial response after lesion/inflammation (Draheim *et al.*, 1999; Prinz *et al.*, 1999; Boucsein *et al.*, 2000). It has to be borne in mind that the cell culture system constitutes an artificial environment, which certainly causes important changes of the physiological microglial phenotype (Biber *et al.*, 2014).

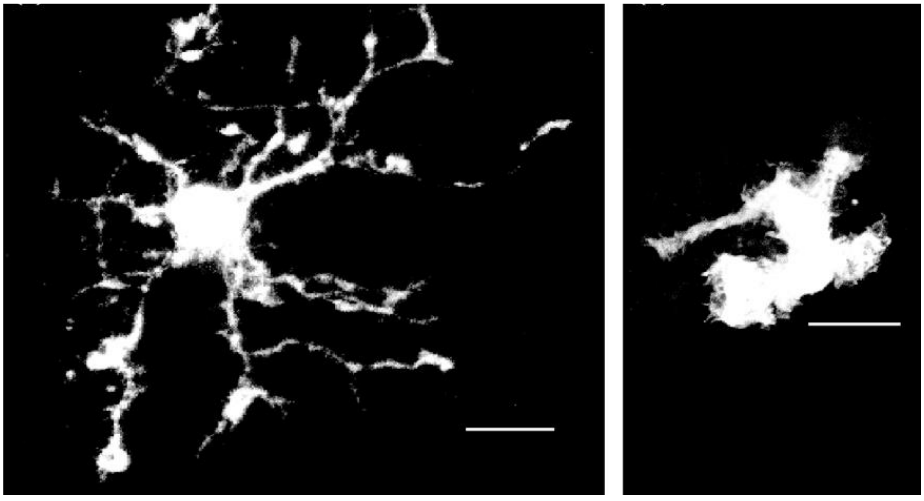

Figure III: Ramified and amoeboid microglia in acute brain slices.

(Left) Ramified microglia in acute slices from rat facial nucleus.

(Right) Activated (amoeboid) microglia from rat facial nucleus after facial nerve axotomy. Adapted from (Pocock and Kettenmann, 2007).

2.4.2 Microglia FACS staining

In 1991 *Jonathon Sedgwick* proposed a tool for specific selection of microglia population in fluorescence-activated cell sorting (FACS). This method was based on fluorescent labeling of the surface markers CD11b and CD45 and it is still routinely used today. CD11b is a component of the heterodimeric Complement Receptor 3 (CR3), highly expressed in different myeloid phagocytes (including microglia). CD45 is a common-leukocyte antigen expressed at variable level in both myeloid and lymphoid leukocytes. Microglia express reduced levels of CD45 compared to other brain macrophages (such as perivascular, meningeal and choroid plexus macrophages) which are strongly CD45 and MHCII-positive. By using this labeling panel, parenchymal microglia were identified as $CD11b^{+}CD45^{low}$ population. A much smaller population of non-parenchymal macrophages appeared as $CD11b^{+}CD45^{hi}$ cells (Sedgwick *et al.*, 1991).

2.4.3 Iba1-staining in immuno-histochemistry

In the mid of the 1990s, the microglial phenotypic marker Iba1 was identified and development of antibodies directed against this protein has revolutionized the way to study microglia in histology (Imai *et al.*, 1996). Iba1 stays for "ionized calcium-binding adapter molecule-1" and is encoded by the gene Aif1 (Allograft Inflammatory Factor-1). Iba1 is essentially an actin-binding protein specifically expressed in microglia and macrophages. It is involved in recruitment and scaffolding of an array of components aiming to stabilize actin filaments. Iba1 contains a Ca^{2+}-binding domain and was described as an important mediator in mechanisms of membrane ruffling and phagocytosis (Ito *et al.*, 1998; Ohsawa *et al.*, 2000; Sasaki *et al.*, 2001; Yamada *et al.*, 2006). The term "membrane ruffling" indicates the formation of a membrane protrusion involving polymerization of actin filaments. Such protrusions (lamellopodia and filopodia) may have functions of cell motility, inter-cellular communication and formation of phagocytic cups (Small *et al.*, 2002; Kress *et al.*, 2007). Iba1 became some the most widespread marker for studying dynamics microglia activation in immuno-histochemistry (Okere and Kaba, 2000; Ito *et al.*, 2001). In comparison with other microglia markers (such as CD11b, F4/80, IB4 and others), labelling of Iba1 has certain advantages, for instance the specificity of the antibody, the high expression levels of the marker and the uniform distribution through the cytoplasm, which allows a precise distinction of both cell body and processes. More recently, a transgenic mouse expressing eGFP under the control of Iba1 promoter was generated (Hirasawa *et al.*, 2005).

2.4.4 Generation of the Cx3cr1^{GFP} and Cx3cr1^{CreER} mouse lines

CX3CR1 is a chemokine receptor which in the brain shows a very high expression level in microglia and other brain macrophages, with basically no expression in neurons or other glial cells. In the 2000, *Steffen Jung* generated the transgenic mouse strain *Cx3cr1^{GFP}*, harboring a GFP coding sequence inserted in-frame within the *Cx3cr1* locus (Jung *et al.*, 2000). GFP knock-in causes on one hand the knock-out of the endogenous *Cx3cr1* gene and, on the other hand, induces GFP expression under the control of the *Cx3cr1* promoter, thus resulting in GFP+ microglia (**Figure IV**). The *Cx3cr1^{GFP}* became soon the most widespread mouse line for studying microglia *in vivo* (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005). Years later the *Cx3cr1^{CreER}* mouse line was generated, which expresses tamoxifen-inducible Cre-recombinase under the control of the *Cx3cr1* promoter (Goldmann *et al.*, 2013; Parkhurst *et al.*, 2013; Yona *et al.*, 2013). This line allowed a temporally defined (tamoxifen-dependent) labelling of CX3CR1+ cells, which helped investigate embryonic brain colonization and post-natal turnover of microglia (Wieghofer and Prinz, 2015; Wieghofer *et al.*, 2015).

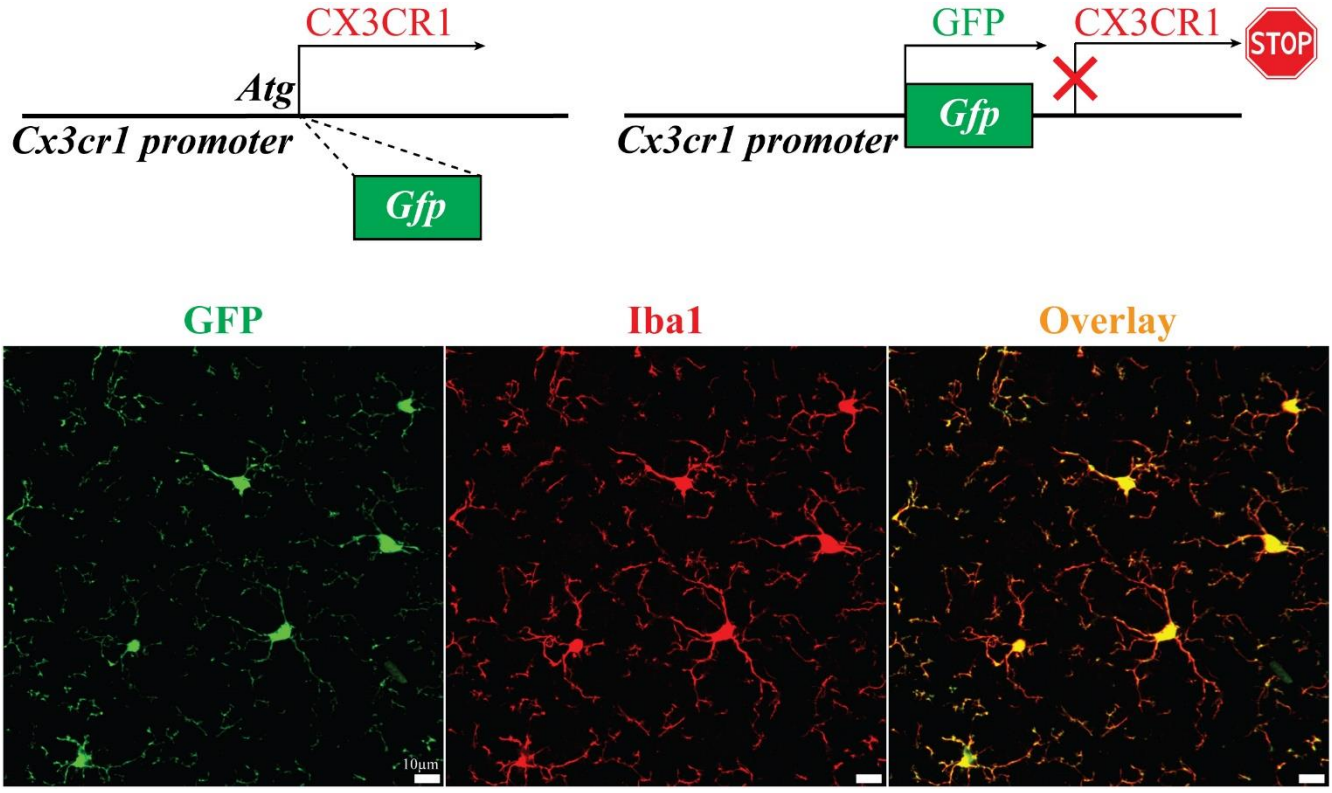

Figure IV: The Cx3cr1$^{GFP/+}$ mouse line. (Upper panel) Scheme of the *Cx3cr1^{GFP}* transgene. **(Bottom panel)** Representative confocal picture in the hippocampus of a *Cx3cr1$^{GFP/+}$* brain with immuno-staining for the microglial cytoplasmic marker Iba1 (from Simone Brioschi). Note the perfect overlay between the GFP (green) and the Iba1 (red) signals.

3. Ontogeny of microglia

Microglia are myeloid cells and as such they belong to the hematopoietic developmental lineage. Hematopoiesis can be distinguished in three main waves, temporally and anatomically distinct, but partially overlapping one to each other. The first wave is the primitive hematopoiesis during the early embryonic period, which occurs within the yolk sac (therefore externally the developing embryo). This stage of embryonic hematopoiesis is short and transitory, and mainly serves to supply the first erythrocytes for the embryonic circulation. Primitive hematopoiesis is soon replaced by the definitive hematopoiesis (second wave), which takes place first in the in the aorta-gonad-mesonephros (AGM) and soon after in the fetal liver (therefore within embryo). Hematopoietic stem cells (HSC) giving rise to all differentiated blood cells are generated at this stage. Shortly before birth, HSCs colonize the bone marrow (third wave) which will be hereafter the main hematopoietic tissue throughout life (Bertrand *et al.*, 2005; Samokhvalov *et al.*, 2007; Cumano and Godin, 2007; Orkin and Zon, 2008; Jagannathan-Bogdan and Zon, 2013).

A number of landmark publications established that microglia originate from the primitive hematopoiesis during the embryonic development (Prinz and Priller 2014; Epelman *et al.*, 2014; Perdiguero and Geissmann 2015; Ginhoux and Prinz 2015; Kierdorf *et al.* 2016). This idea was initially suggested in the late 1999 (Alliot *et al.*, 1999) and then finally confirmed about 10 years later. By means of a fate-mapping study, *Ginhoux and colleagues* revealed that microglial precursors cells are generated from the yolk sac and then colonize the brain rudiment migrating through the embryonic bloodstream between embryonic day E9-10 (Ginhoux *et al.*, 2010). Seeding of yolk sac-derived microglial precursors takes place after establishment of the embryonic circulation, which occurs at about E8.5 (McGrath *et al.*, 2003; Stremmel *et al.*, 2018), but ends with the closure of the blood-brain barrier (BBB) at about E14.5 (Daneman *et al.*, 2010). Importantly, microglia ontogeny does not require the transcription factor MYB (Schulz *et al.*, 2012), which is otherwise necessary for development of HSCs and their progeny (Mucenski *et al.*, 1991). This evidence suggested that bone marrow-derived myeloid cells and microglia do not share the same ontogenetic pathway (Chen *et al.*, 2011; Schulz *et al.*, 2012; Hoeffel *et al.*, 2015; Sheng *et al.*, 2015). Furthermore, recent findings showed that (in addition to microglia) also perivascular and meningeal macrophages are generated in the yolk sac and are not (or just partially) replaced by bone marrow derived monocytes (Goldmann *et al.*, 2016).

Introduction

Likewise other embryonic-derived macrophages (Sieweke and Allen, 2013; Ginhoux and Jung, 2014) microglia are generated from uncommitted $cKit^{+}CD31^{+}CD45^{-}$ erythromyeloid progenitor cells (EMP) of mesodermal origin. These multipotent macrophage progenitors emerge from the yolk sac hemogenic endothelium as early as E7.5–8.5 (McGrath *et al.*, 2015; Ginhoux and Guilliams, 2016) and their differentiation into macrophages is governed by a combination of transcription factors, including Runx1, PU.1 and IRF8 (Rosenbauer and Tenen, 2007; Ginhoux *et al.*, 2010; Kierdorf *et al.*, 2013; Crotti and Ransohoff, 2016; Hagemeyer *et al.*, 2016). At about E9, yolk sac-derived EMPs generate a subpopulation expressing an array of phenotypic markers resembling microglial phenotype (such as CX3CR1, CSF1R, CD45 and F4/80). These cells eventually start colonizing the neural tube from E9.5 onward, giving rise to earliest microglia population which can be found in the embryonic brain starting from E10.5 (Kierdorf *et al.*, 2013). Although origin and differentiation of other types of tissue-resident macrophages are still debated (Gomez Perdiguero *et al.*, 2014; Hoeffel *et al.*, 2015; Sheng *et al.*, 2015; Bonnardel and Guilliams, 2018; Hoeffel and Ginhoux, 2018), it is now broadly accepted that murine microglia arise entirely and exclusively from the yolk sac EMPs. Multiple waves of microglia from different hematopoietic progenitors have been hypothesized in zebrafish model (Xu *et al.*, 2015; Ferrero *et al.*, 2018), however several lines of evidence discourage such a scenario for the mouse brain.

Between E12-16 the embryonic mouse brain shows an uneven microglia distribution, with "spots" of higher cell density localized in the cortical white matter. Gradually, proliferating microglia migrate towards the gray matter, yielding a rather homogeneous pattern at about E18 (Swinnen *et al.*, 2013; Squarzoni *et al.*, 2014). During this period microglia still display an immature amoeboid morphology. The ramified shape typical of mature microglia slowly develops starting from late gestation till the 2^{nd}-3^{rd} postnatal week (Wu *et al.*, 1993; Orłowski *et al.*, 2003; Walker *et al.*, 2014; Miyamoto *et al.*, 2016; Bennett *et al.*, 2016). Key factors mediating recruitment and distribution of embryonic microglia in the developing brain are still uncertain (Kierdorf *et al.*, 2013; Arnò *et al.*, 2014; Mass *et al.*, 2016). Further researches are required for shading some light over this point. Altogether these findings established, once and for all, that microglia ontogeny takes place in the yolk sac during the embryonic period and it is therefore unrelated to the definitive hematopoiesis from either fetal liver or bone marrow. Nevertheless, for long time it was not clear whether adult hematopoiesis may account for the microglial turnover along the life span of the mouse. Indeed, it was postulated that after birth circulating monocytes could enter the brain and differentiate into microglial cells (Kennedy and Abkowitz, 1997; Priller *et al.*, 2001; Simard *et al.*, 2006; El Khoury *et al.*, 2007).

These findings were particularly relevant for cell therapy approaches as bone marrow-derived monocytes could then be exploited as a vehicle for delivering therapeutic transgenes into the brain to help treat a number of brain pathologies (Flügel *et al.*, 2001; Vallieres and Sawchenko, 2003; Djukic *et al.*, 2006; Beers *et al.*, 2006; Lebson, 2010; Koronyo *et al.*, 2015). These studies were primarily based on the bone marrow chimeras with transplantation of fluorescent HSC into a wild-type recipient mouse. However, other works highlighted that bone marrow ablative treatments (such as irradiation or chemotherapy regimen) typically induces a severe disruption of the BBB. In this condition monocytes are allowed to engraft the brain, yet this does not seem to occur in the absence of BBB damage (Ajami *et al.*, 2007; Mildner *et al.*, 2007, 2011; Prinz *et al.*, 2011; Bruttger *et al.*, 2015; Buttgereit *et al.*, 2016; Wang *et al.*, 2016). Moreover, using genetic-based approaches for specific labelling of either microglia or HSC, it was demonstrated that adult hematopoiesis has a negligible contribution to the pool of brain resident microglia (Ajami *et al.*, 2007; Hashimoto *et al.*, 2013; Yona *et al.*, 2013; Goldmann *et al.*, 2013; Gomez Perdiguero *et al.*, 2014; Goldmann *et al.*, 2016; Huang *et al.*, 2018). From these studies emerged that circulating monocytes engraft the CNS only under certain neuroinflammatory conditions which cause an increased permeability of the BBB, for example in the EAE model or after whole body irradiation (Mildner *et al.*, 2007; Ajami *et al.*, 2011; Bruttger *et al.*, 2015). Nonetheless, life-span of blood-derived cells in the injured tissue is supposed to be rather ephemeral and usually these cells are no longer detectable after resolution of inflammation (Brendecke and Prinz, 2015; Greter *et al.*, 2015). These evidences have led to the definition of microglia as "long-living" cells. We should anyway keep in mind that microglia population is slowly, but constantly, renovated along the life-span of an organism. This is due to apoptosis and local proliferation of resident microglia apparently occurring in a rather stochastic manner under homeostasis (Lawson *et al.*, 1992; Askew *et al.*, 2017; Tay *et al.*, 2017; Füger *et al.*, 2017). By contrast, microglia can undergo clonal expansion under pathological condition (Füger *et al.*, 2017; Tay *et al.*, 2017). So far, only one study attempted to determine microglial turnover in the human brain. Although limited by the low number of recruited subjects (only two), this study could show that human microglia divide at a very slow rate, but enough to yield a complete renewal of the entire microglia population across the life (Réu *et al.*, 2017). Interestingly, some recent findings are suggesting that bone marrow-derived monocytes can indeed seed the brain and generate microglia-like cells even in absence of BBB preconditioning. This would happen when the endogenous microglia population is ablated, thus generating empty niches in the brain's parenchyma which can be filled by circulating monocytes (Bennett *et al.*, 2018; Cronk *et al.*, 2018; Lund *et al.*, 2018b).

4. Microglia during brain development

Early development of the mammalian brain is characterized by intensive neurogenesis and synaptogenesis. However, only a fraction of the neuronal structures will eventually be integrated in the functional brain's circuits (Zhao *et al.,* 2008; Ming and Song, 2011). Microglia, by virtue of their phagocytic nature, might play an essential role in the programmed death of supernumerary neurons, as well as their subsequent engulfment and removal (Brown and Neher, 2012; Bilimoria and Stevens, 2015; Paolicelli and Ferretti, 2017). To observe this phenomenon, one of the first approaches was the organotypic slice culture system. In a seminal study from *Marín-Teva and colleagues* it was observed that depletion of microglia from cerebellar cultures using clodronate filled liposomes was associated with increased number of Purkinje cells. Authors further suggested that microglia restricted neuronal proliferation through release of superoxide free radicals generated by the NADPH-oxidase enzyme (Marín-Teva *et al.*, 2004). Very similar results were later on provided by a different group working on hippocampal organotypic cultures (Wakselman *et al.,* 2008), indicating that the mechanism of microglia-mediated neuronal death is probably conserved across different brain regions. More recently, microglia were observed to phagocytose neuronal progenitor cells (NPCs) within the ventricular and sub-ventricular zone, which give rise to the cortical projections and olfactory bulb neurons. This activity was confirmed in cortical samples of rats, monkeys and humans (Cunningham *et al.,* 2013). A similar behavior was also witnessed in embryonic zebrafish CNS (Sieger *et al.,* 2012; Mazaheri *et al.,* 2014). Taken together, these results suggest that microglia actively regulate the overall number of neurons, counterbalancing the enhanced neurogenesis naturally occurring during the neurodevelopmental period. In this specific context, microglia behave as a sort of brain's scavengers, whose activity helps support the physiological development of the CNS.

Relevance of microglia for post-natal brain development was also described *in vivo*, for example in the CSF-1 receptor (CSF1R) knock-out line. CSF1R signaling is essential for macrophage survival, therefore lack of the *Csf1r* gene leads to apoptosis of microglia and macrophages during their development (Pixley and Stanley, 2004; Stanley and Chitu, 2014). At birth, the brain of CSF1R-deficient mice is almost completely devoid of microglia, nevertheless it does not show substantial structural abnormalities compared to wild-types. However, at the 3rd postnatal week brain's morphology appears severely disrupted, displaying enlarged ventricles and clearly misshapen neighboring regions, such as cortex, hippocampus and olfactory bulb. *Csf1r* knock-out mice typically

do not survive longer than 3-4 post-natal weeks. Furthermore, immunohistochemistry revealed higher number of $GFAP^+$ astrocytes and decreased number of $Nogo^+$ oligodendrocytes. Surprisingly, NeuN staining did not show any change in number of mature neurons (Erblich *et al.*, 2011). A later work showed that lack of CSF1R causes an accumulation of apoptotic $Nestin^+$ cells (bona fide neuronal progenitors) within the major neurogenic niches. This indicates that the absence of microglia hampers brain development, presumably because apoptotic cells are not efficiently removed from the brain's parenchyma (Nandi *et al.*, 2012). Notably, pharmacological or genetic-based depletion of microglia during the embryonic stage seemed to produce only minor alterations of the brain's structure (Nakayama *et al.*, 2018; Rosin *et al.*, 2018). This would suggest that the severe phenotype of the CSF1R knock-out mice be not entirely a microglia-dependent effect. Indeed, lack of CSF1R causes a lethal pathological condition known as osteopetrosis, characterized by a severe disruption of the bone and teeth development (Dai *et al.*, 2002; Hume and MacDonald 2012; Chitu *et al.*, 2016). However, the pathological phenotype and reduced life-span can be improved (or even rescued) with bone marrow transplantation from wild-type donor mice (Bennet *et al.*, 2018).

As already mentioned, microglia play a critical role in the disposal of apoptotic cells within the brain's parenchyma. Phagocytosis of immature neurons by microglia does not strictly occur during the perinatal period and it seems to persist in the adult brain. For instance, *Sierra and coworkers* showed that dentate gyrus microglia phagocytose proliferating neural progenitors arising from the sub-granular zone (SGZ). Interestingly, this phenomenon occurs physiologically (and highly efficiently) in both juvenile and adult mice, in absence of any obvious inflammatory reaction (Sierra *et al.*, 2010). Impairment of this microglia-mediated clearance of newborn neurons would lead to an accumulation of apoptotic cells within the neurogenic niches (Abiega *et al.*, 2016; Fourgeaud *et al.*, 2016). Microglia are therefore required in order to ensure a rapid and efficient removal of apoptotic corpses produced through the neurogenesis (Sierra *et al.*, 2014).

Intriguingly, some works also suggested that microglia may provide a trophic support to the developing brain. It was shown that microglia are important for myelination and oligodendrocytes differentiation during the post-natal period (Shigemoto-Mogami *et al.*, 2014; Hagemeyer *et al.*, 2017; Wlodarczyk *et al.*, 2017). Other works provided evidences that microglia are required for a proper development of neuronal connections. For example, *Squarzoni and coworkers* analyzed the developing brain at different time points in mice lacking of embryonic microglia (Squarzoni *et al.*, 2014). To

deplete microglia in the embryos, authors repeatedly administrated anti-CSF1R antibody in pregnant dams during early gestation. This treatment resulted in chronic blockage of CSF1R-signalling, thus causing apoptosis of microglia progenitors prior brain's colonization. In parallel, authors analyzed the brain of *Spi1* knock-out mice that fail to develop yolk sac-derived myeloid cells, including microglia. *Spi1* encodes for PU.1, a critical transcription factor for both embryonic and adult hematopoiesis (Scott *et al.*, 1994, 1997; Olson *et al.*, 1995; McKercher *et al.*, 1996; Anderson *et al.*, 1998; Iwasaki *et al.*, 2005). Both microglia-depletion models revealed that dopaminergic axons within the developing cortex grow abnormally in absence of microglia. By contrast, maternal immune activation (through prenatal LPS injection) yielded an opposite effect. Moreover, mice lacking microglia as well as mice exposed to maternal immune activation, displayed an increased growth of cortical interneurons. Such abnormalities were still observable 20 days after birth, indicating that maldeveloped neural networks are still present in the adulthood. These data suggest that microglia are indeed important for embryonic brain wiring (Squarzoni *et al.*, 2014). Another important contribution comes from the work of *Ueno and colleagues*. These authors temporarily depleted microglia in the early post-natal brain using a transgenic mouse expressing human diphtheria-toxin receptor (DTR) under the control of CD11b promoter. Injection of diphtheria toxin (DT) in P5 mice induced fast (although transient) depletion of all CD11b-expressing cells, including microglia. At this time-point microglia were found in close contact with projecting axons, raising the hypothesis that they may aid axonal guidance through the cortical layers. Upon DTR-mediated microglia depletion, a significant increase of neuronal apoptosis was observed in different cortical layer (especially within layer V). Authors further suggested that microglia support neuronal survival during early life through production of IGF1 (Ueno *et al.*, 2013). These findings indicate that microglia may help shape brain's architecture during the early post-natal period. Nevertheless, data obtained in the DT-depletion models should be interpreted with caution as a sudden death of the entire microglia population seems to trigger a storm of inflammatory mediators, which may thus affect neuronal viability (Waisman *et al.*, 2015). The idea that microglia are important for neuronal survival and function was further corroborated with a very recent work from *Mass and colleagues*. This group introduced a somatic mutation of the BRAF gene in embryonic EMPs by means of Tamoxifen-induced recombination, thus triggering constitutive activation of the RAS-MEK-ERK pathway in microglia. BRAF mutation in microglia was associated with abnormal microglia proliferation, late-onset neurodegeneration and motor deficits. This phenotype might be due to a persistent pro-inflammatory activation of microglia, thus suggesting a possible etiological mechanism for sporadic neurodegenerative diseases in humans (Mass *et al.*, 2017).

To sum up, alterations in microglia during embryonic period or early after birth may severely affect brain development, thus possibly causing neurodevelopmental disorders, cognitive disabilities and neuropsychiatric conditions (Wake *et al.*, 2013; Aguzzi *et al.*, 2013; Nayak et al., 2014; Prinz and Priller, 2014; Ginhoux *et al.*, 2015; Biber *et al.*, 2016; Colonna and Butovsky, 2017; Wolf *et al.*, 2017; Kierdorf and Prinz 2017; Neniskyte and Gross, 2017).

5. Microglial gene-expression signature

The transcriptomic heterogeneity of the different brain cells started to be explored no longer than 10 years ago (Cahoy *et al.*, 2008). Accordingly, our knowledge about microglia gene-expression profile is relatively recent (Crotti and Ransohoff, 2016; Wes *et al.*, 2016; Masgrau et al., 2017). Nevertheless, different works have so far uncovered a unique gene expression signature of microglia compared to other brain cells and/or different tissue macrophages (Gautier *et al.*, 2012; Hickman *et al.*, 2013; Chiu *et al.*, 2013; Butovsky *et al.*, 2014; Zhang *et al.*, 2014; Bruttger *et al.*, 2015). These studies consistently highlighted a pool of genes that in the brain are exclusively expressed in microglia, among which are *Cx3Cr1, Csf1R, Trem2, Siglech, Aif1, Itgam, Itgb5, CD68, P2yr12, P2yr13, Fclrs, Tmem119, Crybb1, Hexb, Olfml3, Rnase4, Slc2a5, Gpr34, Gpr84, Socs3, Tyrobp, Sall1*, and others. Importantly, some of these genes (such as *P2yr12, Fclrs, Crybb1, Hexb, Olfml3* and *Tmem119*) are not expressed by other brain macrophages residing within the perivascular space, meninges and choroid plexus. These proteins might therefore represent promising candidates to specifically target parenchymal microglia (Butovsky *et al.*, 2014; Bennett *et al.*, 2016). However, microglia gene expression signature dramatically changes under neuroinflammatory and neurodegenerative condition, thus hindering a clear distinction among resident microglia and blood-derived myeloid cells (Ajami *et al.*, 2018; Mrdjen *et al.*, 2018; Jordão *et al.*, 2019).

The microglia gene-expression profile gradually evolves across the embryonic and post-natal period following a stepwise developmental program, reaching a final maturation approximately at the second week after birth (Bennett *et al.*, 2016; Matcovitch-Natan *et al.*, 2016; Hammond *et al.*, 2018). Single-cells RNAseq analysis showed that microglial phenotype exhibits a greater degree of heterogeneity across the period of CNS development, spanning from mid-gestation up to the first post-natal weeks. By contrast, adult (or mature) microglia are more homogeneous and express a defined

repertoire of phenotypic markers (Hammond *et al.*, 2019; Li *et al.*, 2019). This maturation process is governed by a combination of key transcriptional regulators (such as Sall1) and cytokines like TGFβ. Indeed, knock-out of these genes resulted in a severe disruption of microglia development (Buttgereit *et al.*, 2016; Wong *et al.*, 2017; Butovsky *et al.*, 2014; Lund *et al.*, 2018; Qin *et al.*, 2018). Very recent findings also highlighted a primary importance of both miRNAs (Varol *et al.*, 2017) and HDACs enzymes (Datta *et al.*, 2018) in development of embryonic microglia. Just recently, different groups were able to produce *in vitro* human and murine microglial cells derived from induced Pluripotent Stem Cell (iPSC) (Muffat *et al.*, 2016; Abud *et al.*, 2017; Douvaras *et al.*, 2017; Haenseler *et al.*, 2017; Pandya *et al.*, 2017; Takata *et al.*, 2017). These new tools might provide further insights into the molecular framework orchestrating microglia development.

Little is known about the molecular cues shaping the identity of the microglial phenotype. A number of recent works are suggesting that a combination of ontogeny and environmental stimuli might sculpt the epigenetic fingerprint of macrophage precursors, thereby activating the developmental specification of tissue-resident macrophage, including microglia (Gosselin *et al.*, 2014; Lavin *et al.*, 2014; Okabe and Medzhitov, 2014; Amit *et al.*, 2015; Lavin *et al.*, 2015; Scott *et al.*, 2016; Mass *et al.*, 2016; van de Laar *et al.*, 2016; Gundra *et al.*, 2017; Guilliams and Scott 2017). In strong support of this theory, it was shown that acutely isolated microglia lose their phenotypic identity in a few hours after exposure to the cell culture condition (Bohlen *et al.*, 2017; Gosselin *et al.*, 2017). Interestingly, cultured microglia transplanted back into the mouse brain reacquired the original gene expression profile in about two weeks (Bohlen *et al.*, 2017; Bennett *et al.*, 2018). Similarly, transplantation of iPSC-derived macrophages into the post-natal mouse brain generated microglia-like cells fully integrated within the host tissue (Takata *et al.*, 2017). These data, although not surprisingly, proofed that brain's environment is critical for the maturation and maintenance of microglial phenotype. Nonetheless, bone marrow-derived monocytes engrafting the brain under non-inflammatory conditions (microglia depletion or whole-body irradiation) exhibit typical microglial features such as ramified morphology, longevity and radio-resistance, however they fail to acquire a transcriptomic profile identical to the yolk sac-derived microglia (Bennett *et al.*, 2018; Lund *et al.*, 2018b; Shemer *et al.*, 2018). These evidences suggest that a combination of both local environment and origin contribute to shape the microglial phenotype.

Such a concept would now rise an additional question. The brain, structured in different regions hosting various populations of neurons and glial cells, is far from being a homogeneous tissue. It is therefore possible that microglial phenotype may vary depending on their location within the brain. So far only one work attempted to define a comprehensive microglial gene-expression from different brain regions, revealing that microglial phenotype might actually be rather heterogeneous (Grabert *et al.*, 2016). For instance, cerebellar microglia seemed skewed towards a more "immune alerted" and "metabolically demanding" phenotype, possibly because of the lower microglial cell density and higher content of white matter. By contrast, microglia in cortex and striatum appeared in a more "quiescent" state, while hippocampal microglia exhibited a more mixed phenotype. Similarly, another work described a heterogeneous gene-expression profile of microglia selectively isolated from cortex, Nucleus Accumbens, Ventral Tegmental Area and Substantia Nigra (De Biase *et al.*, 2017). More precisely, the most variable genes were involved in pathways of vesicles release, mitochondrial function, cell metabolism, oxidative stress, lysosomal activity and transport of metal ions. Along the same line, *Ayata and colleagues* described diverse gene-expression patterns and epigenetic signature between cerebellar and striatal microglia. In particular, cerebellar microglia were highly enriched of transcripts associated to phagocytosis and clearance of apoptotic cells, while striatal microglia exhibited higher expression of genes related to immunological surveillance (Ayata *et al.*, 2018). Very recently, single-cell mass cytometry allowed to identify a region-dependent phenotypic heterogeneity of microglia from human post-mortem brains (Böttcher *et al.*, 2018). These data suggest that environmental cues within specific brain regions may indeed contribute in shaping the phenotype of local microglia.

Additional scientific effort is now demanded to better understand the phenotypic change of both mouse and human microglia during brain diseases. We have just started to explore this new field of microglia research and a handful of recent works have already identified important transcriptomic imbalances in microglia during aging and under neurodegenerative conditions such as Alzheimer Disease (Holtman *et al.*, 2015; Yin *et al.*, 2016; Galatro *et al.*, 2017; Gosselin *et al.*, 2017; Krasemann *et al.*, 2017; Friedman *et al.*, 2018). Importantly, transcriptomic changes in microglia develop in a stepwise fashion along with the disease progression, suggesting that microglial phenotype is somewhat interlinked to the disease severity (Keren-Shaul *et al.*, 2017; Mathys *et al.*, 2017). Notably, singe-cell transcriptomic studies from different groups indicated ApoE (Apolipoprotein E) as one of the most upregulated transcripts in microglia during neurodegeneration (Keren-Shaul *et al.*, 2017; Krasemann *et*

al., 2017; Mathys *et al.*, 2017; Tay *et al.*, 2018), although its actual function is yet debated. Recent findings underlined that microglial gene-expression is the most susceptible to aging, therefore deterioration of microglial phenotype might be mechanistically associated with brain pathologies of senility, Alzheimer's Disease overall (Zhang *et al.*, 2013; Soreq *et al.*, 2017; Olah *et al.*, 2018). Moreover, we should better clarify similarities and differences between human and murine microglia, being the latter commonly used as benchmark for pre-clinical investigation (Smith and Dragunow, 2014). Such a goal is clearly not trivial. Human microglia are obtained from either post-mortem or surgical specimens. This implies a rather low quality of the extracted RNA, which undoubtedly affects the ensuing gene expression analysis (Masgrau *et al.*, 2017).

6. Mechanisms of microglia-neuron communication

6.1 Fractalkine receptor

Fractalkine receptor (CX3CR1) is a membrane G-protein coupled receptor (GPCR) which specifically recognizes the chemokine Fractalkine (CX3CL), and probably represents the most investigated pathway of microglia-neuron communication. Importantly, lack of the CX3CR1 dramatically amplifies microglia reactivity in various models of brain pathology (Cardona *et al.*, 2006), often (but not always) causing the exacerbation of the disease (Limatola and Ransohoff, 2014; Ransohoff and El Khoury, 2015). It is therefore believed that CX3CR1 signaling is important for the maintenance of microglial homeostasis. The human CX3CL was discovered about 20 years ago (Bazan *et al.*, 1997) and, so far, it is the only known member of the CX3C family. One year later the murine ortholog gene was identified and characterized, showing a high degree of conservation with the human counterpart (Rossi *et al.*, 1998). CX3CL is produced by several cell types, including epithelial cells of lung, kidney, intestine and thymus, blood vessels endothelial cells, subcutaneous lymph-nodes and lymphatic vessels. Neurons express CX3CL as well, especially in the hippocampus, striatum, cortical layer II and spinal cord dorsal horn neurons (Kim *et al.*, 2011; Wolf *et al.*, 2013). CX3CL is synthetized in form of membrane-bound protein, but can be subsequently cleaved by the extracellular metallo-proteinases ADAM-10 and ADAM-17, thereby promoting chemoattraction and migration of circulating CX3CR1-expressing cells (Garton *et al.*, 2001; Tsou *et al.*, 2001; Hundhausen *et al.*, 2003; Dreymueller *et al.*, 2012). CX3CR1 is a typical GPCR expressed on subsets of circulating leucocytes as monocytes, NK cells and T-lymphocytes (Jung *et al.* 2000; Geissmann *et al.* 2003; Landsman *et al.*

2009; Geissmann *et al.* 2010). It was shown that CX3CR1 promotes cell adhesion and diapedesis through activated endothelial wall (Imai *et al.*, 1997; Pan *et al.*, 1997; Ancuta *et al.*, 2003; Fong *et al.*, 1998; Schulz *et al.*, 2007). CX3CR1 activates Gi proteins, inhibiting adenylate-cyclase and simultaneously activating the PI3K/AKT and PLC/PKC pathways. CX3CR1 was described to promote migratory capacity of circulating leukocytes due to elevation of intracellular Ca^{2+} and ensuing cytoskeleton reorganization (Al-Aoukaty *et al.*, 1998; Haskell *et al.*, 1999; Sheridan and Murphy, 2013). In the CNS parenchyma, expression of CX3CR1 is restricted to microglia (Harrison *et al.*, 1998; Nishiyori *et al.*, 1998; Jung *et al.*, 2000; Mizutani *et al.*, 2012; Wolf *et al.*, 2013), whereas the ligand was primarily detected in mature neurons, especially in the hippocampus, striatum and cortex (Tarozzo *et al.*, 2003; Kim *et al.*, 2011; Wolf *et al.*, 2013). Our understanding of the CX3C axis in the context of microglia-neuron communication was strongly fostered with the generation of the mouse strain *Cx3cr1*^{GFP}, in which the GFP coding sequence was integrated in-frame within the *Cx3cr1* locus, thus producing a knock-in/knock-out transgene. In their seminal paper, *Jung and colleagues* found prominent expression of GFP in monocytes, subsets of NK, dendritic cells and microglia, but failed to demonstrate any major role of CX3CR1 in the migratory capacity of these cells (Jung *et al.*, 2000). The role of CX3CR1 in microglia was initially investigated in the context of CNS inflammation. A bulk of literature demonstrated a clear interrelatedness between the CX3CR1 pathway and dynamics of pro-inflammatory microglia activation (Biber *et al.*, 2007; Ransohoff and Cardona, 2010; Prinz *et al.*, 2011; Hellwig *et al.*, 2013; Limatola and Ransohoff, 2014). For instance, LPS administration to cultured microglia was shown to downregulate CX3CR1 expression, along with over-expression of TNFα, IL-1β, IL6 and iNOS (Boddeke *et al.*, 1999; Wynne *et al.*, 2010). Coincubation with the CX3CL resulted in a blunted release of classical inflammatory mediators in LPS-stimulated microglia (Zujovic *et al.*, 2000; Mizuno *et al.*, 2003; Lyons *et al.*, 2009). Later, it was showed that *in vivo* LPS injection in CX3CR1 deficient mice dramatically increased the magnitude of microglia pro-inflammatory reaction, resulting in extended neurotoxicity and prolonged sickness behavior (Cardona *et al.*, 2006; Corona *et al.*, 2010, 2013). Furthermore, *Cx3cr1* knock-out led to increased neuronal loss in mouse models of Parkinson Disease and Amyotrophic Lateral Sclerosis (Cardona *et al.*, 2006; Pabon *et al.*, 2011; Garcia *et al.*, 2013). Apparently in contrast with these data, *Cx3cr1* knock-out in different mouse models of amyloid-β deposition (recapitulating certain features of Alzheimer Disease) seemed to mitigated the overall amyloid load and reduced neuronal loss (Fuhrmann *et al.*, 2010; Lee *et al.*, 2010; Liu *et al.*, 2010; Derecki *et al.*, 2014; Meyer-Luehmann and Prinz, 2015). Whether CX3CR1 may indeed play any role in Alzheimer Disease is yet uncertain.

CX3CR1 became also matter of interest regarding the role of microglia during the post-natal brain development. Under CX3CR1-deficient condition, number of microglial cells appeared reduced during the first post-natal week (Paolicelli *et al.*, 2011; Hoshiko *et al.*, 2012). However, whether CX3CR1 (or other chemokine receptors) is required in order to seed the developing mouse brain is still unclear (Kierdorf *et al.*, 2013; Arnò *et al.*, 2014; Mass *et al.*, 2016; Stremmel *et al.*, 2018). Contingently to the reduced microglia density, barrel cortex of CX3CR1-deficient animals showed an altered development of the thalamocortical glutamatergic synapses (Hoshiko *et al.*, 2012). These mice also exhibit microglia deficiency in the CA1 hippocampal region, together with an increased density of PSD95 spines. Electrophysiological studies further confirmed higher abundance of immature excitatory synapses in the hippocampus of *Cx3cr1* knock-out mice (Paolicelli *et al.*, 2011), which then results in reduced glutamatergic connectivity (Basilico *et al.*, 2018) Moreover, *Cx3cr1*-deficient microglia exhibit impaired migration towards ATP gradients (Pagani *et al.*, 2015). These data highlight the relevance of CX3CR1 for microglial physiology and potentially for maturation of synapses in different brain regions, especially during the early post-natal period (Paolicelli *et al.*, 2014).

Given the importance of CX3CR1 for microglia biology, various works attempted to assess whether CX3CR1 deficiency may lead to certain behavioral alterations. Initially, lack of CX3CR1 was described to cause an impaired LTP at the CA1 region, reduced neurogenesis and decreased cognitive skills and motor learning (Rogers *et al.*, 2011). However, other groups presented conflicting findings, namely increased hippocampal plasticity and cognitive performances under CX3CR1-deficient condition, but confirmed reduced neurogenesis in these mice (Maggi *et al.*, 2011; Reshef *et al.*, 2014). A more recent work pointed towards reduced learning and memory ability in absence of CX3CR1. Again, reduced hippocampal neurogenesis was observed in these mice (Sellner *et al.*, 2016). Interestingly, it was described that *Cx3cr1* knock-out mice display an altered connectivity of different brain regions and weaker synaptic transmission. A delayed/impaired development of Cortical-Limbic networks was translated into autism-related behaviors, such as reduced social interaction and increased self-grooming behavior. Importantly, these differences were observable in both juvenile and adult mice, suggesting that lack of CX3CR1 may lead to long-term behavioral consequences, partially resembling symptoms of neuro-psychiatric disorders (Zhan *et al.*, 2014). Recent works also described reduced spine number in granule cells of dentate gyrus and olfactory bulb in *Cx3cr1* knock-out mice (Bolós *et al.*, 2017; Reshef *et al.*, 2017), thus indicating that CX3CR1 signaling in microglia is important for proper synaptic formation in new-born neurons.

6.2 Microglial purinergic receptors

Purinergic receptors represent an extended family of membrane receptors widely expressed in all tissues and cell types, further classified into P1 (metabotropic G_s or $G_{i/o}$), P2Y (metabotropic $G_{q/11}$) and P2X (ionotropic). P1 is activated by adenosine, while P2Y and P2X are primarily activated by ATP or ADP. The nucleotide ATP is highly concentrated within the intracellular compartments, whereas it is quickly degraded once released in the extracellular environment. Release of ATP can occur through different ways: exocytosis, diffusion, active transport and "leakage" upon cell damage/death. Focusing on the brain, both astrocytes and microglia abundantly express all different members of purinergic receptors, except of P2Y12 which is present in microglia only (Sasaki *et al.*, 2003; Abbracchio *et al.*, 2009). Glial cells (especially microglia) are therefore equipped with a huge variety of purinergic receptors for sensing ATP levels in the brain. ATP is physiologically secreted by neurons as a neurotransmitter, hence it represents an important molecule for brain's neurochemistry.

On the other hand, during brain injuries a massive release of ATP occurs. In such a context, ATP acts as a danger signal and triggers a widespread response in glial cells via the purinergic receptors. Accordingly, ATP is usually addressed as a microglial "ON-signal", able to promptly switch microglial phenotype in response to tissue insults (Färber and Kettenmann, 2005; Biber *et al.*, 2007; Kettenmann *et al.*, 2011). Purinergic receptors have been associated with several microglial functions, ranging from release of inflammatory mediators (Chakfe *et al.*, 2002; Cavaliere *et al.*, 2005; Hasko *et al.*, 2005; Färber and Kettenmann, 2006; Choi *et al.*, 2007; Monif *et al.*, 2009; Shieh *et al.*, 2014), phagocytosis (Kettenmann, 2007; Koizumi *et al.*, 2007, 2013) and chemotaxis (Honda *et al.*, 2001; Haynes *et al.*, 2006; Ohsawa *et al.*, 2010; Ohsawa and Kohsaka, 2011).

First evidences of the purinergic system in microglia date back to the early '90s and they were essentially based on patch-clamp and Ca^{2+}-imaging techniques. Several *in vitro* observations revealed that ATP induced membrane depolarization and inward Ca^{2+} currents in microglia (Walz *et al.*, 1993; Haas *et al.*, 1996; Illes *et al.*, 1996; Toescu *et al.*, 1998). This suggested that glial cells may sense neuronal activity and/or neuronal damage through the purinergic receptors. Interestingly, it was observed that cultured microglia treated with LPS become desensitized to both ATP and P2 agonists (Möller *et al.*, 2000; Boucsein *et al.*, 2003; Hoffmann *et al.*, 2003). In line with these results, *in vivo* observations revealed that peripheral LPS injection reduced ATP-dependent microglia migration towards a laser-induced injury (Gyoneva *et al.*, 2014).

Introduction

Microglia migration in a Dunn chemotaxis chamber towards a gradient of ATP appeared primarily dependent on P2X4 and P2Y12 receptors (Ohsawa *et al.*, 2007). Further studies revealed that P2Y12-dependent microglia chemotaxis implies a series of downstream signals such as K^+ outward currents, activation of PI3K/AKT and PLC pathways and increase of intracellular Ca^{2+} (Wu *et al.*, 2007; Irino *et al.*, 2008; Madry *et al.*, 2018). Time-lapse confocal imaging on acute hippocampal slices showed that microglia rapidly migrate towards the soma of injured neurons. Enzymatic hydrolysis of ATP (to inactive AMP) inhibited microglia migration, whereas incubation with exogenous ATP/ADP promoted microglia migration away from injury site (Kurpius et al., 2007). Similarly, exposure to high K^+ concentrations (thus disrupting the physiological K^+ electrochemical gradient) reduced microglia ramification and processes motility (Madry *et al.*, 2018b).

Other studies highlighted that, upon ATP stimulation, P2Y12 receptor promotes membrane trafficking of integrin-β1 (Ohsawa *et al.*, 2010). Expression of α5/β1 integrin is necessary for the interaction between microglia and the extracellular matrix (Smolders *et al.*, 2017). Interestingly, adenosine A(2A) receptors were shown to mediate processes retraction during inflammation, contingently to the downregulation of P2Y12 receptor (Orr *et al.*, 2009). Importantly, these two receptors are stimulated by different ligands, indeed P2Y12 receptor is primarily engaged by ATP/ADP while A2 is activated by adenosine which is its breakdown product. This would suggest that microglial purinergic receptors may promote both processes extension and retraction.

Microglia motility was also described *in vivo* by means of time lapse two-photon imaging. Laser-induced focal cortical ablation evoked rapid migration of microglial processes, which seemingly formed a sort of physical barrier around the lesioned area (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005). A similar microglia migration was achieved by focal application of ATP or P2Y agonists onto the mouse cortex. Consistently, application of either ATP-degrading enzymes or P2Y12 receptors blockers almost abolished microglial chemotaxis (Davalos *et al.*, 2005; Sipe *et al.*, 2016). Deletion of P2Y12 receptor was also shown to reduce microglia migration in a model of kainic acid-induced seizures (Eyo *et al.*, 2018). Taken together, these data highlight ATP/ADP as major chemotactic factors inducing microglia migration.

6.3 Microglia motility

First observations of microglia migrating within cultured brain slices date back to '90s (Brockhaus *et al.*, 1996). Generation of the *Cx3cr1*^{GFP} and Iba1-EGFP mouse lines paved the way to the first *in vivo* imaging of microglia. Microglia were often described as quiescent (or resting) immune cells, but able to promptly switch into an activated (or reactive) state upon injury or infection. However, seminal two-photon studies showed that microglia were unexpectedly active even under homeostasis. Although the cell bodies appeared relatively stable, microglial ramifications revealed a surprising degree of motility. Indeed, microglial processes are continuously extended and retracted at a striking speed, up to several µm per minute in deeply anesthetized mice (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005). Similar processes speed was also reported in different *ex vivo* slice culture systems (Stence *et al.*, 2001; Carbonell, 2005; Paques *et al.*, 2010). Allegedly, microglia are the most active cells of the brain (Hughes *et al.*, 2013).

It was suggested that processes motility may allow each microglial cell to scan and sample the surrounding environment, thus keeping brain's parenchyma under constant immunological surveillance (Hanisch and Kettenmann, 2007). It is becoming increasingly clear that microglia motility is strongly influenced by neuronal activity, however the biological underpinnings of this phenomenon are still largely unknown (Tremblay *et al.*, 2011; Miyamoto *et al.*, 2013; Wake *et al.*, 2013; Sierra et al., 2014; Casano and Peri, 2015). For example, *Wake and colleagues* used *in vivo* two-photon microscopy to study the interactions between microglia and synapses in mouse somatosensory cortex. This analysis revealed that microglial processes contact dendritic spines at least once per hour, with an average contact duration of about 5 minutes. Processes motility was reduced after pharmacological blockage of action potential, thereby indicating that microglial motility was (at least partially) dependent on neuronal activity (Wake *et al.*, 2009). However, it should be kept in mind that such observations were carried out under deep anesthesia, which likely reduces *in vivo* processes motility (Dombeck *et al.*, 2007; Dombeck and Tank, 2014; Miyamoto *et al.*, 2016). Therefore, these values may represent an underestimation of the real microglia motility in awake conditions. Similar findings were obtained by *Tremblay et al.* in the mouse visual cortex. Microglia were again shown to establish frequent contacts with neurons. Strikingly, leaving mice under light deprivation reduced processes motility, which was then normalized after exposing the mice again to normal light (Tremblay et al., 2010). These data indicated that microglia-neurons contacts are influenced by the somatosensory experience.

It is then possible that active neurons released certain molecules promoting microglial motility. This idea has been supported with some *ex vivo* studies showing that processes motility was strongly enhanced upon glutamatergic stimulation (Fontainhas *et al.*, 2011; Dissing-Olesen *et al.*, 2014). To date, the specific contribution of AMPA and NMDA receptors is still controversial and may probably vary depending on the used model. Moreover, the importance of glutamate for the processes motility was further demonstrated in zebrafish models (Li *et al.*, 2012; Sieger *et al.*, 2012). Other studies showed that active glutamatergic neurons exhibit downscaled firing after being contacted by microglial processes, thus suggesting a possible neuro-protective mechanism (Liu *et al.*, 2009; Li *et al.*, 2012; Pascual *et al.*, 2012; Ji *et al.*, 2013; Zhang J. *et al.*, 2014; Eyo *et al.*, 2015). Consistently, studies on organotypic hippocampal cultures reported that microglia depletion increased NMDA-mediated neurotoxicity, supporting the idea that microglia protect neurons from glutamatergic excitotoxicity (Vinet *et al.*, 2012). Such a protective effect seemed to require an ATP-dependent microglia activation and TNFα release (Masuch *et al.*, 2016). Similar findings were also obtained *in vivo*. For example, Kainic Acid (KA) administration in mouse induced microglia hyper-ramification along with generation of epileptic seizures. This effect apparently required NMDA receptor stimulation and ATP release. Importantly, P2Y12 receptor knock-out reduced KA-induced processes extension, but at the same time worsened seizures pathology (Eyo *et al.*, 2014). Another work showed that microglia in the visual cortex acquire a hyper-ramified morphology after visual deprivation. This morphological transition was reduced by both P2Y12R knock-out and pharmacological blockage of P2Y12 receptor (Sipe *et al.*, 2016). These data suggest that glutamatergic neurons promote extension of microglial processes in a P2Y12 receptor-dependent manner. At the same time, microglia may protect neurons from excitotoxic damage upon physical contact with the glutamatergic synapses (Kato *et al.*, 2016).

6.4 Synaptic pruning

Besides the mere immunological surveillance, several lines of evidences suggested that microglia may exert a refinement/remodeling of synaptic connections (Schafer *et al.*, 2013; Wake *et al.*, 2013; Salter and Beggs, 2014; Wu *et al.*, 2015; Hong and Stevens, 2016; Kierdorf and Prinz, 2017). First evidences of microglia engulfing synapses date back to the late ‘60s, with the work of *Georg Kreutzberg* on a rat model of facial nerve axotomy (Blinzinger and Kreutzberg, 1968; Moran and Graeber, 2004).

Introduction

Across the following decades a microglia-mediated displacement/phagocytosis of synapses was described in mouse models of brain inflammation and injury (Svensson and Aldskogius, 1993; Trapp *et al.*, 2007). A similar mechanism was also suggested for the human brain (Graeber *et al.*, 1993). This phenomenon was originally termed as "*synaptic stripping*", indicating a physical displacement of synaptic inputs (Kreutzberg, 1996; Kettenmann *et al.*, 2013). In recent times, the term "*synaptic pruning*" became more popular and is commonly used to indicate a synaptic removal due to engulfment and phagocytosis. Intriguingly, some indications suggested that microglia may also aid formation of new synapses, in both slice culture (Lim *et al.*, 2013) and *in vivo* (Parkhurst *et al.*, 2013; Miyamoto *et al.*, 2016; Reshef *et al.*, 2017). Synaptic pruning is thought to occur primarily during the first post-natal weeks, in order to facilitate removal of supernumerary synapses generated during the early brain development (Chung *et al.*, 2013). During this period weaker synapses are pruned and removed, while others are strengthened and integrated within the neural circuits. Pruning of exuberant synapses is therefore necessary for a proper development of the synaptic connections. Importance of microglia for this process was demonstrated on the *Cx3cr1* knock-out mice, characterized by a reduced microglia number during the critical period of synaptic pruning.

Two independent studies reported an increased density of immature excitatory synapses in these mice, therefore suggesting a deficit of microglia-mediated pruning after birth (Paolicelli *et al.*, 2011; Hoshiko *et al.*, 2012). More recently, a functional MRI study on CX3CR1-deficient mice highlighted a reduced synchrony across several brain regions (especially between neocortex and limbic system), indicating a weaker connectivity among these areas. Behavioral tests showed a deficit of social interaction, indicating the presence of autism-like behaviors in these mice (Zhan *et al.*, 2014). Authors suggested that deficit of synaptic pruning may cause a maldeveloped brain's connectivity and ensuing behavioral alterations. Deficit of synaptic pruning may help explain the etiology of several neurodevelopmental and psychiatric disorders (Neniskyte and Gross, 2017), however, several questions still await an answer. For example, how do microglia discriminate the synapse that have to be eliminated from those that will be preserved? Does the synaptic pruning occur equally in all brain regions or there are defined hot-spots? Do microglia have preference for certain neuronal types? Do microglia prune both pre- and post-synaptic inputs? For example, a recent study on organotypic slice culture showed that microglia tend to prune (almost exclusively) pre-synaptic buttons, whereas spines are indeed contacted, albeit not removed from the dendritic tree (Weinhard *et al.*, 2017). Further studies are warranted to corroborate these findings.

Introduction

Little in know about the immunological signals involved in the synaptic pruning. A growing body of literature indicates that the complement system plays an important role in the elimination synapses (Stephan *et al.*, 2012). The classical complement cascade is initiated with the C1q complement protein binding at a target antigen (typically molecular pattern of a microorganism). C1q is subsequently opsonized by the C3 protein, which also acts as chemoattractant for the innate-immune cells. Eventually the C1q/C3 complex is recognized by the Complement Receptor-3 (CR3) expressed on tissue-resident phagocytes, which in turn engulf and degrade the noxious element through phagocytosis (Stephan *et al.*, 2012). CR3 is a heterodimeric receptor formed by CD11b and CD18 integrins, which in the brain are solely expressed in microglia. Complement proteins are probably produced by multiple cell types, including neurons and astrocytes (Bialas and Stevens, 2013), however recent evidences indicate microglia as a major source of C1q (Zhang *et al.*, 2014; Fonseca *et al.*, 2017; Liddelow *et al.*, 2017). During the early post-natal period, C1q was found in colocalization with glutamatergic synaptic inputs, while C1q deficient mice exhibited a higher number of glutamatergic synapses (Stevens *et al.*, 2007). Higher abundance of excitatory synapses was also found in mice lacking either CR3 or C3 (Schafer *et al.*, 2012). These data indicate that early after birth microglia mediate elimination of supernumerary synapses through the C1q/C3/CR3 cascade.

Synaptic pruning has often been investigated during brain development. Nevertheless, a pathological form of synaptic pruning could take place in the adult brain. For example, it was suggested that increased synaptic pruning may occur during aging. It is known that, in both rodents and humans, C1q levels tend to increase in the aged brain (Stephan *et al.*, 2013). Moreover, C3 deficient mice exhibited higher synaptic density and better learning and memory ability compared to wild-types (Shi *et al.*, 2015). This suggests that microglia-mediated synaptic pruning might contribute to the loss of synapses and cognitive decline during senility. Furthermore, growing evidences indicate that exaggerated synaptic pruning may occur during pathology. Indeed, microglia exhibited augment synaptic pruning under different neuro-inflammatory conditions (Trapp et al. 2007; Chen et al. 2014; Chen et al. 2012; Bisht et al. 2016; Vasek et al. 2016; Bialas et al., 2017), as well as in mouse models of Alzheimer Disease (Hong et al., 2016; Shi et al. 2017; Paolicelli et al., 2017), Frontotemporal Lobar Degeneration (Lui *et al.*, 2016) and Rett Syndrome (Schafer *et al.*, 2016). Presence of altered synaptic pruning was also recently suggested for psychiatric illnesses as Autism Spectrum Disorders and schizophrenia (Fernández de Cossío *et al.*, 2016; Kim *et al.*, 2016; Sekar *et al.*, 2016). Very recently, impaired synaptic pruning and behavioral alterations were also observed in the Trem2 knock-out mice

(Filipello *et al.*, 2018). Altogether, pathological synaptic pruning may represent a common trait of several brain diseases (Chung *et al.*, 2015; Li and Barres, 2017; Salter and Stevens, 2017).

Albeit the complement complexes C1q/C3 have been repeatedly indicated as a prototypical "eat-me" signal for microglia, it is yet unclear how these molecules can be activated onto the synaptic membrane. Moreover, molecular cues inhibiting synaptic pruning ("don't eat me" signals) need to be better characterized. For example, one of the first proposed molecules was the Major Histocompatibility Complex-I (MHC-I). It was indeed observed that mice lacking MHC-I exhibit an exaggerated synaptic removal (and ensuing synaptic loss) upon neuronal injury (Huh *et al.*, 2000; Oliveira *et al.*, 2004; Cullheim and Thams, 2007; Thams *et al.*, 2008). Furthermore, MHC-I deficient mice exhibit altered synaptic pruning of retinal ganglion inputs onto the thalamus (Lee *at al.*, 2014). It is possible that microglia be equipped with a repertoire of molecules for the recognition the neuronal MHC-I, however this mechanism has never been thoroughly investigated. More recently, the CD47-SIRPα axis was suggested as a candidate "don't eat me" signal for synaptic pruning. It was shown that juvenile SIRPα-deficient mice exhibit a reduced number of glutamatergic synapses, thus indicating that SIRPα is important for synaptic maturation (Toth *et al.*, 2013). Interestingly, CD47 and SIRPα are highly expressed in synapses and microglia respectively during brain development and both CD47 and SIRPα knock-out mice display exaggerated synaptic pruning early postnatally (Lehrman *et al.*, 2018). Further studies are needed to understand the real contribution of the CD47-SIRPα communication pathway in both brain development and disease.

7. Neuron-derived factors are crucial for microglial survival and phenotype

Microglial survival is strictly dependent on CSF1-receptor signaling. Genetic deletion (Ginhoux *et al.*, 2010; Erblich *et al.*, 2011) or pharmacological blockage (Elmore *et al.*, 2014; Waisman *et al.*, 2015; Spangenberg *et al.*, 2016) of CSF1R results in massive microglia apoptosis. In the brain, CSF1R is believed to be expressed in microglia only, although CSF1R immunoreactivity on NPCs and immature neurons has been described during early brain development (Nandi *et al.*, 2012). Another work reported CSF1R-promoter activity in mature neurons upon excitotoxic damage (Luo *et al.*, 2013), supporting the idea that CSF1R could be expressed in neuroectodermal cells under certain circumstances. However, these evidences cannot be confirmed in the MacGreen mouse line (*Csf1r-eGFP*), which exhibit GFP expression only in macrophages and microglia (Sasmono *et al.*, 2003). Furthermore, RNA sequencing data showed enrichment of CSF1R transcript in microglia, while expression in other cell types was virtually absent (Zhang *et al.*, 2014). To date, the hypothesis that neurons could express CSF1R (either during development or under pathological conditions) is still controversial (Chitu *et al.*, 2016).

In the brain, constant stimulation of CSF1R in microglia is ensured by two different cytokines (CSF1 and IL34) primarily secreted by neurons (Nandi *et al.*, 2012; Zelante and Ricciardi-Castagnoli, 2012; Chitu *et al.*, 2016). Of note, expression of CSF1 has also been detected in microglia, especially during the late embryonic stage and under pathological condition (Zhang *et al.*, 2014; Bruttger *et al.*, 2015; Matcovitch-Natan *et al.*, 2016; Keren-Shaul *et al.*, 2017). Lack of either CSF1 or IL34 causes a partial deficiency of microglia, without obvious consequences on mouse viability (Węgiel *et al.*, 1998; Erblich *et al.*, 2011; Greter *et al.*, 2012; Nandi *et al.*, 2012; Wang *et al.*, 2012). By contrast, overexpression of CSF1 in astrocytes induced microglia proliferation and increased the overall microglia number (De *et al.*, 2014). Despite the low degree of sequence homology, presence of domains with analogue 3D structure allows both cytokines to engage CSF1R at the same binding site (Lin *et al.*, 2008; Stanley and Chitu, 2014). To date, CSF1 is supposed to bind exclusively to CSF1R, whereas IL34 was shown to interact also with receptor-type protein-tyrosine phosphatase-ζ (RPTP-ζ), a membrane protein of unknown function expressed on both neural progenitors and glial cells (Nandi *et al.*, 2013).

Unlike the CSF1R knock-out, genetic ablation of either CSF1 (Erblich *et al.*, 2011; Nandi *et al.*, 2012) or IL34 (Greter *et al.*, 2012; Nandi *et al.*, 2012; Wang *et al.*, 2012) results in a partial microglia deletion, estimated in about 30% and 50%, respectively. This was explained by the fact that both cytokines show a non-overlapping expression pattern across the brain, resulting in a non-redundant (but rather complementary) effect on microglia survival (Ryan *et al.*, 2001; Wei *et al.*, 2010; Greter *et al.*, 2012; Wang *et al.*, 2012). Indeed, CSF1 expression was primarily found in cortical layer VI, corpus callosum, CA3 hippocampal region, cerebellum and spinal cord, whereas IL34 was found more abundant in neocortex, olfactory bulb, striatum and hippocampus (Greter *et al.*, 2012; Nandi *et al.*, 2012; Wang *et al.*, 2012). Moreover, expression of both CSF1 and IL34 commences early during embryonic brain development, however, the first tends to decrease after the third postnatal week, while the second is apparently maintained throughout the life-span (Nandi *et al.*, 2012).

An additional critical factor for microglial development and function is TGFβ-receptor, which in the brain is prominently expressed in microglia. Indeed, TGFβ1 deficiency causes a dramatic decrease of microglia number along with alteration of microglial phenotype (Butovsky *et al.*, 2014; Buttgereit *et al.*, 2016). Similarly, deletion of TGFβ-receptor in $CX3CR1^{+}$ cells produces a severe disruption of microglial phenotype and progressive limb paralysis in mice (Lund *et al.*, 2018; Zöller *et al.*, 2018). Moreover, mice deficient for *Lrrc33* (NROSS), a macrophage surface molecule important for TGFβ1 signaling, show progressive paralysis and premature death along with dramatic alterations in microglia (Wong *et al.*, 2017; Qin *et al.*, 2018). Together, these evidences show that TGFβ signaling is crucial for microglia homeostasis.

8. Microglia in psychiatric diseases

Some lines of evidence suggest that microglial may play a role in certain neurological conditions, especially of psychiatric nature (Li and Barres, 2017; Mondelli *et al.*, 2017; Tay *et al.*, 2018). The current chapter aims to address the most important findings and controversies regarding microglial dysfunction and possible implications in animal behavior. Before moving to such a controversial matter, it is necessary to point out few important forewarnings.

8.1 Caveats in microglia research

First, expression of Cre recombinase under the control of a microglial promoter is probably one of the most useful strategy to study microglia (Wieghofer and Prinz, 2015; Wieghofer *et al.*, 2015). For example, LysM (*Lyz2*) was broadly considered as a myeloid-specific gene, therefore the $LysM^{Cre}$ mouse line became a very popular tool for genetic targeting of macrophages and microglia (Clausen *et al.*, 1999). However, recent works showed that this gene is transiently expressed in neurons (Wang *et al.*, 2015; Orthgiess et al., 2016), thus putting into question the reliability of $LysM^{Cre}$ for microglia research (Blank and Prinz, 2016). More recently the $Cx3cr1^{Cre}$ (constitutive) and $Cx3cr1^{CreER}$ (tamoxifen-inducible) lines were generated and, in comparison to the $LysM^{Cre}$, exhibited an improved efficiency and specificity of recombination (Goldmann *et al.*, 2013; Parkhurst *et al.*, 2013; Yona *et al.*, 2013). Nevertheless, neuronal recombination was also observed in $Cx3cr1^{Cre}$ mice (Haimon *et al.*, 2018), while the $Cx3cr1^{CreER}$ mice seemed to undergo spontaneous recombination in absence of tamoxifen administration (Fonseca *et al.*, 2017; Weber *et al.*, 2018). Similarly, the $Csf1r^{Cre}$ (constitutive) and $Csf1r^{Mer\text{-}Cre\text{-}Mer}$ (tamoxifen inducible) lines showed recombination in endothelial cells (Plein *et al.*, 2018), thus arguing against the specificity of these fate-mapping systems for the macrophage lineage. In conclusion, approaches of microglia-specific targeting have certainly been improved in the last years, however further optimizations are still demanded.

Second, the antibiotic minocycline has been for a long time considered as a putative "microglia inhibitor". Based on this pre-concept, many *in-vivo* studies claimed that microglia activation was "blocked" by minocycline treatment. Minocycline may indeed blunt microglia reaction under inflammation, however, this drug does not have any acknowledged specifically for microglia.

Therefore, confounding effects due to undesired targeting of other brain cells cannot be excluded (Möller *et al.*, 2016).

Third, a popular tool for microglia depletion *in-vivo* is the administration of Diphtheria Toxin (DT) in transgenic mice expressing Diphtheria-Toxin Receptor (DTR) under the control of a microglia-specific promoter. Using this system, certain studies described alterations in brain development and mouse behavior (Parkhurst *et al.*, 2013; Ueno *et al.*, 2013; Miyamoto *et al.*, 2016). Although often ignored, cell death caused by DT toxicity induces a peak of pro-inflammatory mediators (Akazawa *et al.*, 2004; Agarwal *et al.*, 2012; Bruttger *et al.*, 2015) which may potentially affect the physiological brain functions, regardless of the contingent vanishing of microglia (Waisman *et al.*, 2015). Given the risk of possible experimental artifacts, a critical appraisal of these data is always recommended.

8.2 Microglia and animal behavior

First indication that mutated microglia might be a cause of neurological disorders date back to the early 2000s. Loss-function mutations in DAP12 and TREM2 were associated with the Nasu-Hakola disease, a rare form of leukodystrophy characterized by a progressive pre-senile dementia together with a spectrum of psychiatric symptoms. Moreover, rare Trem2 allelic variants have been correlated with increased risk of late-onset Alzheimer Disease (Guerreiro *et al.*, 2013; Jonsson *et al.*, 2013; Colonna and Wang, 2016; Sims *et al.*, 2017; Yeh *et al.*, 2017). DAP12 serves as an adaptor protein for intracellular signaling of TREM2 and CSF1R (McVicar and Trinchieri, 2009; Colonna and Wang, 2016; Ulrich *et al.*, 2017), genes that in the brain are exclusively expressed in microglia. In mice it was shown that DAP12 knock-out causes a series of brain's abnormalities, such as hypomyelination and synaptic degeneration. Moreover, traits of schizophrenia-like behavior were observed in these mice, (Kaifu *et al.*, 2003; Roumier, 2004). In line with these findings, Trem2-deficient mice exhibited reduced synaptic pruning during brain development and a spectrum of behavioral alterations resembling autism (Filipello *et al.*, 2018). Interestingly, a very recent study investigated gene-expression of human microglia in different neurological diseases, including schizophrenia. Microglia isolated from the anterior pre-frontal cortex of schizophrenic patients exhibited upregulation of about 600 microglia-enriched transcripts. Moreover, one third of the genetic risk factors for schizophrenia resulted prominently expressed in microglia (Gosselin *et al.*, 2017).

A second example is the case of the Hoxb8 knock-out mice, a model of obsessive-compulsive disorder (OCD) which develops a very aggressive form of pathological grooming. This behavioral alteration is typically manifested with compulsory removal of the fur from certain body areas and presence of self-inflicted lesions. Additionally, HoxB8 mutant mice exhibit alteration of cortico-striatal circuits, with excess of dendritic spines, along with structural and functional deficit of synaptic connections (Nagarajan *et al.*, 2017). *Chen et al.* reported that in the brain Hoxb8 was prominently expressed in microglia, therefore suggesting a potential contribution of microglia in the pathology. In support of this hypothesis, it was shown that transplantation of wild-type bone marrow into Hoxb8 knock-out recipient rescued the pathological grooming behavior. Conversely, transplantation of Hoxb8 knock-out bone marrow into wild-types increased the time spent in self-grooming, although at a way lesser extent compared to the non-chimeric knock-out (Chen *et al.*, 2010). A similar scenario was recently described the progranulin (*Grn*) knock-out mice. In humans, *Grn* mutations have been linked to frontotemporal lobar degeneration and lysosomal-storage disorder characterized by accumulation of lipofuscin deposits in the brain (Baker *et al.*, 2006; Chen-Plotkin *et al.*, 2010; Roberson *et al.*, 2013; Petkau and Leavitt, 2014; Kao *et al.*, 2017). Progranulin-deficient mice have reduced life-span, accompanied by behavioral disturbances resembling OCD pathology (Yin *et al.*, 2010), namely pathological grooming with open skin lesions and reduced social interaction. Progranulin is highly expressed in myeloid cells and *Grn* null-mutation seemed to induce several folds upregulation of complement proteins in microglia (along with a general alteration of microglial gene-expression). Interestingly, crossing *Grn* and *C1q* knock-out mice mitigated the OCD-like behavior along with a significant extension of mouse survival (Lui *et al.*, 2016). Another work showed that *Grn* ablation in microglia and macrophages (by means of the *Cx3cr1*^{Cre} transgene) was sufficient to phenocopy the self-grooming behavior observed in the constitutive *Grn* knock-out line (Krabbe *et al.*, 2017).

Microglia were also suggested as major players in the Rett Syndrome. *Derecki et al* showed that Mecp2 knock-out mice (a mouse model of Rett Syndrome) exhibited increased life-span along with a general improvement of the pathological course after receiving transplantation of wild-type bone marrow (Derecki *et al.*, 2012). Moreover, reactivating expression of a functional Mecp2 allele in microglia (using either the LysM^{Cre} or *Cx3cr1*^{CreER} transgenes) significantly delayed disease onset and reduced neurological symptoms in otherwise Mecp2 knock-out mice (Derecki *et al.*, 2012; Cronk *et al.*, 2015). These studies indicated that lack of Mecp2 in microglia has remarkable importance for Rett Syndrome.

However, a different group failed to reproduce beneficial effect of the bone marrow transplantation in Mecp2 knock-out recipient. Likewise, preserving Mecp2 expression in the sole myeloid lineage did not produce any beneficial effect compared to the complete Mecp2 knock-out (Wang *et al.*, 2015). Another study showed that Mecp2 knock-out mice exhibit highly phagocytic microglia, which was associated with exaggerated synaptic pruning and ensuing synaptic loss. However, this phenomenon was independent of Mecp2 expression in microglia. Therefore, microglia may indeed play an important role in the pathogenesis of Rett Syndrome, however, lack of Mecp2 seems unrelated to the pathological phenotype of microglia (Schafer *et al.*, 2016).

Role of microglia in mouse learning abilities represents a second example of discrepancy in literature. Findings and controversies regarding the CX3CR1-deficient mice have been already addressed in a dedicated chapter, therefore the current paragraph will focus exclusively on the microglia depletion models. An important breakthrough in the field came with the work of *Parkhurst et al.* who generated a transgenic mouse expressing DTR in microglial cells only. Accordingly, DT administration in young adult mice allowed selective (although transient) depletion of microglia, leaving other macrophage populations unaffected. Authors showed that a short period of microglia deficiency resulted in a diminished performance at different learning tasks, such as motor learning and hippocampal-dependent memory. These behavioral alterations were ascribed to an impairment of spine turnover. Authors suggested that microglia mediate continuous synapses remodeling through release of BDNF, especially during learning processes. As a main message, microglia-derived BDNF is a key factor promoting learning-dependent spine formation and associated learning ability (Parkhurst *et al.*, 2013). Soon after, *Elmore et al.* announced a different tool for depleting microglia, which was based on the chronic pharmacological inhibition of CSF1R. Compared to the former study, using this method it was possible to deplete microglia for a long period of time. Surprisingly, even after months of depletion no overt behavioral phenotype could be observed. Authors concluded that presence of microglia is superfluous for cognitive abilities and other behavioral skills (Elmore *et al.*, 2014).

Although later works actually reported cognitive and behavioral alterations in mice after transient depletion of microglia using the CSF1R inhibitor (Torres *et al.*, 2016; Rosin *et al.*, 2018), the issue remain confused and still highly debated. Nevertheless, microglia depletion may produce remarkable effects under pathological conditions. Indeed, two studies using the CSF1R inhibitor in mouse models of Alzheimer Disease (AD) reported that microglia-depletion reduced learning and memory deficits

(Olmos-Alonso *et al.*, 2016; Spangenberg *et al.*, 2016). Another recent work showed a remarkable reduction of the amyloid-beta load when microglia were depleted via administration of CSF1R inhibitor prior the pathology onset, thus suggesting a crucial (unexpected) role of microglia in amyloid-beta deposition (Sosna *et al.*, 2018). For the sake of comprehensiveness, it should be noted that an earlier study showed indistinguishable neuropathological features between microglia-depleted and non-depleted AD mice (Grathwohl *et al.*, 2010). Divergent findings are likely owing to the use of different transgenic models and microglia-depletion strategies. Similarly, improved psychiatric-like behavior was described in *Cnp* knock-out mice exhibiting mild white matter abnormalities (Janova *et al.*, 2018). Conversely, microglia depletion in a ALS (Amyotrophic Lateral Sclerosis) mouse model produced detrimental outcomes (Spiller *et al.*, 2018). Further studies are required to shed more light on the role(s) played by microglia under different pathological conditions.

8.3 Are microglia important players in depressive disorders?

Major Depressive Disorder (MDD) is one of the most common neuropsychiatric illness, affecting about 5% of the worldwide population (Kessler and Bromet, 2013). In developed countries the percentage of individuals experiencing depression at least once in life was estimated around 15% (Krishnan and Nestler, 2008). Chronic exposure to psychological stress likely represents a major etiological factor, however, the underlying neuropathological features are still poorly understood (Kendler et al., 1999; Otte et al., 2016; van Calker et al., 2018). Symptomatology of MDD is complex and highly heterogeneous, encompassing depressed mood, anxiety, panic, sleep alteration, social avoidance, lack of motivation/interests, anhedonia, cognitive disturbances and, also, tendency to suicide (American Psychiatric Association, 2013). Most of the pharmacological treatments primarily aim to increase brain's levels of serotonin and noradrenalin. However, conventional therapies may require months-long treatments before symptoms recovery and an important percentage of patients appears completely unresponsive (Krishnan and Nestler, 2008). A comprehensive understanding of the disease in its multiple facets will help develop more effective therapeutic strategies (Papakostas and Ionescu, 2015). In this regard, microglial cells have gained growing attention (Dantzer *et al.*, 2008; Yirmiya et al., 2015; Mondelli et al., 2017; Tay et al., 2018). Studies on human post-mortem samples described a form of microgliosis in patients suffering of Major Depressive Disorder (MDD) (Steiner *et al.*, 2008; Torres-Platas *et al.*, 2014). Furthermore, PET studies reported increased uptake of TSPO

(Translocator Protein 18KDa) radioligand in the brain of MDD patients (Setiawan *et al.*, 2015; Holmes *et al.*, 2017; Setiawan *et al.*, 2018). Although the biological and clinical significance of increased TSPO expression in the brain is still uncertain (Rupprecht *et al.*, 2010), these findings suggest the presence of pro-inflammatory microglia in brain of MDD patients, especially within regions associated with the depressive symptoms. Moreover, important alterations in microglia gene-expression profile were also reported in schizophrenic patients (Gosselin et al., 2017).

Consistently, evidences of activated microglia were found in different animal models of chronic stress, especially in brain regions associated with the stress-response, such as nucleus accumbens, prefrontal cortex and hippocampus (Sugama *et al.*, 2007; Tynan *et al.*, 2010; Hinwood *et al.*, 2012; Wohleb *et al.*, 2014). Although still unclear, it is possible that a maladaptive immunological response to environmental stressors might cause prolonged and exaggerated microglial reactivity in certain brain regions (Delpech *et al.*, 2015; Wohleb *et al.*, 2016; Bisht *et al.*, 2016). Not surprisingly, microglial reaction in rodent models of chronic-stress appeared qualitatively different depending on the experimental set up and investigated time points. For example, rats undergoing chronic-restraint stress or corticosterone treatment revealed a tendency towards microglia hyper-ramification (Hinwood *et al.*, 2013; Walker *et al.*, 2014; Caetano *et al.*, 2016). A similar behavior was observed in mouse models of repeated forced-swim stress (Hellwig *et al.*, 2016; Llorens-Martín *et al.*, 2016). In contrast, mice under chronic-mild stress showed a biphasic microglial response, characterized by an initial proliferation and pro-inflammatory skew, followed by microglia atrophy and degeneration at later stages (Kreisel *et al.*, 2014). Additionally, studies on repeated social defeat models indicated a role of both pro-inflammatory monocytes and microglia in anxiety-related behaviors (McKim *et al.*, 2017; Wohleb *et al.*, 2014). Interestingly, independent groups described a clear resilience of *Cx3cr1* knock-out mice to stress-induced behavioral alterations (Wohleb *et al.*, 2013; Rimmerman *et al.*, 2015; Hellwig *et al.*, 2016; Milior *et al.*, 2016; Rimmerman *et al.*, 2017; Winkler *et al.*, 2017). Although still debated, it was suggested that disruption of certain microglia-neuron communication pathways may be the cause of the observed beneficial effects. In support of this view, a recent work showed that TLR2/4 double knock-out mice are remarkably resilient to the Repeated Social Defeat Stress. TLR2/4 are primarily expressed in microglia, therefore authors suggested that TLRs ligands (such as DAMPs) may be released by neurons during chronic-stress and in turn activate neighboring microglia, inducing release of cytokines and promoting phagocytosis (Nie *et al.*, 2018).

Together, these findings suggest that microglia may play a determinant role in the physiopathology of several neurological conditions including psychiatric disorders (Colonna and Butovsky, 2017; Wolf *et al.*, 2017), however, their true function remains yet elusive. Possibly, targeting microglia may delay onset, slow progression or simply ameliorate symptoms of different brain diseases, including psychiatric disorders as MDD (Prinz and Priller, 2014; Biber *et al.*, 2016). More studies are demanded to better explore the molecular network between microglia and other brain cells (especially neurons) during chronic-stress. Identification of key molecules and pathways associated with the stress-induced microglia activation may ultimately pave the way towards new therapeutic avenues for clinical depression.

Aim of the study

The scope of my project was to characterize microglial alterations in the hippocampus using a mouse model of Repeated Forced Swim Stress (RFSS). With this work, I aimed to answer the following questions:

1) Do microglia exhibit obvious changes under RFSS?

- Count the number of microglial cells in the hippocampus
- Assess microglial morphology in the hippocampus
- Compare microglial cells density and morphology among different brain regions

2) Are microglia important for the behavioral phenotype in this model?

- Test the *IL34* knock-out line (deficient of microglia) at the RFSS paradigm
- Test the *Cx3cr1*^{Cre}*:TNFa*$^{flox/flox}$ line (with TNFa deletion in microglia) at the RFSS paradigm

3) Do hippocampal microglia exhibit phenotypic and functional alteration under RFSS?

- Assess expression of cytokines, chemokines and neurotrophic factors
- Assess expression of macrophage-activation markers (CD11b and CD68)
- Assess microglial processes motility

4) Does this model exhibit synaptic alterations in the hippocampus?

- Count of glutamatergic synapses in the hippocampus

5) Does the microglial P2Y12-receptor play any role in this model?

- Test the *P2y12r* knock-out line at the RFSS paradigm
- Assess microglial morphology and number of glutamatergic synapses in *P2y12r*-deficient mice

Materials and Methods

1. Animals and Ethics

All animal experimentations were approved by both the local authorities of the Regional Council of Freiburg (Regierungspräsidium) and the animal welfare committee of the University of Freiburg. All interventions on laboratory animals (including breeding, housing, handling, testing and sacrifice) were performed in accordance with the international guidelines of the Federation for Laboratory Animal Science Associations (FELASA). All mice used here were maintained under C57BL/6 background. Mice were bred under specific pathogen-free conditions, in a temperature- and humidity-controlled facility with a 12 hours light-dark cycle. Food and water were available ad libitum. Unless otherwise specified, both genders were used at the age of 10-12 weeks old. P2y12-receptor knock-out mice were provided by Prof. Marco Idzko (Clinic of Pneumology, University of Freiburg Medical Center, Germany); IL34 knock-out mice were provided by Prof. Marco Colonna (Department of Pathology and Immunology, Washington University of St. Louis, USA); *Cx3cr1*^{Cre} *: TNFα*$^{flox/flox}$ mice were provided by Prof. Steffen Jung (Weizmann Institute, Rehovot, Israel); *Cx3cr1*$^{GFP/+}$ mice (for in vivo two-photon imaging experiment) were provided by Dr. Martin Fuhrmann (DZNE Bonn, Germany).

2. Behavioral tests

Activity and behavior of mice were observed using an automatic video tracking system for recording and analysis (VideoMot2 system V6.01, TSE), unless otherwise specified. All behavioral experiments were performed in the morning starting from 9:00 am. An equal number of both males and females were used for all behavioral studies. In experiments comparing either wild-type vs. mutant, or untreated vs. treated groups, all animals were littermates.

Repeated Forced Swim Stress

To induce chronic stress in mice I used a previously described protocol (Sun *et al.*, 2011; Serchov *et al.*, 2015), which represents a modified version of the widely used Forced Swim Test (FST). Mice were subjected to repeated forced swimming in a transparent glass cylinder (15 cm diameter)

containing 20 cm of water (23 – 24 °C), 10 minutes daily for 5 consecutive days (day 1 - 5, induction phase). During this period mice were video recorded, and the time spent in immobile position and swum distance (expressed in seconds and cm respectively) were scored by means of the VideoMot2 mouse-tracking software. After each session, mice were returned to their home cage.

Tail Suspension Test (TST)

TST is a commonly used test to assess depressive-like behavior and antidepressant treatment response in mice. A TST session was given to the mice 3 days before the first day of Repeated Forces Swim Stress (day -3, TST basal) and one day after the last session of Repeated Forces Swim stress (day 6, TST Stress). Each mouse was suspended by a tape wrapped around the tail to an aluminum bar (1 cm height, 1 cm width, 60 cm length) located horizontally over an empty white box (30 x 50 x 20 cm). Mice were hanging upside down, at about 20 cm above the bottom of the box, for a time of 6 minutes. During this period mice were video recorded, and the time spent in immobile position (expressed in seconds) was manually scored with a stopwatch by a blind examiner.

Morris Water Maze (MWM)

A circular tank (120 cm diameter) was filled with water equilibrated at the room temperature (23 – 24 °C) and dyed with a soluble white plaint. A submerged (not visible) platform was located into a target quadrant at about 1 - 2cm below the water level. Before starting the test, mice were placed on the submerged platform for 30 seconds. During the training phase mice were carefully lied into the water (facing towards the wall of the tank) and let free to swim for the following 60 seconds. Animals that were unable to reach the platform were manually guided to the platform. The overall learning session consisted of 4 trials / day for 5 up to 7 days. After this period animals were returned to their home cage. The latency time to the platform was determined as average swimming time of the 4 daily trials. When a mouse failed to locate the platform, the resulting latency time was arbitrarily scored as 60 seconds. The probe session was performed 24 hours after the last training session. To do so, the submerged platform was removed, and mice's swimming path was recorded for 60 seconds. To assess the long-term memory retention, percentage of time in the target quadrant and number of entries in the platform area were calculated. Average speed (cm / s) was also measured to assess mice's swimming ability.

3. Clopidogrel and LPS treatments

Clopidogrel

Plavix capsules containing 70 mg clopidogrel were finely pulverized and dissolved in 28 ml of saline solution, yielding a final concentration of 2,5 mg/ml. To maximize the solubility of the drug, clopidogrel solutions were maintained overnight under shaking at 500 rmp. Wild-type mice were daily injected ip. with 50 mg/kg of clopidogrel for 5 days. The exact injected volume was determined by weighting each mouse before the first injection. The treatment started on the first day of the induction phase (day 1) and ended on the last day (day 5). Mice were injected every day at 9:00 am in the morning and the FST session was performed four hours later. No side effects were reported during the 5 days of treatment.

Lipopolysaccharide (LPS)

Purified lipopolysaccharide endotoxin (LPS) from E. coli (serotype 0111:B4, Sigma) was diluted in saline solution at final concentration of 0.1 μg/μl. The solution was injected ip. in wild-type mice at the dosage of 0,5 mg/kg. The exact injected volume was determined by weighting each mouse before the injection. In control mice, an equal volume of saline solution was injected. After the injection, mice were returned to their home cage. No obvious sickness behavior was observed upon LPS injection. Mouse behavior was scored 24 hours post-injection.

4. Immunohistochemistry

Mice were overdosed through intraperitoneal (ip.) injection of ketamine hydrochloride (Ketavet, Pfizer; dosage 300 mg/kg body weight) and xylazine (Rompun, Bayer HealthCare; dosage 30 mg/kg body weight). After loss of the paw-pinch reflex mice were transcardially perfused with 25 ml of ice-cold PBS. Dissected brains were fixed in 4% PFA (paraformaldehyde) overnight at 4ºC and subsequently cryoprotected in 30 % sucrose solution for at least 48 hours at 4 ºC. Brains were cut into 60 μm think coronal sections at the cryostat (approximately between bregma -2.1 and -2.9). Slices were either used immediately for immunohistochemistry or stored at -20 ºC in freezing media (30 % Ethylene glycol, 30 % Glycerol, 10 % phosphate buffer 0.2 M, 30 % MilliQ water).

Materials and Methods

Free-floating sections were pre-incubated for 2 hours (room temperature, under shaking) with the blocking solution (5 % horse or goat serum, 0.5 % Triton X-100, diluted in PBS). Subsequently, sections were incubated 24 - 48 hours (4 ºC, under shaking) with the primary antibody solution (1 % horse or goat serum, 0.5 % Triton X-100, diluted in PBS). Slices were washed at room temperature 3 x 10 minutes in PBST (0.1 % Tween20 diluted in PBS) and then additional 3 x 10 minutes in PBS. Sections were then incubated overnight (4 ºC, under shaking) with the secondary (fluorochrome-conjugated) antibodies solution (1 % horse or goat serum, 0.5 % Triton X-100, secondary antibody 1:1000, DAPI 1:4000, diluted in PBS). After washing as described above (3 x 10' PBST + 3 x 10' PBS), sections were mounted on Superfrost glass slides with either aqueous fluorescent mounting medium (DAKO, indicated for water-immersion objective) or ProLong Diamond antifade mounting medium (Thermo Scientific, indicated for oil-immersion objective) and eventually covered with high precision 170 ± 5 μm think coverslips (Lab Supply Ltd). The complete antibody list can be found in **Table 1.**

Antigen	Company	Host	Dilution	Secondary Antibody
Iba1	Wako	Rabbit	1:500	Life Technology anti-Rabbit-Alexa 647 (1:1000)
CD11b	eBioscience	Rat	1:500	Abcam anti-Rat-Alexa 488 (1:1000)
VGLUT1	Millipore	Mouse	1:500	Life Technology anti-Mouse-Alexa 488 (1:1000)
Homer1	Millipore	Rabbit	1:500	Life Technology anti-Rabbit-Alexa 647 (1:1000)
Synaptophysin	Millipore	Mouse	1:500	Life Technology anti-Mouse-Alexa 488 (1:1000)
GFAP	Abcam	Mouse	1:500	Life Technology anti-Mouse-Alexa 488 (1:1000)
CD68	Serotec	Rat	1:200	Abcam anti-Rat-Alexa 488 (1:1000)
CD34	eBioscience	Mouse	1:200	Life Technology anti-Mouse-Alexa 488 (1:1000)

Table 1: Complete antibody list

5. Confocal imaging

Immuno-stained 60 μm think brain cryo-sections were analyzed by confocal laser scanning ZEISS LSM 510 META microscope. For the Iba1 and CD11b staining, 20 μm think z-stack images were obtained with either 25X / 0.8NA or 63X / 1.2NA water-immersion objectives (Zeiss). Microscope settings: 1.6 μs pixel dwell, resolution 2048 x 2048 pixels, averaging number 4, z-step 0.5 μm, unidirectional acquisition mode, color depth 12 bit. For the Homer1 and VGLUT1 staining, 10 μm think z-stack images were obtained with 63X / 1.4NA oil-immersion objectives (Zeiss). Microscope settings: 1.6 μs pixel dwell, resolution 2048 x 2048 pixels, averaging number 4, z-step 0.3 μm, unidirectional acquisition mode, color depth 12 bit, 1,8X digital zoom (imaged area 2,500 μm^2). Confocal images were initially deconvolved by Huygens Professional software (https://svi.nl) using default parameters settings.

6. Image analysis and microglia 3D reconstruction

Images were analyzed in IMARIS software version 7.5.3 (www.bitplane.com). Number of microglial cells per stack was assessed by means of the object detection tool (allow ellipsoid, default auto threshold, object diameter ≥ 10 μm) in 25X magnified images (sections = 450 x 450 μm field, 20 μm thick). If needed, automatic cell count was manually corrected. Number of both VGLUT1- and Homer1-positive synaptic inputs was assessed by means of the object detection tool (allow ellipsoid, default auto threshold, object diameter ≥ 1μm) in 63X magnified images plus 1,8X digital zoom (sections = 50 x 50 μm field, 10 μm thick). Number of glutamatergic synapses per field of view was determined via automated count of colocalized objects (number of VGLUT1 / Homer1 spots with a distance < 1 μm). Number of Iba1- and CD11b-positive voxels per stack was assessed by means of the surface reconstruction tool (default auto threshold) in 63X magnified images (sections = 180 x 180 μm field, 20 μm thick). Microglia morphometric analysis was performed on 3D cell reconstructions, generated by means of the filament tracer tool (no loops allowed, start-end points calculated via spot detection, cell body diameter ≥ 10 μm, processes diameter ≥ 0.5 μm, default auto threshold). If needed, automatic spot detection was manually corrected. Cells were considered eligible for the analysis only when the cell body and all the processes were clearly visible and located within the stack volume. Analyzed morphological parameters: processes area, total process length, number of branch points,

number of terminal points and number of Sholl intersections per μm. For each experiment 10-12 randomly chosen microglial cells were analyzed.

7. Real-Time PCR

After perfusion, Hippocampi and Cortex were surgically dissected and the homogenized with a tissue potter. Microglia enriched fraction was collected in a 70% / 25% / 0% percoll gradient (centrifuged 30 minutes at 1000 g, 4 °C without break). After centrifugation, myelin debris settled at the interface 25 % / PBS, while the fraction enriched in microglia was collected at the interface 25 % / 75%. Microglia was then resuspended in 10 ml PBS and pelleted by centrifugation (200 g, 4° C). Microglia pellet was used for ensuing RNA extraction. Microglia-derived RNA was extracted and purified by means of the Illustra RNAspin Isolation Kit (GE Healthcare). Purified RNA was precipitated overnight at -20 °C in a solution containing 75 mM Sodium Acetate, 50 ng/ul Glycogen and 75 % Ethanol. The day after RNA was pelleted (15 minutes centrifugation at 12000 g, 4 °C) and subsequently washed two times with 500 μl 70 % ethanol. RNA pellet was then dried at room temperature and resuspended in 30 μl of ultrapure water. RNA samples were copied into cDNA using a retro-transcription master mix (Promega). Aliquots of 200 ng cDNA were stored at -20 °C till use. Amplification mix for each target contained ~2 ng/μl microglia-enriched cDNA, 1:2 diluted iQ SYBR green Supermix (Bio-Rad), 50 pmol of exon-spanning primers. Each reaction had a final volume of 10 μl with ~20 ng of microglia-enriched cDNA. Each sample was run in triplicate for both GAPDH (housekeeping gene) and the target genes. Quantitative real time PCR was run in 96 - well plates using a C1000 Thermal Cycler (Bio-Rad). Results were analyzed using the Bio-Rad CFX Manager 3.0 software. To estimate the relative gene-expression level the $2^{-(\Delta-\Delta Ct)}$ method was applied (assuming an amplification yield of the 100 %). The complete primers list can be found in **Table 2.**

Materials and Methods

Target	Primers sequence	Biological function
iNOS	Forward: TCAACTGCAAGAGAACGGAGAA Reverse: TGAGAACAGCACAAGGGGTTT	Nitric-oxide synthesis, vasodilation, inflammation
CCL2	Forward: CCACAACCACCTCAAGCACT Reverse: TAAGGCATCACAGTCCGAGTC	Monocytes chemoattractant protein
NOX2	Forward: TCATTCTGGTGTGGTTGGGG Reverse: AGTGCTGACCCAAGGAGTTT	Superoxide ions synthesis, oxidative-stress
TNFα	Forward: CCCACGTCGTAGCAAACCA Reverse: ACAAGGTACAACCCATCGGC	Pro-inflammatory cytokine
IL6	Forward: AGTTGACGGACCCCAAAA Reverse: AGCTGGATGCTCTCATCAGG	Pro-inflammatory cytokine
IL1b	Forward: TGCCACCTTTTGACAGTGATG Reverse: ATGTGCTGCTGCGAGATTTG	Pro-inflammatory cytokine
CIITA	Forward: GATGTGGAAGACCTGGATCG Reverse: TGCATCTTCTGAGGGGTTTC	MHC-II trans-activator, MHC-II expression
IL10	Forward: GAAAATAAGAGCAAGGCAGTGG Reverse: GTCCAGCAGACTCAATACACAC	Anti-inflammatory cytokine
BDNF	Forward: GCCGCAAACATGTCTATGAGGGTT Reverse: TTGGCCTTTGGATACCGGGACTTT	Neurotrophic factor
TGFβ1	Forward: CTGCTGACCCCCACTGATAC Reverse: GCCCTGTATTCCGTCTCCTT	Transforming Growth Factor Beta, cytokine
CX3CR1	Forward: CCATCTGCTCAGGACCTCAC Reverse: CACCAGACCGAACGTGAAGA	Chemokine receptor, microglial marker
CD11b	Forward: CGGTGGCAGTGTGAAGCTCTTCTC Reverse: GGCGCCTATGATCCGCTGGCT	Integrin αM, cell adhesion, phagocytosis
Iba1	Forward: CAGGGATTTGCAGGGAGGAAA Reverse: AGTTTGGACGGCAGATCCTC	Membrane ruffling, microglial marker
GAPDH	Forward: TGTCCGTCGTGGATCTGA Reverse: CCTGCTTCACCACCTTCTTG	Glyceraldehyde-dehydrogenase, glycolysis

Table 2: Complete primers list

8. Flow cytometry (FACS)

Perfused brains were dissected and homogenized as described above. Myelin was depleted by centrifugation on 35 % percoll gradient overlaid with PBS (30 minutes at 1000 g, 4 °C without break). Myelin fraction was discarded while the cellular fraction was collected in the pellet. Brain pellets devoid of myelin underwent the follows staining steps (on ice): 5 minutes incubation with fixable viability dye eFluor780 (1:1000, Thermo Fisher); 15 minutes incubation with FC-receptors blocker (1:100, eBioscience); 30 minutes incubation with anti-mouse CD45-APC (1:300, eBioscience) and anti-mouse CD11b-BV421 (1:300, BioLegend). Intracellular staining for VGLUT1 was carried out according to the protocol of the Cell Fixation/Permeabilization Kits (BD Bioscience). After surface labeling, samples were fixed for 20 minutes on ice with 250 µl of the BD Cytofix/Cytoperm solution (containing 4 % PFA). Pellets were then washed twice with the BD Perm/Wash buffer and stained overnight at 4 °C with 1:100 anti-mouse VGLUT1-PE antibody (Millipore, FCMAB335PE). The following day samples were washed twice with the BD Perm/Wash buffer, resuspended in 200 µl of FACS buffer (0,5 % BSA, 1 mM EDTA, 0,1 % Na-Azide, diluted in PBS) and then analyzed in flow cytometry. FACS experiments were carried out with the BD FACS Canto II and data were analyzed with FlowJo V10. Cells populations were hierarchically gated as follows (Y/X axes): $CD11b_{log}$ / FSC-A (brain's myeloid and non-myeloid cells) → FSC-A / FSC-W (singlets) → eFluor780 / FSC-A (viable cells) → CD11b / CD45 (microglia) → compensated-PE histogram (PE MFI and % of PE+ events).

9. Serum corticosterone dosage

Approximately 100 ul of blood was collected from the retro-orbital venous plexus 9 days before the Repeated Forced Swim Stress began. On day 6 (24 hours after the last Repeated Forced Swim Stress session) additional 800 ul of blood was collected through transcardial puncture prior to perfusion. These timepoints were selected to assess the corticosterone levels under Basal and Stress condition respectively. Serum was separated from the whole blood by means of MiniCollect® Z Serum Sep. Tubes (Greiner Bio-one) and stored at -80 °C till analysis. Corticosterone concentration was determined by ELISA with the Corticosterone enzyme immunoassay kit (Arbor Assays). Optical absorbance was measured with a µQuant spectrophotometer (Bio-Tek Instruments). **Note:** This test was performed by Laura Kracht (Department of Neuroscience UMCG, 9713AV Groningen, The Netherlands).

10. Two-photon *in vivo* imaging

In vivo brain imaging was carried out in two-photon microscopy using *Cx3cr1*$^{GFP/+}$: *Thy.1-YFP* double transgenic mice, which allow simultaneous visualization of both microglia and neurons (Fuhrmann *et al.*, 2010; Tremblay *et al.*, 2010). Implantation of the cranial window was performed by Felix Nebeling (Martin Fuhrmann group, DZNE Bonn) three months prior the experiment. A detailed description of the cranial surgery, window implantation and awake brain imaging has been previously published (Gu *et al.*, 2014; Schmid *et al.*, 2016). Briefly, tested mice underwent a craniotomy of ~3 mm Ø was, performed using a dental drill perpendicularly to the position of the dorsal hippocampus (Bregma anteroposterior/mediolateral = −2,2 mm / +1,8 mm). The cortex beneath was removed by aspiration with a syringe till the external capsule of the hippocampus was reached. A cranial window was then implanted onto the skull and sealed with dental acrylic resin. Brain imaging was performed with a LaVision Trim ScopeII (OPO) two-photon microscope under awake condition to avoid drug-related effects on microglial processes motility. For this experiment, 5 mice underwent 40 minutes two-photon imaging in the hippocampus using a 10X / 0,95NA water immersion objective. 100 x 100 x 80µm (x/y/z) z-stacks (z-step 0,4 µm) were acquired every 5 minutes (9 stacks per muse). Stack position was carefully tuned to encompass the Stratum Oriens, Pyramidal Cell Layer and Stratum Radiatum of the CA1 region. At the end for the imaging session, mice were returned to their home cage. The same mice were imaged again after application of our repeated Forced Swim Stress protocol.

Five mice were used for this experiment. For a gross location of the region of interest, a 400 µm^2 was acquired. Having defined the optimal z-stack position, the field of view was magnified four times (100 µm^2) and 80 µm think z-stacks were acquired every 5 minutes, for an overall period of 40 minutes time-lapse imaging (9 stacks per mouse). To relocate the same imaged region before and after chronic-stress, blood vessels and YFP+ pyramidal neurons were used as territory landmark (for a rough identification and fine adjustment respectively). Images were acquired in oversampling using a z-step of 0.4 µm, thus yielding 200 images per stack. Row single images were manually assembled into a z-stack projection using ImageJ (Images > Stacks > Images to Stack). Flickered images were carefully removed from the stack. Signal spillover between GFP and YFP channels were manually corrected by using the image calculator tool of ZEN Black 2.3 SP1 (Zeiss). Moreover, images were processed using the median filter function (kernel size x = 5, y = 5, z = 1) in ZEN Black 2.3 SP1 (Zeiss).

Next, stack images were registered in ImageJ using both StackReg plugin (Plugins > Registration > StackReg; https://imagej.net/StackReg) and Template Matching plugin (Plugins > Template Matching > Alline Slices in stack; https://sites.google.com/site/qingzongtseng/template-matching-ij-plugin). Each 80 µm thick z-stacks was then split into 5 smaller z-stack (~15 µm think) and converted into Z-projection (Images > Stacks > Z Project). The position of these z-stack (within the original 80 µm thick section) was arbitrarily chosen according to the distribution of microglia. Each z-stack should contain at least one clearly visible microglial cell, with both soma and processes entirely included between the section's edges. This step is very critical, as it is necessary to generate Z-projection with the same depth and thickness, for each time-point. Last, Z-projection from the same z-position, but different time-point were assembled into as time-lapse image using ImageJ (Images > Stacks > Images to Stack) and then exported as AVI video files. Microglia motility was assessed with IMARIS on 3D reconstructed microglia using the filament tracer tool. For each cell, processes length (µm) was analyzed every 5 minutes. Processes speed for each timepoint was determined as $\Delta\mu m_{(t1-t0)}/5'$ (t1 = length at time-point 1; t0 = length at time-point 0). This measurement was performed separately for primary and terminal processes. Average speed of primary and terminal processes was determined by mediating the speed at each time-point. For this analysis, 4 microglial cells per mouse were selected at different z position in the stack (20 cells overall).

11. Statistics

Graphs and statistics were produced with Graph-Pad Prism 5 software. Statistical difference between two groups was determined with the paired or unpaired two-tailed Student's T-test. To assess effect of treatments or genotypes the One-Way or Two-Ways Analysis of Variance (ANOVA) with Bonferroni post hoc test were calculated. A significance difference was assigned with a p-value < 0,05 (*p<0,05, **p<0,01, ***p<0,001). All data are displayed as Mean ± SEM (standard error of the mean).

Results

1. Presence of microglial reactivity in the hippocampus of RFSS mice

The hippocampus has been repeatedly described as one of the mainly affected brain regions in Major Depressive Disorder (Rajkowska *et al.*, 1999; Ressler and Mayberg, 2007; Sahay and Hen, 2007; Drevets *et al.*, 2008; MacQueen and Frodl, 2011; Small *et al.*, 2011; Price and Drevets, 2012). For example, MRI studies on depressed patients indicated the presence of reduced hippocampal volume (Campbell *et al.*, 2004; Videbech and Ravnkilde, 2004). Consistently, a recent fMRI study identified alterations of the ventral hippocampus connectivity as a common hallmark in clinically depressed patients (Drysdale *et al.*, 2017). In mouse models, it has been shown that Dentate Gyrus Granule Cells are the critical target of conventional anti-depressant drugs (Samuels *et al.*, 2015), and optogenetic excitation of the Dentate Gyrus in the ventral hippocampus remarkably reduced anxiety-like behaviors (Kheirbek *et al.*, 2013). Moreover, a recent study on a mouse model of chronic anxiety showed that stress-related activity patterns tend to converge on the ventral hippocampus (Hultman *et al.*, 2018). In addition, alteration of synaptic plasticity and neuronal atrophy in the hippocampus were often reported in the literature, both in mouse models of depression as well as in patients (for review Christoffel *et al.*, 2011). To date, molecular mechanisms of depression and stress-related disorders are still obscure. In the last decade, this specific field of research has expressed a growing interest for microglia (for review Yirmiya *et al.*, 2015), however, despite the mounting research effort, it is still unclear how microglia respond to chronic-stress and whether microglial cells may represent a potential therapeutic target.

I carried out all my studies on a mouse model of Repeated Forced Swim Stress (RFSS). This model was previously used in my lab (Serchov *et al.*, 2015; Hellwig *et al.*, 2016; Normann *et al.*, 2017) and we already knew that this chronic-stress paradigm evokes microglia reactivity in the hippocampus of wild-type mice (Hellwig *et al.*, 2016). The RFSS paradigm was designed as follows: 1) on day -3, ten wild-type mice underwent a first TST session; 2) from day 1 to day 5, the mice were exposed to the RFSS protocol consisting of 10 minutes FST/day (otherwise termed as "induction phase"); 3) on day 6, the mice underwent a second TST session (**Figure 1A**). For simplicity, the 1st TST session will be termed as "Basal" as it measures the basal immobility time under homeostatic condition, while the 2nd TST session will be termed as "post-RFSS" because it measures the immobility time 1 day after the RFSS paradigm (see material and method for more details).

Results

All the mice here tested were sacrificed on day 6 (within 1 hour from the 2nd TST session) and brains were immediately collected for immuno-histochemical and biomolecular analysis. In agreement with the previous literature (Sun *et al.*, 2011; Serchov *et al.*, 2015; Mul *et al.*, 2016), all the tested mice exhibited a progressive increase of immobility time (IT) during the 5 days of induction phase, typically reaching a plateau condition between day 4 and day 5. (**Figure 1B**). As a further readout, swum distance decline was also measured (not shown). Consistently, mice also showed a significant IT increase at the TST (Basal versus post-RFSS) (**Figure 1C**). A significant IT increase at the TST is commonly interpreted as evidence of stress-induced depressive-like behavior (Cryan *et al.*, 2005).

Importantly, mice undergoing RFSS exhibited significantly increased levels of corticosterone, a steroid hormone secreted under psychological stress. Blood withdrawal could not be performed in my lab as not part of the available animal license. The concentration of corticosterone in the serum (ng/ml) was assessed in ELISA by Laura Kracht (UMCG Groningen, The Netherlands). Blood samples were collected from 8 wild-type mice at two timepoints: 1 week before the beginning of the RFSS paradigm (Basal) and on the 5th day of the RFSS session (post-RFSS) (**Figure 1D**). These results indicate that the RFSS paradigm causes chronic-stress in mice.

I first aimed to characterize the morphology of hippocampal microglia in RFSS mice. Unstressed littermates served as controls (CTRL). To do so, I performed an immunostaining for Iba1 (microglia marker) on brain cryo-sections and I analyzed microglia in confocal imaging. In my study I focused on the three main hippocampal regions, namely DG, CA3 and CA1. Moreover, I also analyzed three different control brain regions (Striatum Caudato-Putamen nucleus, Somatosensory Cortex and Cerebellum) which are not involved in the physiopathology of chronic-stress (hereafter CTRL regions) (**Figure 1E**). In agreement with the previous literature (Tynan *et al.*, 2010; Kreisel *et al.*, 2014; Hellwig *et al.*, 2016; Milior *et al.*, 2016), confocal imaging on Iba1-stained brain slices revealed the presence of reactive microglia in the hippocampus of RFSS mice. More precisely, microglia appeared hypertrophic and exhibited more complex ramifications (**Figure 1F**), similarly to the hyper-ramified microglia described by both our group (Hellwig *et al.*, 2016) and others (Hinwood *et al.*, 2013; Llorens-Martín *et al.*, 2016). To quantify Iba1 immunoreactivity I analyzed the number of Iba1+ voxels in each z-stack (z = 20µm; x/y = 180µm). The analysis was performed with Imaris software after applying an auto threshold as of default setting. In RFSS mice, the Iba1 immunoreactivity was significantly increased in all the investigated hippocampal regions, whereas no difference was observed in selected the CTRL regions (**Figure 1G**).

I then counted the number of microglial cells bodies in each section in a semi-automated manner. The analysis was performed with Imaris software via the object detection tool. The measurement was set to detect Iba1+ objects with a diameter of at least 10µm (function "allow detection of ellipsoid" activated). To my experience, this setting can generate an accurate quantification of the microglial cell bodies within the imaged volume. In case the algorithm failed to detect one cell, or the same cell was detected twice, the automatic detection was manually corrected. Knowing the area of the imaged field (450µm^2), I could estimate the density of microglial cells per mm^2. Doing this calculation, I could not observe any significant change in the microglial cells number between RFSS and control mice (**Figure 5H**). Next, I wanted to analyze microglial morphology in 3D cell reconstruction (**Figure 5I**).

Results

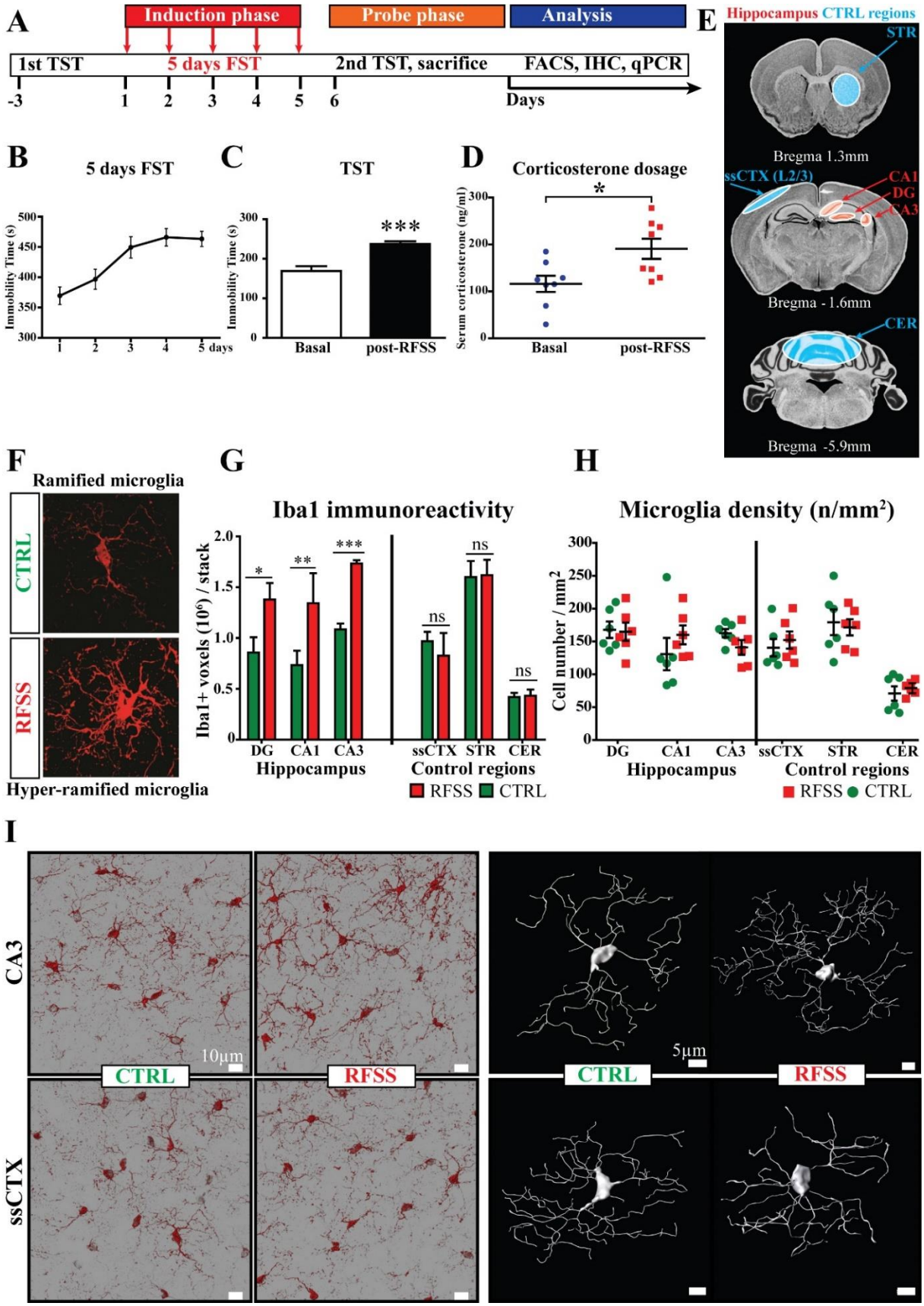

Figure 1 legend on the next page

Figure 1: Presence of microglial reactivity in the hippocampus of RFSS mice. (A) Experimental plan. **(B)** Induction phase, progressive increase of the immobility time along 5 days of RFSS (N=10 mice). **(C)** Probe phase, increased immobility at the TST after 5 days of RFSS (N=10 mice, paired T-test, ***p<0.001). **(D)** Corticosterone concentration (ng/ml) assessed by ELISA on peripheral blood serum sampled 1 week before and after 5 days of RFSS (N=8 mice, paired T-test, *p<0.05) **(E)** Anatomical localization of the investigated brain regions, namely Hippocampus (DG, CA1 and CA3) and CTRL brain regions (STR = Striatum Caudate Putamen; ssCTX = Somatosensory Cortex layer II/III; CER = Cerebellum) **(F)** Representative confocal image of typical normally ramified and hyper-ramified microglia (cropped from a 63X image). **(G)** Iba1 immunoreactivity quantified as number of $Iba1^+$ voxels per stack (N=6 z-stacks from 3 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test, *p<0.05, **p<0.01, ***p<0.001). **(H)** Estimation of the microglial cells number per mm^2 (N=6 z-stacks from 3 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test). **(I)** Representative confocal image of Iba1-stained microglia and 3D cell reconstruction showing stress-induced hyper-ramification at the CA3, but not in the ssCTX (63X objective). **Data in Figure 1D were provided by Laura Kracht (UMCG Groningen, The Netherlands)**

2. Microglia exhibit a hyper-ramified morphology in the hippocampus of RFSS mice

3D cell reconstruction and morphometric analysis of microglia were performed using the Imaris filament reconstruction tool, as previously published by our group (Hellwig *et al.*, 2016). I considered eligible for the analysis only the microglial cells with both cell body and processes clearly visible within the z-stack. The filament reconstruction algorithm was set to detect both the processes origin (cell body, diameter ≥ 10μm) and the individual processes (diameter ≥ 0.5μm) and the default auto threshold was applied. In case of underestimation or overestimation of the processes detection, the error was manually corrected. After a 3D cell reconstruction was successfully accomplished, the following parameters were scored:

- Processes area: sum of the area covered by each process, expressed in μm^2.
- Processes length: sum of the length of each process, expressed in μm.
- Number of branching points: sum of the points at which one bigger process bifurcates generating two smaller processes, expressed as absolute number.
- Number of terminal points: sum of the end-point of each process, expressed as absolute number.
- Number of Sholl intersections: After having selected a cell of interest, the software virtually creates a series of concentric spheres, the diameter of which progressively increases by 1μm at each next sphere. The cell body is considered as the beginning point (diameter = 0μm). Typically, one microglial cell can be fully included within a sphere of 40-50μm of diameter. The software calculates automatically the number of intersections between all the processes and each sphere. On the X-axis I plotted the diameter of the sphere (ergo the distance from the cell body), while on the Y-axis I plotted the corresponding number of intersections. In short, the Sholl analysis provides a measure about the complexity of microglial ramification.

Similarly to our previous work (Hellwig *et al.*, 2016), the Sholl analysis showed a significant increase of microglia ramification in the hippocampus of RFSS mice (**Figure 2A**). By contrast, I could not observe any difference within the chosen CTRL regions (**Figure 2B**). Moreover, a significant increase of processes area, processes length, number of branch points and number of terminal points was detected in hippocampal microglia, but not in microglia from CTRL regions (**Figure 2C**). To summarize this part, our model of RFSS exhibited a form of "*microglia activation*" (at least in the hippocampus), characterized by increased processes ramification. According to the previous literature (Tynan *et al.*, 2010; Hinwood *et al.*, 2013; Walker *et al.*, 2014; Hellwig *et al.*, 2016), I will refer to this morphological phenotype as "*hyper-ramified microglia*".

3. No evidences of monocytes infiltration or astrocytes activation in RFSS mice

To test whether RFSS increased monocytes recruitment to the brain I analyzed the $CD11b^{+}CD45^{hi}$ population (bona-fide extra-parenchymal macrophages and blood monocytes) by flow cytometry in both RFSS and control mice (gating strategy in **Figure 3A**). At the present stage, I cannot perform a fate-mapping experiment, which would certainly be more informative. However, I presume that the conventional CD11b/CD45 staining may serve well as a gross preliminary assessment. In this experiment I analyzed separately hippocampus and cortex from both RFSS and control mice **(Figure 3B)**. Neither the $CD11b^{+}CD45^{hi}$ population (for simplicity $CD45^{hi}$ MPs), nor the $CD11b^{+}CD45^{lo}$ population (microglia) were significantly changed between the two groups (**Figure 3C**). Surprisingly, a higher % $CD45^{hi}$ MPs was found in the hippocampus of both cohorts, as compared to the cortex. I may hypothesize that during the dissection of the hippocampi, part of the Choroid Plexus was unintentionally resected and contaminated the samples.

To test whether chronic-stress affected astrocytes I performed an immunostaining for the GFAP (astrocyte marker) on brain cryo-sections and I analyzed astrocytes in confocal imaging. Quantification of astrocyte number and GFAP immunoreactivity was carried out with the same method used for microglia. No obvious changes in astrocytes density (n/mm^2) or GFAP immunoreactivity (GFAP+ voxels per z-stack) was observed, at least in the hippocampus (**Figure 3D**). Moreover, immunostaining for the cell-cycle marker Ki67 did not reveal presence of proliferating cells in the hippocampus, neither in control nor in RFSS mice (not shown). Very little is known about astrocyte's reaction under chronic-stress, however this topic (although intriguing) is beyond the primary scope of this project.

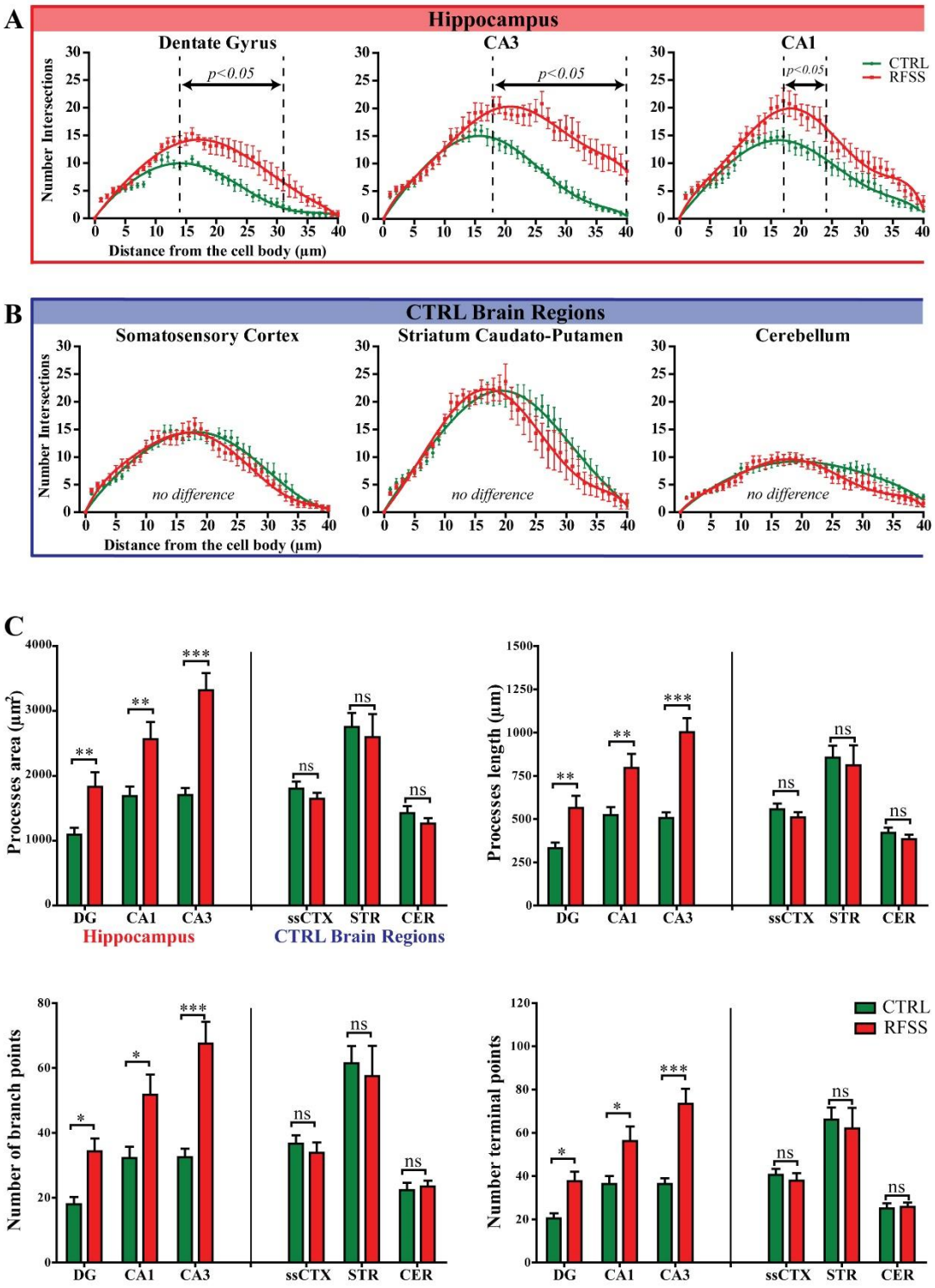

Figure 2: Microglia exhibit a hyper-ramified morphology in the hippocampus of RFSS mice. (A) Sholl analysis of microglia from DG, CA3 and CA1 (N=12 cell from 3 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test, the dashed lines define the region with a statistical significance). **(B)** Sholl analysis of microglia from CTRL brain regions, namely Somatosensory Cortex, Striatum Caudate Putamen nucleus and Cerebellum (N=12 cell from 3 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test, no significant difference). **(C)** Morphological analysis on 3D reconstructed microglia; assessed parameters: processes area, processes length, number of branch points and number of end points (N=12 cell from 3 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test, *p<0.05, **p<0.01, ***p<0.001).

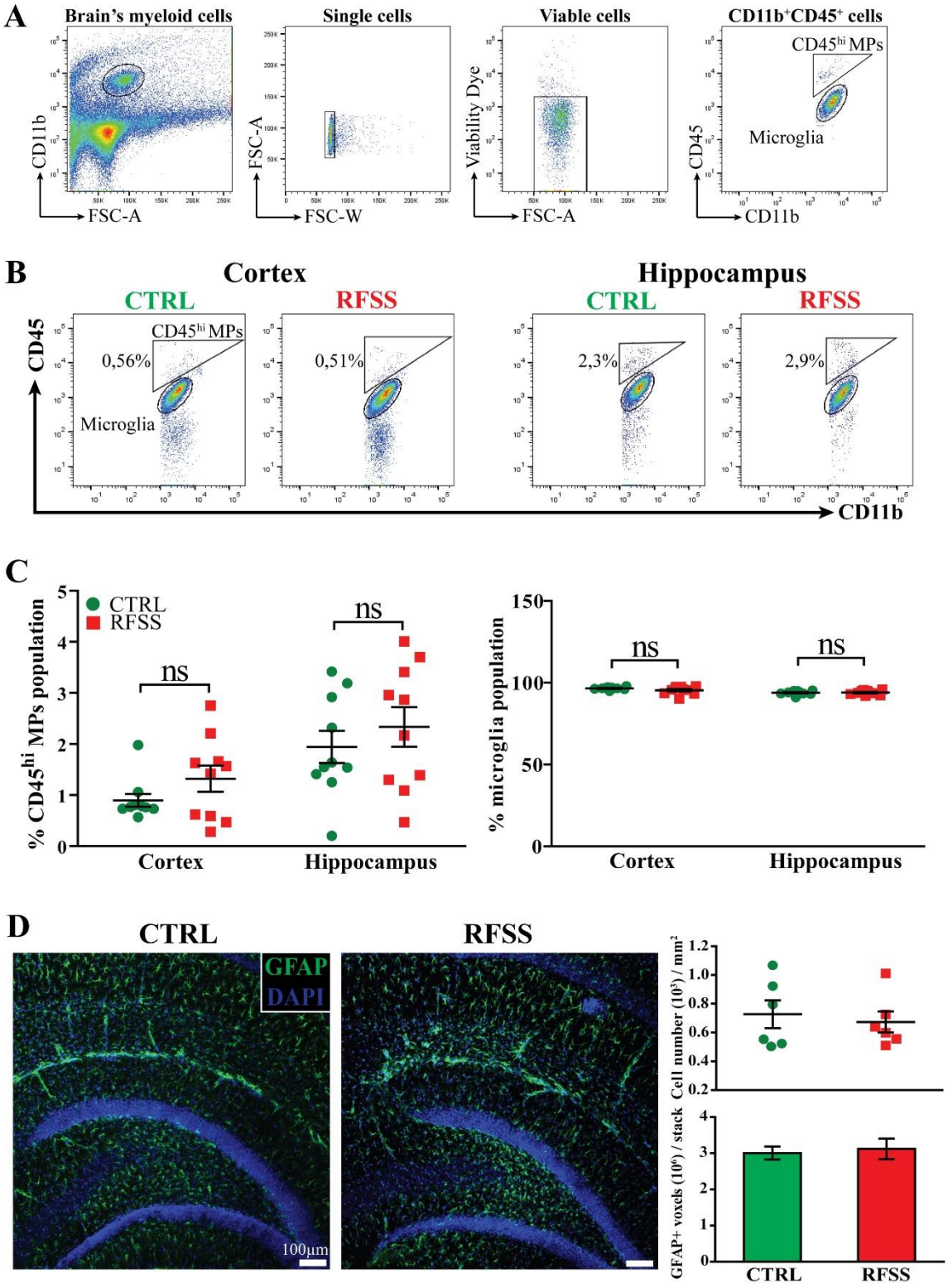

Figure 3: No evidences of monocytes infiltration or astrocytes activation in RFSS mice (A) FACS gating strategy of brain CD11b^{+}CD45^{+} cells. **(B)** Representative FACS plot of microglia and CD45^{hi} macrophages (CD11b^{+}CD45^{lo} and CD11b^{+}CD45^{hi} respectively) in both hippocampus and cortex. **(C)** Quantification of the % of cells (out of all single/viable brain myeloid cells) for both microglia and CD45^{hi} population (N=10 mice/group). **(D)** 10X confocal image of GFAP immunostaining in the hippocampus of control and RFSS mice. Total number of GFAP^{+} voxels per stack and number of GFAP^{+} astrocytes per mm^{2} were counted (N=6 z-stacks from 3 mice/group, unpaired T-test).

4. Microglia deficiency reduces RFSS-induced behavioral changes, but does not affect learning and memory

To determine whether microglia are important for stress-induced behavioral alterations, I exposed IL34 knock-out mice ($IL34^{LacZ/LacZ}$) to our RFSS protocol, hence I assessed depressive-like behavior as previously described in Figure 1A. This transgenic mouse line is known to exhibit an important microglia deficiency (Greter *et al.*, 2012; Wang *et al.*, 2012). Indeed, counting for the Iba1+ cells in different brain regions (Dentate Gyrus = DG; Somatosensory Cortex = ssCTX; Medial Prefrontal Cortex = mPFC; Cerebellum = CER and Spinal Cord = SC) I could confirm the partial lack of microglia under IL34-deficient condition (microglial cells density n/mm^2 displayed in **Figure 4**). More precisely, I measured a ~32% reduction of the microglia population of in the DG, ~65% in the ssCTX and ~74% in the mPFC. According to the previous literature (Greter *et al.*, 2012; Wang *et al.*, 2012) microglia population was unaffected in CER and SC (**Figure 5A**). In our RFSS paradigm, IL34 knock-out mice performed similarly to wild-type littermates during the induction phase (12 mice per group). Indeed, both genotypes exhibited an almost identical IT increase (**Figure 5B**) and swum distance decline (**Figure 5C**) along the 5 days of RFSS. Nevertheless, the probe phase showed a significant IT reduction at the TST in IL34 knock-out mice compared to wild-type littermates (**Figure 5D**). These data suggest that lack of microglia is protective under chronic-stress, therefore presence of microglia is required to fully evoke stress-induced depressive-like behavior. Interestingly, microglia deficiency did not seem to affect learning and memory skills. Both IL34 knock-out mice and wild-type littermates (12 mice per group) were tested at the Morris Water Maze, a commonly used test to assess hippocampal-dependent spatial memory in rodents (data in Figure 5 E-H were generated by Dr. David Wozniak, Washington University School of Medicine, Saint Louis, USA). Both genotypes exhibited a similar latency time to locate the submerged platform during 5 days of training, indicating comparable learning ability (**Figure 5E**). 24 hours after the last training day (day 6) mice underwent a probe phase in absence of submerged platform. Both genotypes showed identical swimming time in the target quadrant (expressed as % of the total swimming time) (**Figure 5F**) and number of entries into the platform area (**Figure 5G**). Importantly, IL34 knock-out did not affect the swimming speed of the mice (**Figure 5H)**. These data indicate that a partial microglia deficiency (estimated around 30% less in the hippocampus) does not affect learning and memory ability in mouse. At present, we cannot claim that microglia certainly play no role at all for the mouse's cognition. On the other hand, these data demonstrate that the observed stress-resilient phenotype is not due to the impairment of learning skills.

Results

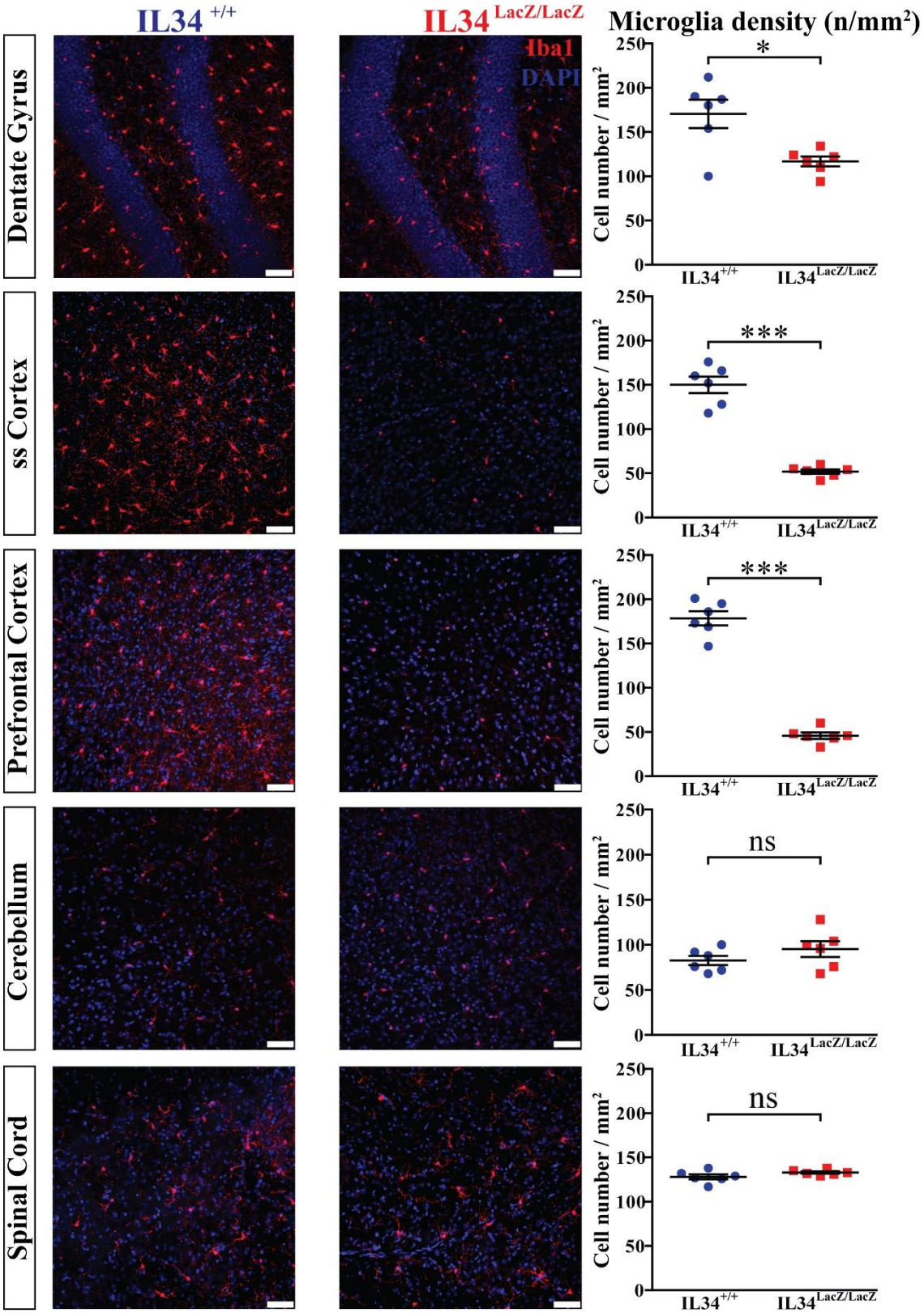

Figure 4: Microglial cell density is reduced defined CNS regions of IL34 knock-out mice. Confocal imaging reveals a significant reduction of microglia number in discrete brain regions (Dentate Gyrus, Somatosensory Cortex and Medial Prefrontal Cortex) under IL34 knock-out condition (25X objective). Microglia population was apparently unaffected in Cerebellum and Spinal Cord (N=6 z-stacks from 3 mice/group, unpaired T-test, *p<0.05, ***p<0.001). Scale bar = 100µm.

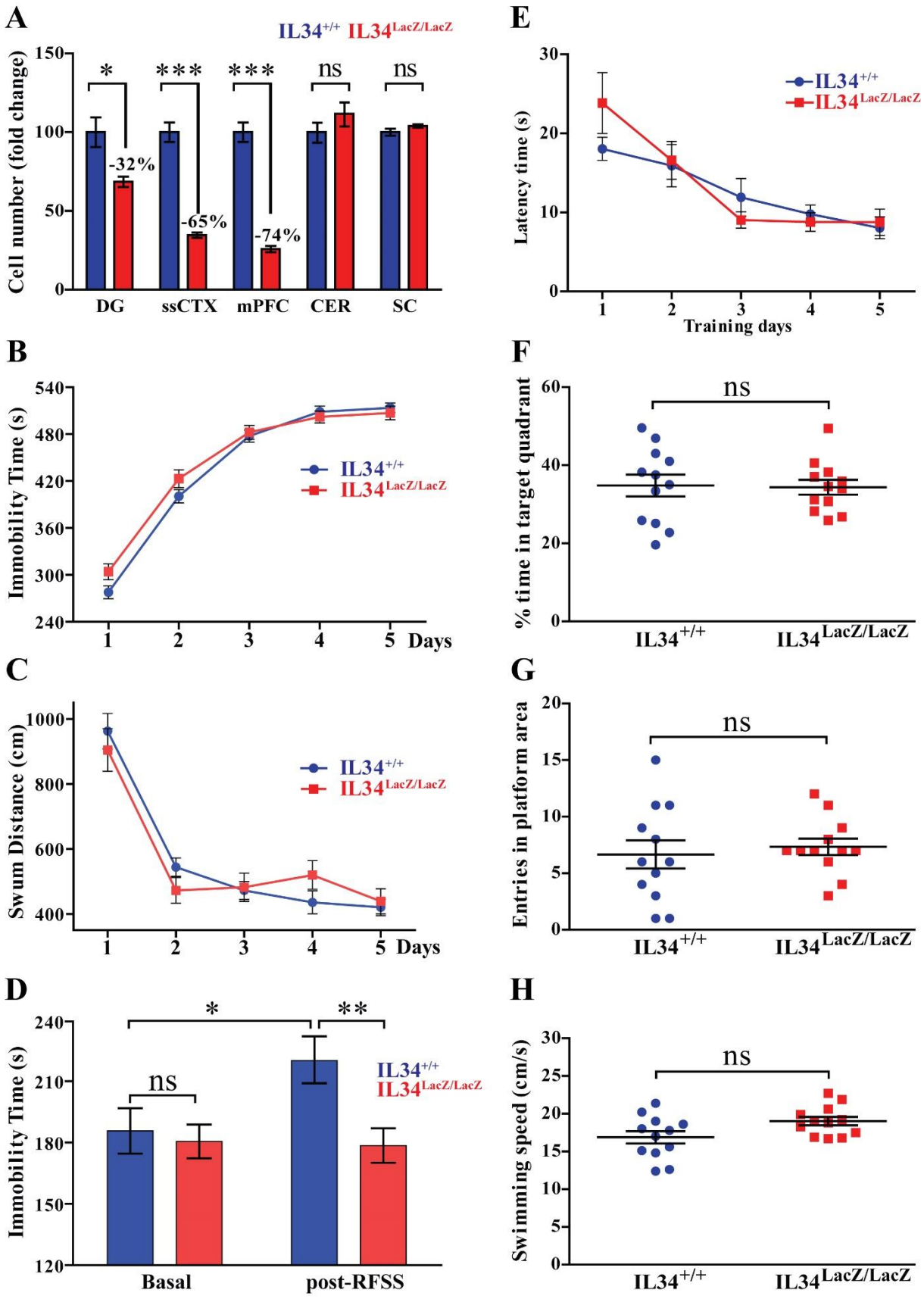

Figure 5 legend on the next page

Figure 5: Microglia deficiency reduces RFSS-induced behavioral changes, but does not affect learning and memory
(A) IL34-deficient mice displayed reduced microglia population in DG (Dentate Gyrus), ssCTX (Somatosensory Cortex), mPCF (medial Prefrontal Cortex), but not in CER (Cerebellum) and SC (Spinal Cord) (N=6 z-stacks from 3 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test, *p<0.05, ***p<0.001). **(B)** Immobility time and **(C)** swum distance along 5 days of RFSS for both IL34 knock-out mice and wild-type littermates (12 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test). **(D)** Immobility at the TST for both IL34 knock-out and wild-type littermates (12 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test, *p<0.05, **p<0.01). **(E)** Morris Water Maze test (learning phase), latency time (s) to reach the platform position (N=12 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test). **(F)** Morris Water Maze test (probe phase), percentage of swimming time in the target quadrant (N=12 mice/group, unpaired T-test). **(G)** Number of entries in the platform area (probe phase). **(H)** Average swimming speed (cm/s). **Data in Figure 5E - H were generated by David Wozniak (Washington University School of Medicine, Saint Louis, USA).**

5. Microglia-derived TNFα is irrelevant for both RFSS-induced behavioral changes and learning and memory

I next wanted to assess whether microglia may promote RFSS-induced behavioral changes by secretion of pro-inflammatory factors, such as TNFα. In both human and mouse, increased levels of TNFα have been repeatedly associated to depression, especially under chronic low-grade peripheral inflammation (Simen *et al.*, 2006; Dowlati *et al.*, 2010). Consistently, TNFα blockers were reported to improve depressive symptoms in both rodent models and patients (Bayramgürler *et al.*, 2013; Krügel *et al.*, 2013; Kappelmann *et al.*, 2016). Besides its pro-inflammatory function, brain TNFα exerts pleiotropic effects on synapses. Indeed, glia-derived TNFα was shown to be important for regulating the strength of excitatory neurotransmission (Beattie *et al.*, 2002; Stellwagen *et al.*, 2005; Stellwagen and Malenka, 2006; Kaneko *et al.*, 2008; Riazi *et al.*, 2008; Pribiag and Stellwagen, 2013; Lewitus *et al.*, 2016), while monocyte-derived TNFα was also shown to modulate synaptic remodeling in a model of acute peripheral inflammation (Garré *et al.*, 2017). Very recently, TNFα deletion in microglia markedly reduced the pathological grooming in a genetic moue model of obsessive-compulsive disorder (Krabbe *et al.*, 2017). Our lab also showed that microglia-derived TNFα protects neurons from NMDA-mediated excitotoxicity in hippocampal organotypic cultures (Masuch *et al.*, 2016). Since microglia are the primary source of TNFα in the brain (Zhang *et al.*, 2014), I wanted to better elucidate the role of microglia-derived TNFα during chronic-stress in our RFSS paradigm.

To specifically ablate TNFα in microglia the double-transgenic line $TNF\alpha^{flox/flox}$: $Cx3cr1^{Cre/+}$ was generated. It was previously shown that $Cx3cr1^{Cre/+}$ mice lacked TNFα expression in $CX3CR1^{+}$ cells, including blood monocytes and microglia (Wolf *et al.*, 2017). Cre-mediated recombination of the floxed TNFα locus was confirmed via genomic PCR in FACS sorted microglia (not shown, experiment performed by Yochai Wolf, Weizmann Institute, Rehovot, Israel).

Results

In *ex vivo* culture, microglia from Cre+ mice failed to increase TNFα expression after 4 hours incubation with 10µg/ml LPS (**Figure 6A**, data provided by Yochai Wolf, Weizmann Institute, Rehovot, Israel). Similarly, *in vivo* ip injection of 0,5mg/kg LPS increased TNFα expression in acutely isolated microglia from Cre-, but not from Cre+ mice (**Figure 6B**). These data confirm that TNFα was successfully ablated in microglia expressing Cre-recombinase. Of note, these mice did not exhibit any visible phenotype. Moreover, TNFα-deficient microglia did not show altered cell density, morphology or capacity to upregulate cytokines expression (except TNFα) in response to ip. injected LPS (not shown). Next, I tested the response of the $TNF\alpha^{flox/flox}$: $Cx3cr1^{Cre/+}$ mice to chronic-stress, in comparison to $TNF\alpha^{flox/flox}$: $Cx3cr1^{+/+}$ littermates (12-14 mice per group). It is important to note that TNFα deletion was present in all *Cx3cr1*-expressing cells, namely microglia, but also brain extra-parenchymal macrophages and circulating monocytes. I however decided to use this transgenic line for a preliminary assessment. Further experiments with a tamoxifen-inducible $Cx3cr1^{CerER/+}$ line (with improved specificity for microglia) would have been justified in case of convincing results. As first instance, I wanted to achieve the highest recombination yield. For this reason, I decided to start my study with the non-inducible $Cx3cr1^{Cre/+}$ moue line, which does not require tamoxifen treatment.

In out RFSS paradigm, Cre+ mice (with TNFα-deficient microglia) and Cre- mice (with TNFα-proficient microglia) exhibited similar IT increase (**Figure 6C**) and swum distance decline (**Figure 6D**) during the 5 days of RFSS. Furthermore, no difference at the TST was observed (**Figure 6E**). These data suggest that TNFα produced by microglia is irrelevant for stress-induced behavioral alterations (at least in this model).

Given the importance of glia-derived TNFα for regulation of excitatory synaptic strength (Stellwagen and Malenka, 2006) I assessed learning and memory ability of both Cre+ and Cre- mice at the Morris Water Maze task (30 mice per group). Both genotypes exhibited identical latency time to reach the submerged platform during 7 training days (**Figure 6F**). At the probe phase (24 hours after the last training day) mice from both genotypes spent similar time (expressed in %) in the target quadrant (**Figure 6G**). Consistently, no difference in number of entries in the platform area was observed (**Figure 6H**). Not surprisingly, TNFα deletion did not alter the swimming speed (**Figure 6I**), indicating that both genotypes have comparable swimming ability. In summary, Cre^+ and Cre^- mice did not exhibit any difference in learning and memory, suggesting that microglia-derived TNFα is probably redundant for cognitive skills. Nevertheless, TNFα from microglia may be important under pathology.

Results

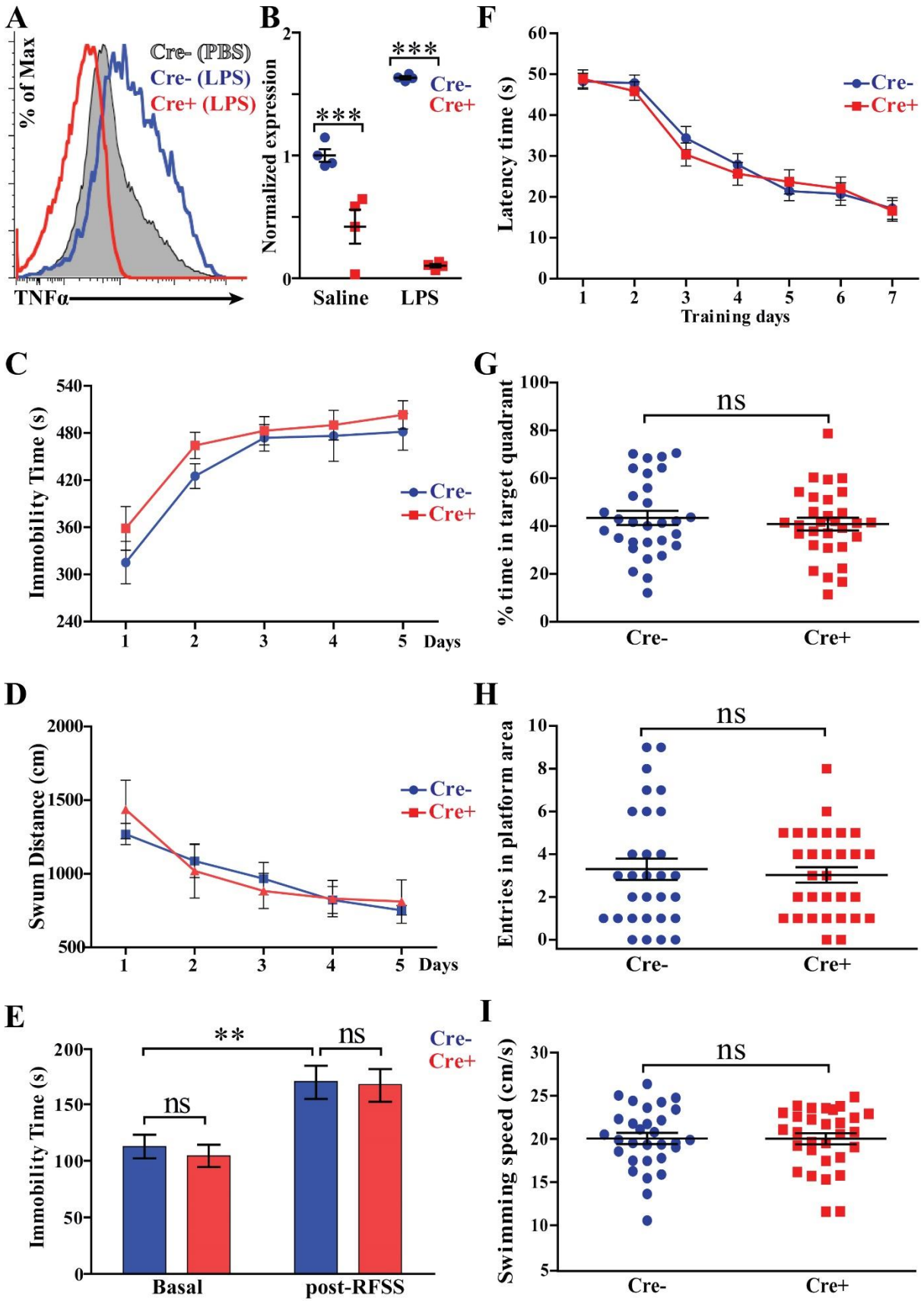

Figure 6 legend on the next page

Figure 6: Microglia-derived TNFα is irrelevant for both RFSS-induced behavioral changes and learning and memory. (A) Flow cytometry confirms absence of TNFα protein in Cre^+ microglia incubated ex-vivo with LPS for 4 hours. **(B)** qPCR shows lack TNFα transcript in Cre^+ microglia, acutely isolated 24 hours after Saline or LPS ip. injection (N=4 triplicate samples/group, Two-Ways ANOVA and Bonferroni post-hoc test, ***p<0.001). **(C)** Immobility time and **(D)** swum distance along 5 days of RFSS for both Cre^+ and Cre^- littermates (12-14 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test). **(E)** Immobility at the TST for both Cre^+ and Cre^- littermates (N=12-14 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test). (F**)** Morris Water Maze test (learning phase), latency time (s) to reach the platform position (N=30 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test). **(G)** Morris Water Maze test (probe phase), percentage of swimming time in the target quadrant (N=30 mice/group, unpaired T-test). **(H)** Number of entries in the platform area (probe phase). **(I)** Average swimming speed (cm/s). **Data in Figure 6A were provided by Yochai Wolf (Weizmann Institute, Rehovot, Israel).**

6. Upregulation of microglial pro-inflammatory genes in RFSS mice

The literature suggests that under chronic-stress microglia release a plethora of pro-inflammatory mediators, especially cytokines and chemokines, which may affect brain's homeostasis and function (Delpech *et al.*, 2015; Yirmiya *et al.*, 2015). I then isolated microglia from both cortex and hippocampus on percoll gradient and assessed expression of the main pro-inflammatory cytokines in qPCR (cut-off at fold-change ≥ 2; *p<0.05). At the mRNA level, RFSS mice showed upregulation of both IL6 and IL1β in hippocampal microglia, but not TNFα. This may explain why I found no behavioral difference between wild-type (Cre-) and conditional TNFα knock-out mice (Cre-) (see Figure 6 A-E). By contrast, none of these cytokines was upregulated in cortical microglia. The sole exception was the chemokine CCL2, which was upregulated in both hippocampal and cortical microglia from RFSS mice (**Figure 7A**). Of note, CD11b was upregulated in hippocampal, but not in cortical microglia, while expression of Iba1 and CX3CR1 was unchanged in both regions (**Figure 7B**). Stress-induced CD11b overexpression was also assessed by confocal imaging (**Figure 7C**). Of note, the CD11b staining mostly labels microglial processes rather than the cell body (**Figure 7D**). Using Imaris software I measured CD11b+ voxels (**Figure 7E**) and % of CD11b+ volume per stack (**Figure 7F**). This measurement was carried out applying the same method described in Figure 1G (z = 20µm; x/y = 180µm). According to these data, RFSS mice exhibit a significant increase in CD11b immunoreactivity in DG and CA1 (but not in CA3). No increase in CD11b immunoreactivity was observed in the ssCTX. In summary, RFSS-induced phenotypic changes of microglia are region specific, thus discrete brain regions likely host heterogeneous microglial phenotypes. Hyper-ramified microglia are probably characterized by increased cytokines and chemokines release. Interestingly, RFSS did not affect the levels of Iba1 mRNA, therefore the increased Iba1 immunoreactivity may stem from a morphological change of microglia (i.e. hyper-ramification) rather than Iba1 upregulation.

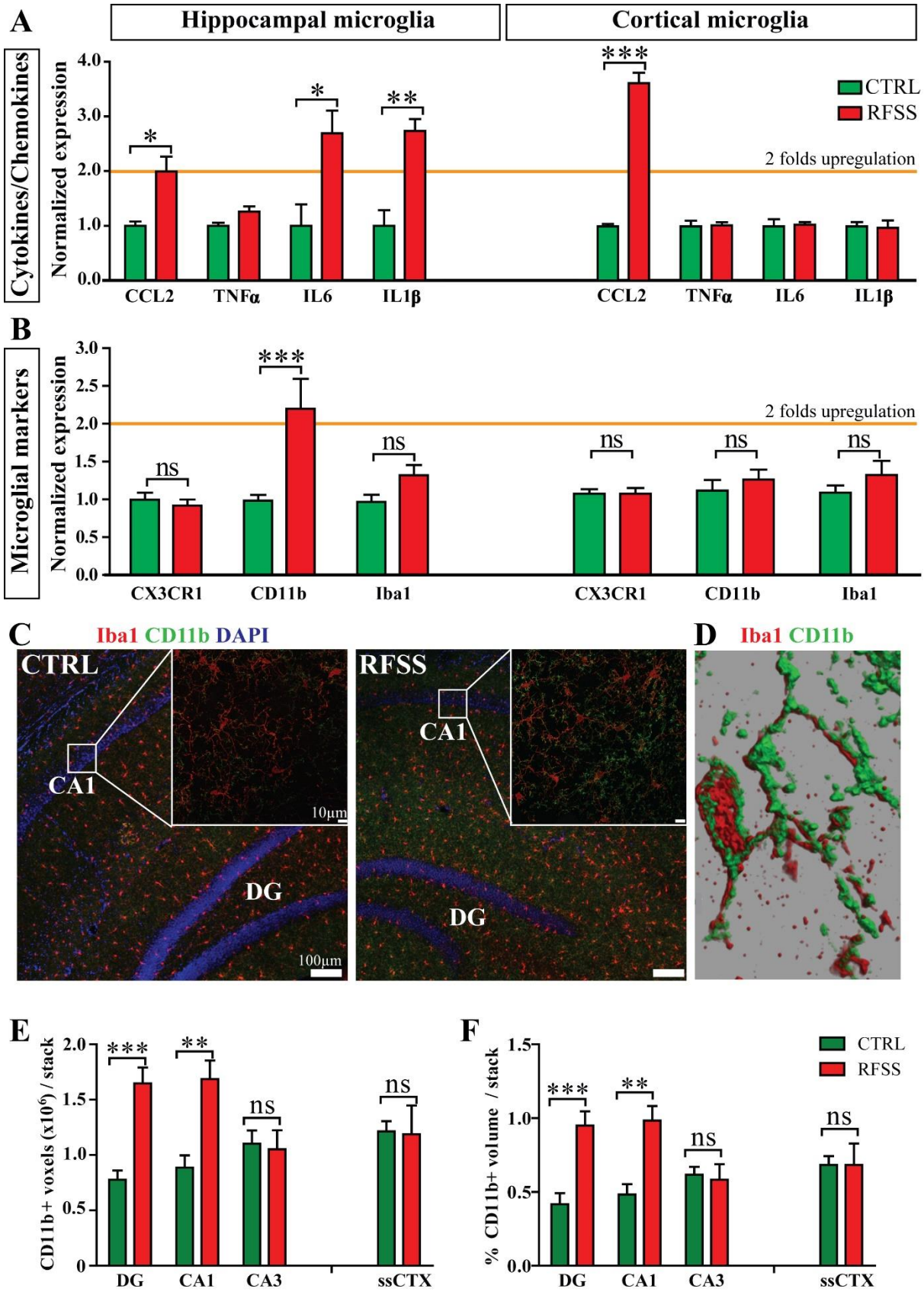

Figure 7 legend on the next page

Figure 7: Upregulation of microglial pro-inflammatory genes in RFSS mice (A) qPCR for canonical pro-inflammatory cytokines/chemokines in percoll enriched microglia from either Hippocampus or Cortex (N=4 triplicate samples/group, Two-Ways ANOVA and Bonferroni post-hoc test, *p<0.05, **p<0.001, ***p<0.001). **(B)** qPCR for canonical microglial markers in percoll enriched microglia from either Hippocampus or Cortex (N=6 triplicate samples/group, Two-Ways ANOVA and Bonferroni post-hoc test, ***p<0.001). **(C)** CD11b immunostaining in the hippocampus (10X objective). The insets show a higher magnification image in the CA1 (63X objective) **(D)** CD11b staining mostly colocalize with microglial processes. (**E**) Quantification of the $CD11b^+$ voxels per stack. (**F**) Quantification of the % of $CD11b^+$ volume per stack (N=6 z-stacks from 3 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test, **p<0.01, ***p<0.001).

7. CA1 microglia do not show increased interaction with brain blood vessels in RFSS mice

It was previously reported that CD11b in microglia is required to sense vascular leakage during neuroinflammatory diseases, thus promoting chemoattraction of microglia towards the injured vessels (Davalos *et al.*, 2012). Moreover, one recent study provided evidences of increased BBB permeability in a model of chronic-stress (Menard *et al.*, 2017). I then analyzed in confocal imaging both Iba1+ microglia and CD34+ blood vessels in the CA1 of control (**Figure 8A)** and RFSS mice (**Figure 8B**). Using Imaris software I measured the Iba1/CD34 double-positive voxels (per stack) (**Figure 8C**) and the % of Iba1+ volume overlapping with the CD34+ vessels (per stack) (**Figure 8D**). My analysis did reveal any difference between control and RFSS mice. I would then exclude that hyper-ramified microglia be primarily associated with possible disruption of vessels integrity. Further studies are needed to better assess the presence of vascular pathology in this model of chronic-stress.

8. CA1 microglia exhibit increased phagosomes size in RFSS mice

Microglia are the professional phagocytes of the brain, therefore I asked whether hyper-ramified microglia could exhibit an increased phagocytic activity. The LAMP family member CD68 glycoprotein is highly enriched in the phagosomes and lysosomes of myeloid phagocytes, and therefore it is often used as marker of "*phagocytic microglia*" (Safaiyan *et al.*, 2016). I then analyzed in confocal imaging both Iba1+ microglia and CD68+ phagosomes in the CA1 of control (**Figure 9A)** and RFSS mice (**Figure 9B)**. Using Imaris software, I assessed number and size of CD68+ phagosomes per stack. My data show no difference in the overall number of microglial phagosomes between RFSS and control mice (**Figure 9C**). On the other hand, RFSS mice showed a significant increase of the CD68+ average volume (**Figure 9D**). I may then suggest that RFSS-induced hyper-ramified microglia in the hippocampus might be characterized by increased phagocytosis, consistently with the findings from a previous study (Llorens-Martín *et al.*, 2016). Additional studies are needed to confirm this hypothesis.

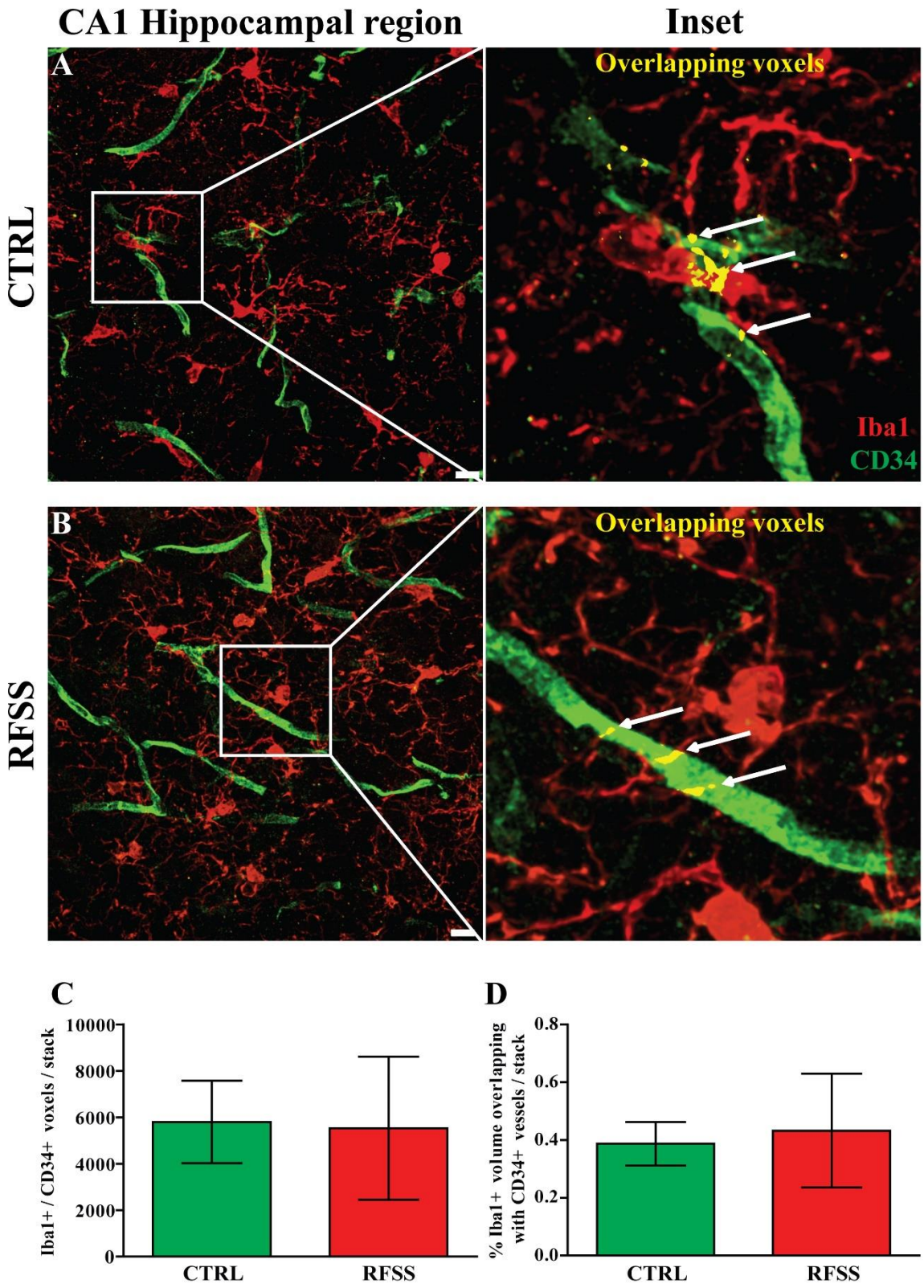

Figure 8: CA1 microglia do not show increased interaction with brain blood vessels in RFSS mice. Representative Iba1/CD34 immunostaining in the CA1 of **(A)** control and **(B)** RFSS mice. White arrows indicate the automatically detected overlapping voxels. **(C)** Iba1/CD34 double-positive voxels per stack. (**D**) % of the Iba1+ volume overlapping with the CD34+ voxels per stack (N=4 z-stacks from 2 mice/group, unpaired T-test). Scale bar = 10µm.

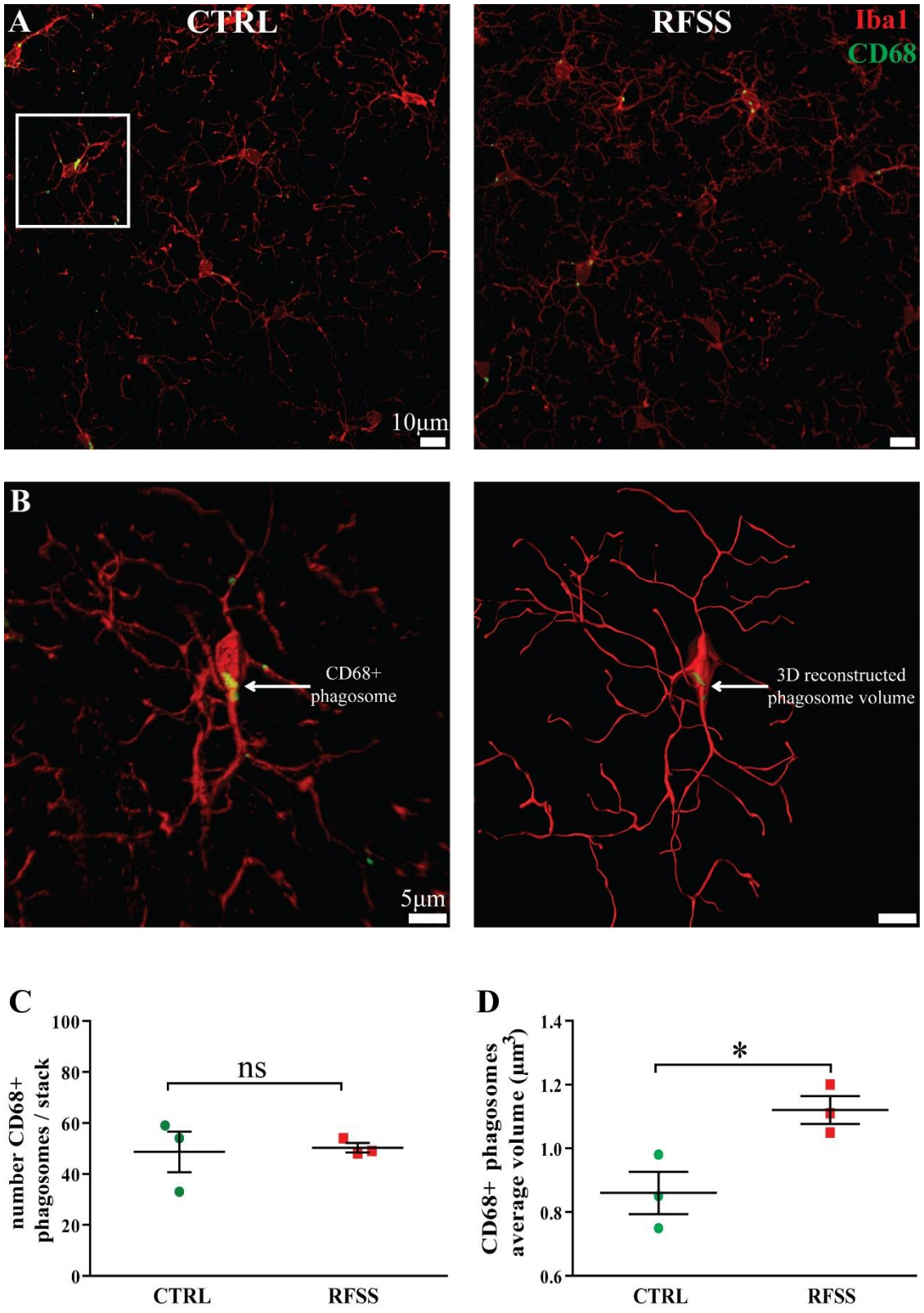

Figure 9: CA1 microglia exhibit increased phagosomes size in RFSS mice. (A) Representative Iba1/CD68 immunostaining in the CA1 of control and RFSS mice. **(B)** 3D reconstruction of a microglial cells (box in A) exhibiting a CD68+ phagosome within the cell body (white arrow). **(C)** Quantification of the number and (**D**) average volume of the CD68+ phagosomes per stack (N=3 z-stacks from 3 mice/group, unpaired T-test, *p<0.05). Scale bar = 5µm.

9. CA1 microglia exhibit reduced processes motility in RFSS mice

Microglia were repeatedly described as highly motile cells, continuously contacting neighboring synapses with their finely ramified processes (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005; Wake *et al.*, 2009). I then asked whether the RFSS paradigm, alongside the microglia hyper-ramification, may also affect processes motility in the hippocampus. To monitor this phenomenon, I performed *in vivo* two-photon imaging of CA1 microglia in *Cx3cr1*$^{GFP/+}$: *Thy1-YFP* mice, before and after RFSS. This transgenic mouse line allows to simultaneously visualize GFP+ microglia and YFP+ pyramidal neurons (Fuhrmann *et al.*, 2010). I personally performed these experiments in the lab of Dr. Martin Fuhrmann (DZNE, Bonn). Brain surgeries and implantation of the cranial windows were performed by Felix Nebeling three months before the experiment started (DZNE, Bonn). The hippocampal two-photon imaging was performed under the supervision of Felix Nebeling (DZNE, Bonn). I personally performed the behavioral testing, prepared the time-lapse images (using both Zeiss ZEN and ImageJ software) and analyzed the data (using Imaris software).

Initially, mice were tested at TST on day -3 (TST Basal). Homeostatic microglia motility was then assessed by two-photon imaging on day -2 (Imaging Basal). From day 1 till day 5 mice underwent the RFSS protocol. After the last FST session (day 5) microglia motility was again assessed in two-photon imaging (Imaging post-RFSS). On day 6, mice were again probed at TST (TST post-RFSS) (**Figure 10A**). Results of both FST and TST (**Figure 10B** and **C** respectively) showed that all the tested mice significantly increased immobility time after 5 days of RFSS, indicating that the used transgenic line was suceptible to the RFSS paradigm. Importantly, Ketamine is known to induce fast-acting antidepressant effects in both human patients (Berman *et al.*, 2000; Abdallah *et al.*, 2015) and mice (Zanos *et al.*, 2016; van Calker *et al.*, 2018), including this specific stress model (Serchov *et al.*, 2015). Recently, isoflurane was shown to reduce microglial motility in acute brain slices via inhibition of K^+-permeable channels in microglia (Madry *et al.*, 2018). To exclude any possible anesthesia-dependent bias in our analysis, I performed *in vivo* microglia imaging in awake (not anesthetized) mice, freely running on a rotating mat (**Figure 10D**). Using this two-photon setting, z-stack series were acquired through the CA1 hippocampal region, encompassing Stratum Oriens, Pyramidal Cell Layer and Stratum Radiatum (**Figure 10E**). Positions of the pyramidal neurons were used as a territory landmark to relocate the same region during Imaging Basal and Imaging post-RFSS (**Figure 10F**). Fine structures such as microglial processes and dendritic spines were clearly visible (**Figure 10G**).

CA1 microglia were imaged for an overall period of 40 minutes, acquiring 80µm-thick z-stacks every 5 minutes (yielding videos of 9 frames per mouse). Time-lapse z-stack series were mounted using ImageJ. The measurement of the microglial processes' motility was carried out using Imaris software. For this analysis, four microglial cells per mouse were randomly chosen throughout the imaged volume (20 cell in total per group). Microglial cells on the edges of the imaging field were excluded. Processes speed was determined in a semi-automated manner on 3D reconstructed cells. Processes length was measured using the filament reconstruction tool as described in Figure 2. Processes speed was then determined by measuring Δ(processes length)/5min at each timepoint. This analysis was performed separately for primary processes (those in direct contact with the cell body) and terminal processes (all those processes after the 1st branch point) and resulting speed values were averaged for each cell. Overall, I analyzed 436 primary and 1197 terminal processes (in average ~11 primary processes and ~30 terminal processes per cell), many of those were continuously extended and retracted across the imaging period (**Figure 10H**). To simplify the analysis, I did not distinguish between protracting and retracting processes speed, which was eventually reported as positive value (n)µm/min.

Before the RFSS paradigm (Imaging Basal), terminal processes exhibited a higher speed as compared to primary processes during the entire imaging period (**Figure 13I**). Interestingly, after the RFSS paradigm (Imaging post-RFSS) primary and terminal processes exhibited a very similar speed (**Figure 13J**). During the Imaging Basal, I estimated an average speed of 3,2±0,3µm/min for the primary processes and 5,7±0,6µm/min for the terminal processes and these values are coherent with those reported in the literature (Nimmerjahn *et al.*, 2005). These data show that, under homeostasis, terminal processes display 81,4% increased speed compared to the primary processes. During the Imaging post-RFSS, I measured an average speed of 2,7±0,3µm/min and 2,4±0,2µm/min for primary and terminal processes respectively. These data indicate that after the RFSS paradigm CA1 microglia exhibit a significantly reduced speed of the terminal processes (**Figure 13K**). In conclusion, RFSS led to a significant reduction of processes motility in CA1 microglia. More studies are needed to determine whether this phenomenon may cause substantial alterations in the homeostatic functions of hippocampal microglia.

Results

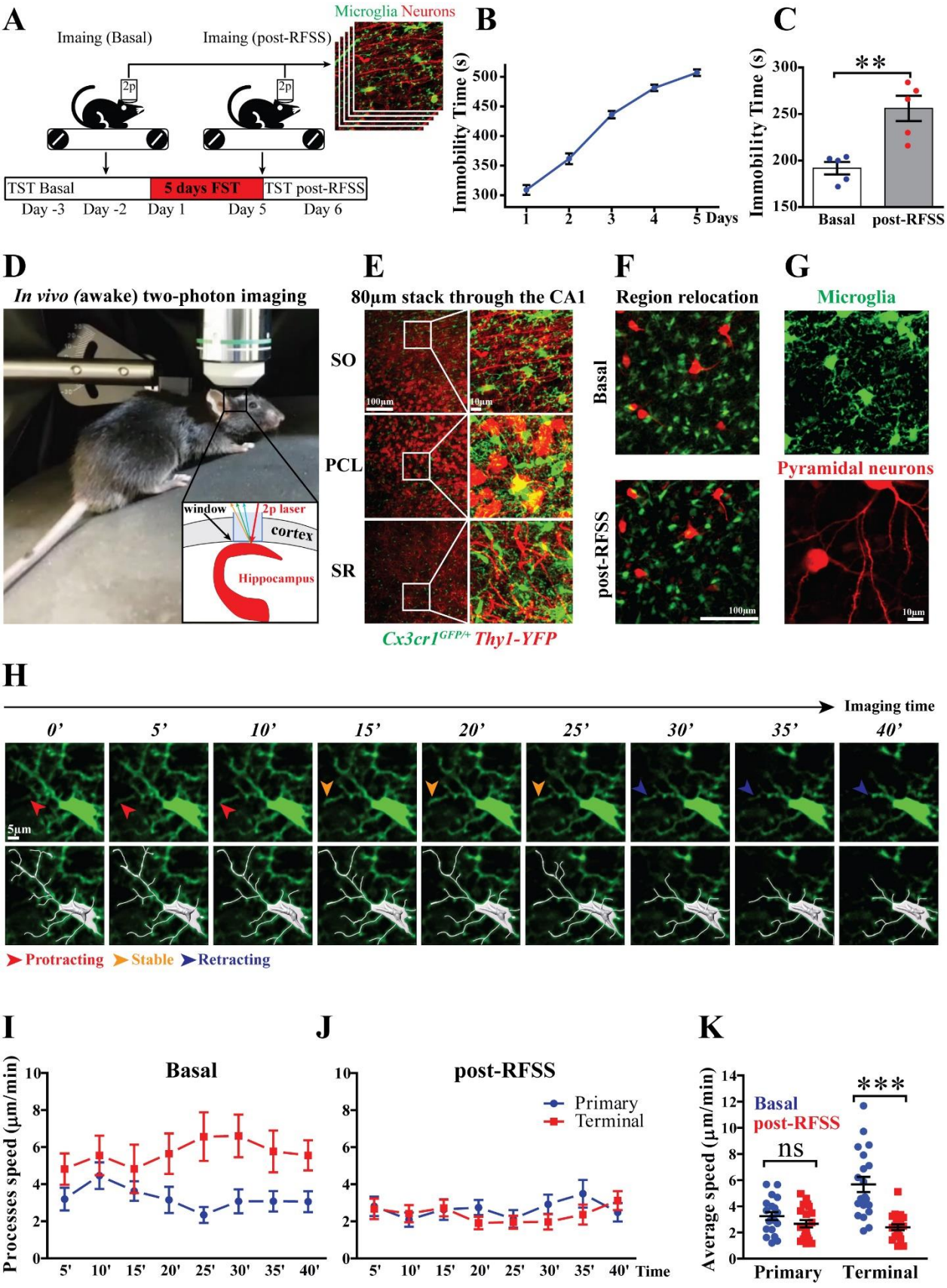

Figure 10 legend on the next page

Figure 10: CA1 microglia exhibit reduced processes motility in RFSS mice. (A) Experimental plan. **(B)** Progressive increase of immobility time during 5 days of RFSS. **(C)** Significant increase of immobility at the TST after 5 days of RFSS (N=5 mice/group, paired T-test, **p<0.01). **(D)** Picture of a tested mouse (*Cx3cr1$^{GFP/+}$: Thy1-YFP*) during a session of awake two-photon imaging. **(E)** An 80µm thick z-stack acquired through the CA1 hippocampal region (SO = Stratum Oriens; PCL = Pyramidal Cell Layer; SR = Stratum Radiatum). In these mice, both GFP+ microglia and YFP+ neurons could be separately visualized. (**F**) Neurons served as a landmark to relocate the region of interest. **(G)** Representative 100µm^2 magnified image showing both GFP+ microglia and YFP+ neurons. (**H**) Representative time-lapse series of a microglial cell showing protraction and retraction of the processes. Processes speed was determined through 3D filament reconstruction for each time-point. **(I)** Motility of microglial primary and terminal processes during Imaging Basal (unstressed condition). **(J)** Motility of microglial primary and terminal processes during Imaging post-RFSS (after 5 days of RFSS). **(K)** Average speed (µm/min) of both primary and terminal processes comparing Imaging Basal and Imaging post-RFSS (N=20 microglial cells from 5 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test, ***p<0,001).

10. Loss of glutamatergic synapses in the CA1 of RFSS mice

Chronic-stress was shown to induce synaptic atrophy in hippocampus and mPFC (Christoffel *et al.*, 2011; Duman and Aghajanian, 2012). Moreover, altered synaptic plasticity was previously described in the RFSS model (Serchov *et al.*, 2015; Normann *et al.*, 2017). To investigate possible evidences of synaptic loss in this model, I first stained for the pan-synaptic marker synaptophysin and I assessed the number of CA1 synaptophysin-positive synapses in confocal imaging (**Figure 11A**). My quantification showed the overall number of synapses was not significantly changed between control and RFSS mice (p=0.3) (**Figure 11B**). Likewise, the estimation of the average synaptic size showed no difference between the two groups (**Figure 11C**). It should be noted that the hippocampal circuits host heterogeneous neuronal populations (mostly glutamatergic, but also GABAergic, Dopaminergic, Noradrenergic, Serotoninergic and Cholinergic). Synaptophysin is expressed in all mature neurons (regardless the nature of the released neurotransmitter) and it does not help distinguish among different synaptic types. It is yet possible that RFSS paradigm caused an important loss of specific synaptic subsets, which could not be properly appreciated under synaptophysin staining.

A growing number of evidences point towards a dysregulation of the glutamatergic neurotransmission as a major hallmark in stress-related disorders (Moghaddam, 2002; Popoli *et al.*, 2012; Sanacora *et al.*, 2012; Musazzi *et al.*, 2013; Rial *et al.*, 2016; Thompson *et al.*, 2015). I then decided to focus specifically on the glutamatergic synapses in the CA1. To do this I performed a double staining for both VGLUT1 and Homer1, marking glutamatergic pre- and post-synaptic puncta respectively.

Results

As already described by other groups (Schafer *et al.*, 2012, 2016; Vasek *et al.*, 2016), VGLUT1/Homer1 colocalized signals were considered as glutamatergic synapses comprising both pre- and post-synaptic inputs in close proximity. I then counted the number of glutamatergic synapses in both CA1 hippocampal region and Cortical Layer II/III (CTRL region) (**Figure 12A)**. This analysis was carried out using the Imaris object detection tool. To minimize the false positive counts, I restricted my detection to the spots with a diameter of at least 1µm (**Figure 12B**). VGLUT1/Homer1 colocalizations were automatically quantified with the Imaris colocalized object plugin (distance between objects < 1µm). Doing so, I could observe a significant reduction of glutamatergic synapses in the CA1 of RFSS mice, while no difference was found in the Cortical Layer II/III (**Figure 12C**).

To confirm the immunohistochemistry data, I performed VGLUT1 intracellular staining in dissected hippocampal and cortical tissue and assessed VGLUT1 immunoreactivity in flow cytometry (**Figure 12D**). Gating on the $CD11b^{-}CD45^{-}$ population (brain's non-myeloid cells) I measured a significant reduction of the VGLUT1-positive events in the hippocampus from RFSS mice, while no difference was found in the cortex (**Figure 12E**). I then wanted to assess VGLUT1 immunoreactivity within the $CD11b^{+}CD45^{lo}$ population (microglia) (**Figure 12F**). Interestingly, hippocampal microglia from RFSS mice exhibited a significant increase of VGLUT1-positive events, while cortical microglia did not (**Figure 12G**). This may suggest that hyper-ramified microglia promoted synaptic loss owing to augmented synaptic engulfment (synaptic pruning). However, we cannot exclude that microglia may locally secrete factors inducing either atrophy or displacement of glutamatergic inputs, as suggested by others (Zhang J. *et al.*, 2014).

Several works pinpointed the importance of microglia for remodeling of synaptic connection, in both postnatal (Stevens *et al.*, 2007; Paolicelli *et al.*, 2011; Schafer *et al.*, 2012; Zhan *et al.*, 2014; Miyamoto *et al.*, 2016) and adult brain (Wake *et al.*, 2009; Tremblay *et al.*, 2010; Sipe *et al.*, 2016; Paolicelli *et al.*, 2017; Reshef *et al.*, 2017). I could hypothesize that microglia hyper-ramification may promote loss of glutamatergic synapses during the RFSS paradigm, possibly via a synaptic pruning mechanism. Indeed, exaggerated synaptic pruning has been already suggested as a possible mechanism to explain synaptic disfunction in different psychiatric disorders (Wake *et al.*, 2013; Zhan *et al.*, 2014; Delpech *et al.*, 2015; Kim *et al.*, 2016; Sekar *et al.*, 2016; Bialas *et al.*, 2017; Li and Barres, 2017). The underlying mechanism(s) leading to synaptic loss during chronic-stress certainly deserves a deeper investigation and should be better addressed with future studies.

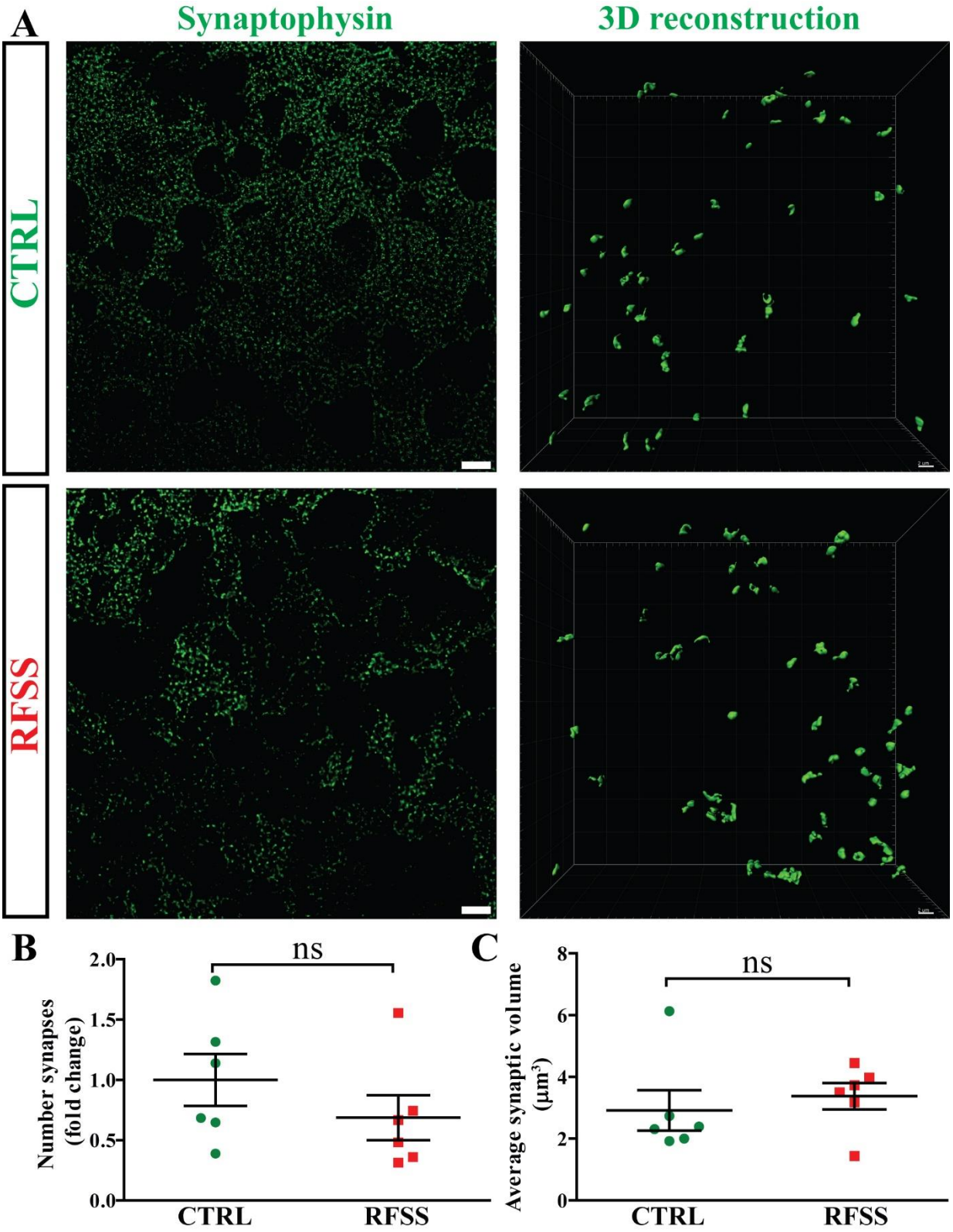

Figure 11: Non-significant change in the overall number of CA1 synapses in RFSS mice. (A) Representative anti-synaptophysin immunostaining and synapses 3D reconstruction in the CA1 in control and RFSS mice. **(B)** Quantification of the number and (**C**) average volume of the synaptophysin-positive synapses per stack (N=6 z-stacks from 3 mice/group, unpaired T-test).

Results

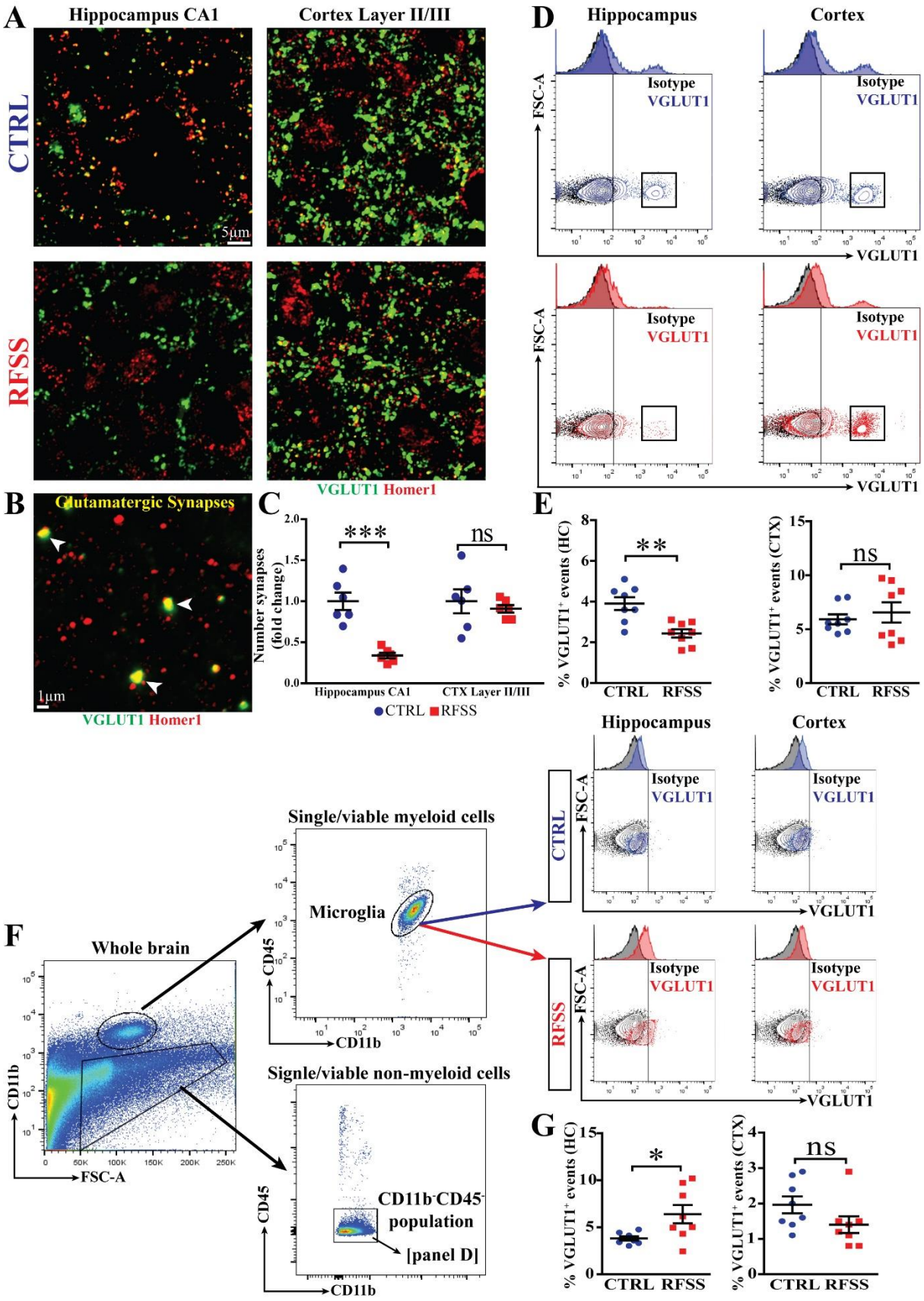

Figure 12 legend on the next page

Figure 12: Loss of glutamatergic synapses in the CA1 of RFSS mice (A) Representative confocal image of VGLUT1/Homer1 double-staining in the CA1 and Somatosensory Cortex Layer II/III. **(B)** High magnification of the colocalized signals representative of glutamatergic synapses (arrow heads). **(C)** RFSS-induced change in number of the glutamatergic synapses assessed in the CA1 and Cortical Layer II/II respectively, Control mean set as value = 1 (N=6 z-stacks from 3 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test, ***p<0.001). **(D)** Representative FACS plot of VGLUT1 staining in the brain's non-myeloid cells (single/viable $CD11b^{-}CD45^{-}$) from either Hippocampus or Cortex. **(E)** Quantification of the VGLUT1-positive events in the chosen gate displayed in panel D (N=8 mice/group, unpaired T-test, **p<0.01). **(F)** Representative FACS plot of VGLUT1 staining in microglia (single/viable $CD11b^{+}CD45^{low}$) from either Hippocampus or Cortex. The gating strategy for the $CD11b^{-}CD45^{-}$ population (analyzed in panel D) is also displayed. **(G)** Quantification of the VGLUT1-positive microglial cells (N=8 mice/group, unpaired T-test, *p<0.05).

11. P2Y12 knock-out mice are resilient to RFSS-induced behavioral changes and show reduced synaptic loss

ATP is known to play a major role in motility/chemotaxis of microglial processes (Davalos *et al.*, 2005). Given that hippocampal microglia exhibited altered motility after the RFSS paradigm, I asked whether purinergic signaling may represent a key pathway for microglia hyper-ramification and possibly for the RFSS-induced behavioral alterations. Indeed, previous studies showed that stress exposure induces a rapid release of both glutamate (Reznikov *et al.*, 2007; Yuen *et al.*, 2009; Treccani *et al.*, 2014; Musazzi *et al.*, 2015; Wang *et al.*, 2015) and ATP (Orellana *et al.*, 2015; Iwata *et al.*, 2016; Rial *et al.*, 2016). Extracellular ATP may then upregulate expression of cytokines (especially IL1β) in microglia via stimulation of purinergic receptors (Iwata *et al.*, 2016; Madry *et al.*, 2018).

Microglia express a variety of purinergic receptors (Biber *et al.*, 2007; Kettenmann *et al.*, 2011). For a preliminary study, I decided to focus on P2Y12-receptor (hereafter P2Y12) because of three main reasons: first, P2Y12 is exclusively expressed in microglia (Butovsky *et al.*, 2014); second, P2Y12 signaling is crucial for microglial processes motility (Haynes *et al.*, 2006; Sipe *et al.*, 2016; Madry *et al.*, 2018); third, a possible implication for P2Y12 in stress-related disorders was so far unexplored. I then tested both P2Y12 knock-out mice and wild-type littermates using our RFSS paradigm (8 mice per group). Interestingly, P2Y12 knock-out mice performed significantly better then wild-types during the 5 days of RFSS (at least on the first two days) (**Figure 13A**). At the probe phase, wild-type mice exhibited a significant IT increase at the TST after RFSS (as expected). However, IT increase was essentially abolished in P2Y12 knock-out mice (**Figure 13B**). Hence, I performed a morphological analysis of CA1 microglia from both P2Y12-deficient and wild-type littermate mice as described in Figure 2 (**Figure 13C**).

Results

Consistently with the previous literature (Haynes *et al.*, 2006; Sipe *et al.*, 2016; Madry *et al.*, 2018), lack of P2Y12 had no obvious effects on microglial morphology under homeostatic (control) condition. After RFSS, both knock-out and wild-type microglia significantly increased processes ramification. Nonetheless, microglial hyper-ramification was more pronounced in wild-type as compared to P2Y12 knock-out mice. More precisely, P2Y12 knock-out microglia exhibited a slightly lower number of Sholl intersections (**Figure 13D**) and reduced processes length compared to wild-type microglia. It should, however, be noted that all the other morphological parameters (processes area, number of branch points and number of end points) were not significantly changed (**Figure 13E**). This may suggest that P2Y12 probably plays a minor role in microglia hyper-ramification. Alternatively, other microglial purinergic receptors (for example P2Y13-receptor) compensated for the absence of P2Y12. Next, I assessed the number of glutamatergic synapses in the CA1 as described in Figure 12. Under homeostatic (control) condition, the number of glutamatergic synapses was unchanged between wild-type and P2Y12 knock-out mice. In RFSS mice, a significant reduction in number of glutamatergic synapses could be observed in both genotypes. Nevertheless, RFSS-induced synaptic loss was significantly reduced under P2Y12-deficient condition (**Figure 13F**). These data suggest that lack of P2Y12 signaling had partially reduced microglia hyper-ramification, mitigated loss of glutamatergic synapses and improved behavioral outcome after chronic-stress exposure.

To further corroborate these data, I wanted to determine whether pharmacological blockage of P2Y12 may phenocopy the beneficial effects of the genetic knock-out. Clopidogrel (alias Plavix) is a specific irreversible P2Y12-inhibitor commonly used in clinic to prevent thrombosis (Bennett, 2001). A previous study showed a remarkable inhibition of microglial processes extension *in vivo* injecting mice ip. with 50mg/kg of clopidogrel (Sipe *et al.*, 2016). To maintain a chronic inhibition of P2Y12 throughout the entire period of the RFSS paradigm, I daily injected wild-type mice with 50mg/kg clopidogrel, starting from day 1 till day 5 of the RFSS protocol (**Figure 13G**). Saline injected wild-type mice were used as control (6 mice per group). During the 5 days of RFSS, no difference in IT increase between clopidogrel and saline injected mice was observed (**Figure 13H**). At the probe phase, saline injected mice exhibited a significant IT increase at the TST (as expected). By contrast, clopidogrel treated mice showed no increase of IT, which was even reduced as compared to the basal condition (**Figure 13I**). In conclusion, both deletion and pharmacological blockage of P2Y12 significantly ameliorated stress-induced depressive-like behavior in this model, thus unmasking a previously unappreciated role for the P2Y12-dependent pathway under chronic-stress.

Results

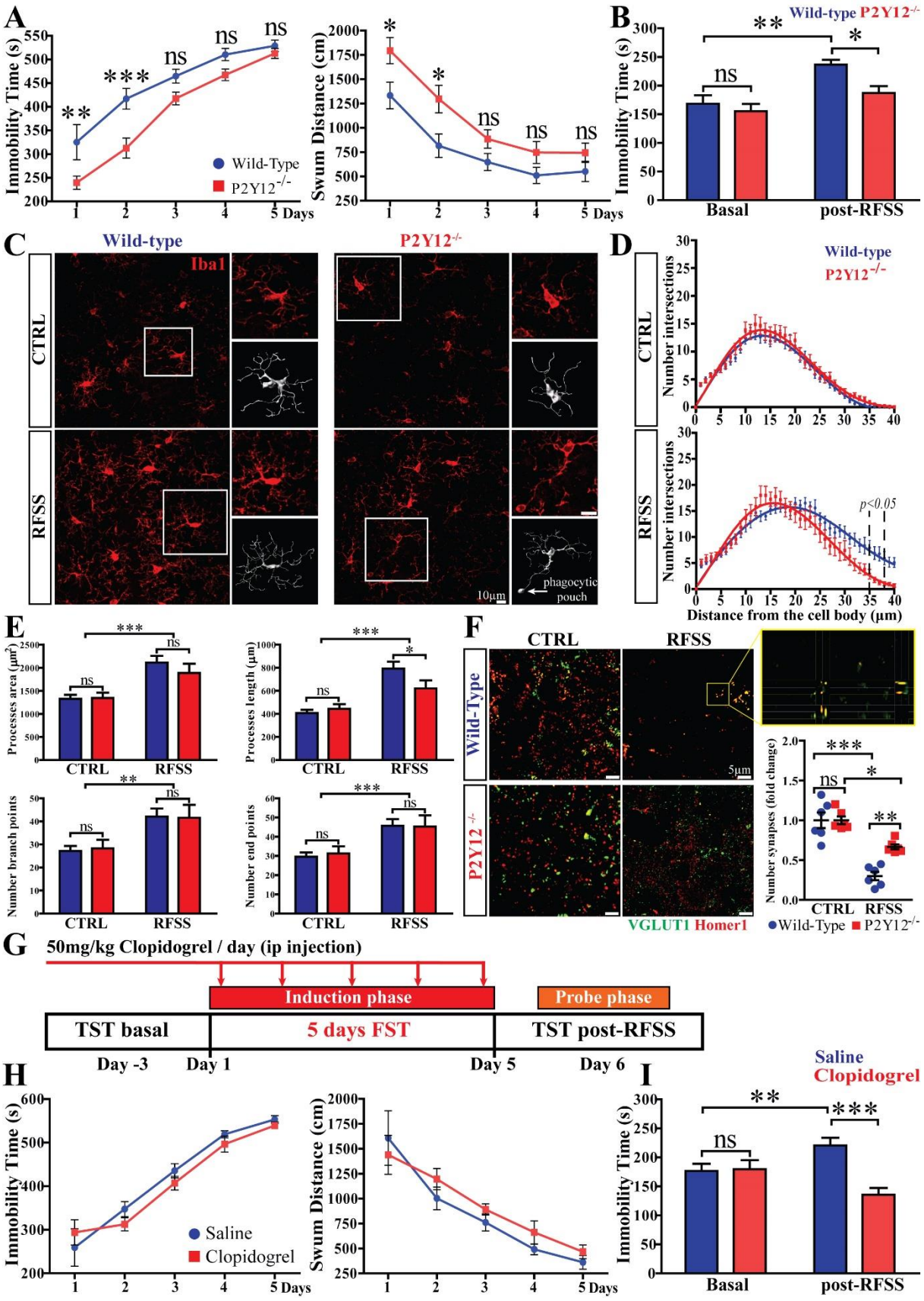

Figure 13 legend on the next page

Figure 13: P2Y12 knock-out mice are resilient to RFSS-induced behavioral changes and show reduced synaptic loss. **(A)** Immobility time and swum distance along 5 days of RFSS for both P2Y12 knock-out mice and wild-type littermates (N=8 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test, *p<0.05, **p<0.01, ***p<0.001). **(B)** Immobility at the TST for both P2Y12 knock-out mice and wild-type littermates (N=8 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test, *p<0.05, **p<0.01). **(C)** Confocal imaging of microglia in the CA1 from both P2Y12 knock-out mice and wild-type littermates (the arrow indicates a phagocytic pouch on a terminal process of the 3D reconstructed microglia). **(D)** Microglia Sholl analysis (N=12 cell from 3 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test, the dashed lines define the region with a statistical significance of at least *p<0.05). **(E)** Morphological analysis on 3D reconstructed microglia; assessed parameters: processes area, processes length, number of branch points and number of end points (N=12 cell from 3 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test, *p<0.05, **p<0.01, ***p<0.001). **(F)** Confocal imaging of VGLUT1/Homer1 double-staining in the CA1 showed reduced loss of glutamatergic synapses in P2Y12 knock-out mice compared to wild-type littermates (N=6 z-stacks from 3 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test, *p<0.05, **p<0.01, ***p<0.001). Note that only VGLUT1/Homer1 colocalized spots were considered for the quantification **(G)** Experimental plan including daily injection of 50mg/kg Clopidogrel during the induction phase. **(H)** Immobility time and swum distance for both Clopidogrel- and Saline-injected littermate mice along 5 days of RFSS (N=6 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test). **(I)** Immobility at the TST for both Clopidogrel- and Saline-injected littermates (N=6 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test, **p<0.01, ***p<0.001).

12. Peripheral LPS challenge reverts microglia hyper-ramification and improves behavior in RFSS mice

Altogether, this study suggests that microglia could exert a detrimental function during chronic-stress, possibly upon acquisition of a hyper-ramified morphology, which may (directly or indirectly) promote loss of glutamatergic synapses. A previous work (and to my knowledge the only one) showed that peripheral Lipopolysaccharide (LPS) injection in chronically stressed mice improved depressive-like behavior (Kreisel *et al.*, 2014). Although the cellular and molecular mechanisms were not investigated, authors suggested that a pro-inflammatory challenge promoted a trophic phenotype of microglia, which in turn ameliorated mouse behavior. These findings were rather surprising since it is known that an acute inflammatory reaction can induce temporary sickness and depressive-like behavior in naïve (unstressed) mice (Dantzer *et al.*, 2008). We then decided to assess whether LPS could produce any beneficial effect in the RFSS model used for this study. To test this hypothesis, I applied a single low-dose of LPS in wild-type mice after 5 days of RFSS, hence I score depressive-like behavior (at both FST and TST) along with the microglia morphology in the hippocampus. Saline injected mice were used as control (10 mice per group). Briefly, TST basal (day -3) and induction phase (RFSS from day 1 to day 5) were carried out as described in Figure 1. On day 6, a dose of 0,5mg/kg LPS (or Saline) was injected ip. in RFSS mice. These mice were again probed at FST and TST 24 hours post-injection (day 7) (**Figure 14A**). In comparison to saline-injected controls, LPS-injected mice showed a remarkable reduction of IT at both FST (**Figure 14B**) and TST (**Figure 14C**). Interestingly, these findings mirror those from *Kreisel and colleagues.*

I then analyzed microglial morphology and microglial cell density in the hippocampus as described in Figure 1 and 2. As already known, LPS promoted microglial processes retraction in both control and RFSS mice (**Figure 14D** and **14E**), whereas the microglial cell number was unaffected using this LPS dosage (**Figure 14F**). These data indicate, albeit without proving, that LPS-induced de-ramification of microglia and promoted recovery of depressive-like behavior. Nonetheless, it is very likely that LPS caused a transient change in the microglial secretome, which may have improved the performance at the behavioral tests. 24 hours post-injection I could still detect a significant upregulation of several cytokines and microglial pro-inflammatory markers (such as CCL2, CITTA, iNOS, NOX2, TNFα, IL6, IL1β and IL10) (**Figure 14G**). Moreover, peripheral LPS injection in RFSS mice induced down-regulation of TGFβ and up-regulation of BDNF in microglia (**Figure 14H**). More studies are needed to better understand the underlying mechanisms of the anti-depressant effects of LPS.

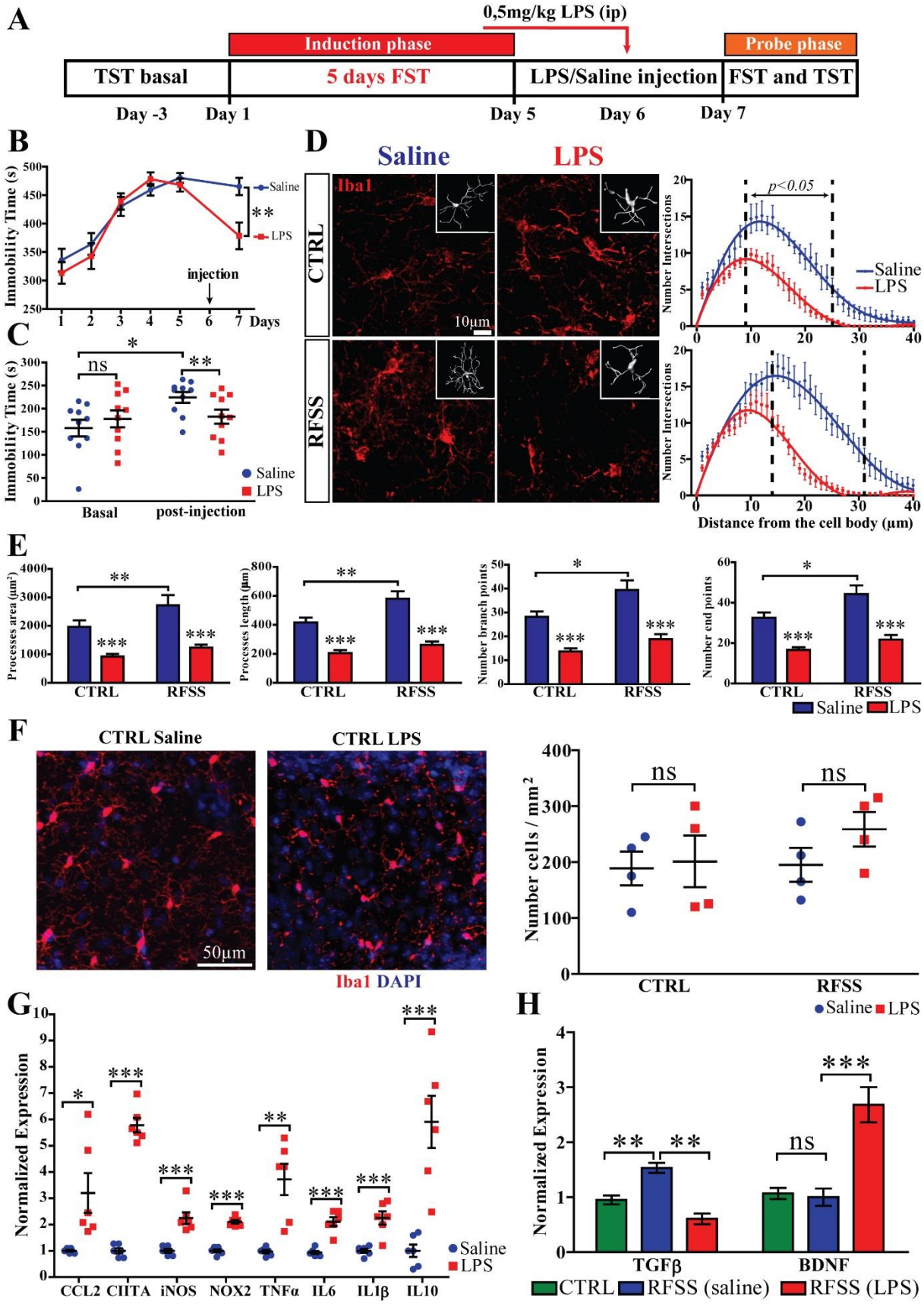

Figure 14 legend on the next page

Figure 14: Peripheral LPS challenge reverts microglia hyper-ramification and improves behavior in RFSS mice. (A) Experimental plan. LPS-injected mice exhibited reduced immobility time compared to Saline-injected littermates **(B)** at the FST and **(C)** at the TST (10 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test, *p<0.05, **p<0.01). **(D)** LPS injection caused microglial processes retraction in both Control and RFSS mice (N=10 cells from 3 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test, the dashed lines define the region with a statistical significance of at least *p<0.05). **(E)** Morphological analysis on 3D reconstructed microglia; assessed parameters: processes area, processes length, number of branch points and number of end points (N=10 cell from 3 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test, *p<0.05, **p<0.01, ***p<0.001). **(F)** LPS injection did not cause significant changes in number of microglial cells in the hippocampus (N=4 z-stacks from 2 mice/group). **(G)** Expression level of different cytokines and pro-inflammatory markers analyzed in qPCR on whole-brain microglia acutely isolated 24 hours post-injection (N=6 triplicate samples/group, Two-Way ANOVA and Bonferroni post-hoc test, **p<0.01, ***p<0.001). **(H)** TGFβ and BDNF expression level analyzed in qPCR on whole-brain microglia acutely isolated 24 hours post-injection (N=6 triplicate samples/group, One-Way ANOVA and Bonferroni post-hoc test, **p<0.01, ***p<0.001).

13. Microglia hyper-ramification in the Amygdala, medial Prefrontal Cortex and Nucleus Accumbens of RFSS mice.

These data show that microglia tend to acquire a hyper-ramified morphology after chronic-stress. However, with the present work I have primarily focused on the main hippocampal areas (DG, CA1 and CA3). I then asked whether such a morphological transition was indeed a peculiarity of the hippocampal microglia, and not a rather diffuse phenomenon. To answer this question, I investigated microglial morphology (as described in Figure 1 and 2) in three additional brain regions, notoriously involved in the emotional response to psychological/environmental stress, namely medial Prefrontal Cortex (mPFC), Nucleus Accumbens (NAc) and Amygdala (Amy) (**Figure 15A**). Several works have described alteration in the physiology of these brain regions during chronic-stress (Rajkowska *et al.*, 1999; Ressler and Mayberg, 2007; Sahay and Hen, 2007; Drevets *et al.*, 2008; MacQueen and Frodl, 2011; Price and Drevets, 2012).

Confocal imaging suggests presence of microglia reactivity within the investigated regions after 5 days of RFSS (**Figure 15B**). A significant increase of Iba1+ voxels per stack was detected in Amy and mPFC, whereas just a non-significant tendency was found in the NAc (**Figure 15C**). Again, the number of microglial cells was unchanged in RFSS mice as compared to controls (**Figure 15D**). 3D cell reconstruction and sholl analysis indicate an important microglia hyper-ramification in Amy, mPFC, and, till a lesser extent, in the NAc (**Figure 15E**). Consistently, microglia exhibited a significant increase of processes area, length, number of branch and terminal points in all the investigate regions (**Figure 15F**).

Results

Altogether, these data show that RFSS-induced microglia hyper-ramification is not solely restricted to the hippocampus as other stress-responsive regions exhibit a similar phenomenon. I may then hypothesize that RFSS-induced microglia hyper-ramification be an altered microglial phenotype which appears exclusively within well-defined brain regions or circuits associated with the stress-response. Given the technical difficulties to isolate microglia from mPFC, NAc and Amy, expression levels of pro-inflammatory cytokines/chemokines could not be assessed. Further studies are needed to evaluate both presence of synaptic loss and possible P2Y12-dependent effects in these regions.

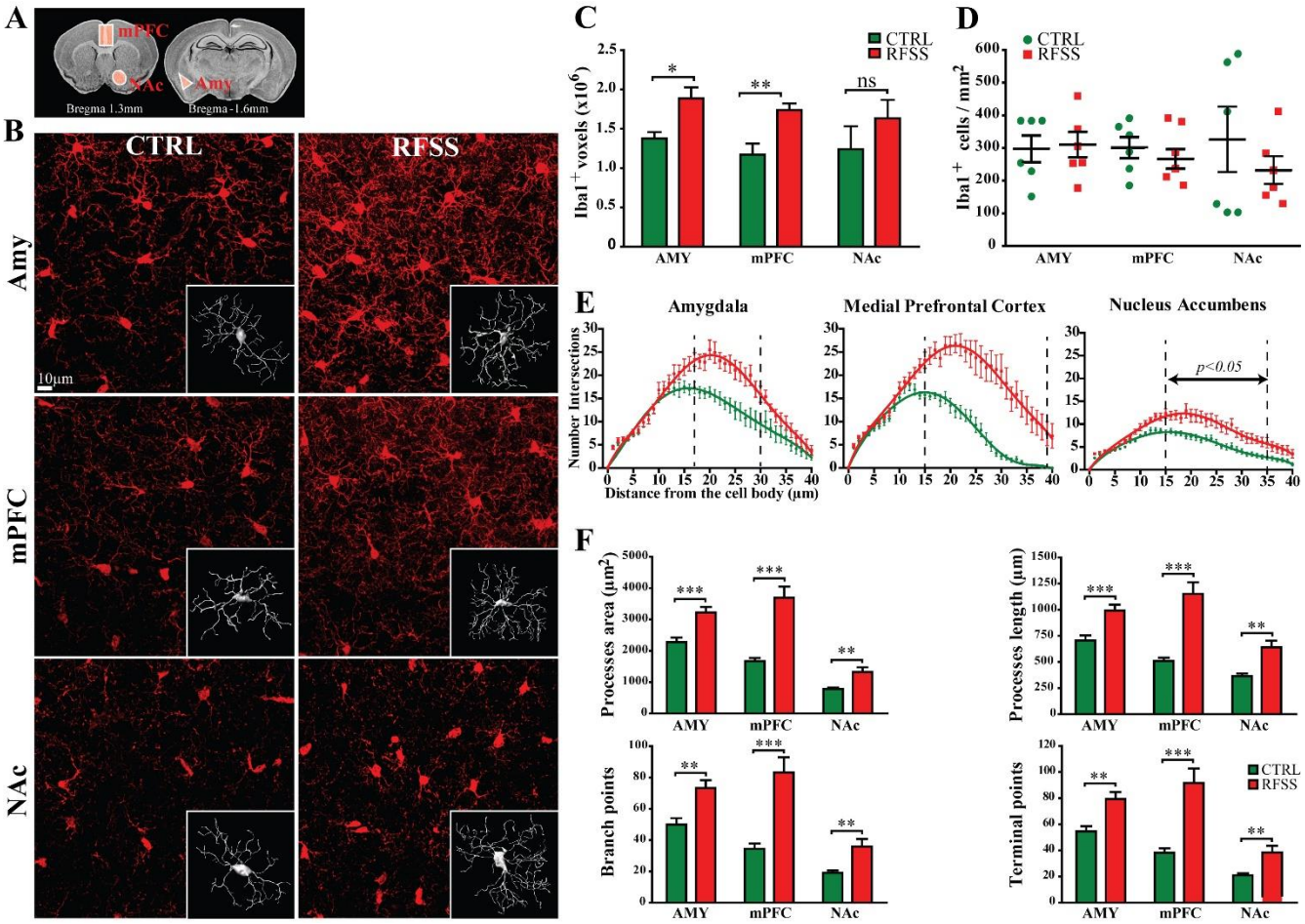

Figure 15: Microglia hyper-ramification in the Amy, mPFC and NAc of RFSS mice. (A) Anatomical localization of the investigated brain regions, namely Amygdala (Amy), medial Prefrontal Cortex (mPFC) and Nucleus Accumbens (NAc). **(B)** Representative confocal image of Iba1-stained microglia and 3D cell reconstruction showing RFSS-induced hyper-ramification in the selected brain regions. **(C)** Iba1 immunoreactivity quantified as number of $Iba1^+$ voxels per stack (N=6 z-stacks from 3 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test, **p<0.01, ***p<0.001). **(H)** Estimation of the microglial cells number per mm^2 (N=6 z-stacks from 3 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test). **(E)** Sholl analysis of microglia from Amy, mPFC and NAc (N=12 cell from 3 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test, the dashed lines define the region with a statistical significance of at least *p<0.05). **(F)** Morphological analysis on 3D reconstructed microglia; assessed parameters: processes area, processes length, number of branch points and number of end points (N=12 cell from 3 mice/group, Two-Ways ANOVA and Bonferroni post-hoc test, **p<0.01, ***p<0.001).

Discussion

1. A maladaptive brain-immune network may represent a common substrate for stress-related disorders

Psychological/environmental stress is known to trigger a reaction of the innate-immune system, in both animals and humans. This often results in prolonged elevation of circulating inflammatory biomarkers including cytokines/chemokines, C-reactive protein and myeloid leukocytes (Miller *et al.*, 2009; Powell *et al.*, 2013; Gold, 2015; Hodes *et al.*, 2015; Miller and Raison, 2016; Wohleb *et al.*, 2016; Leday *et al.*, 2017; Leighton *et al.*, 2017; Köhler *et al.*, 2017). In the wild, animals regularly fight with prays, predators or competitors for food, reproduction and territory. The fight or flight response has evolved a strong interplay with the immune-system to promote a priming state of the peripheral immunity while facing with a potentially harmful situation. This would help convey immunological protection against infection and injuries inflicted during physical conflicts. The same may happen in humans, perhaps as a form of maladaptive immunological reaction to psychological and social distress (Raison and Miller, 2003; Gold, 2015; Wohleb *et al.*, 2015; Miller and Raison, 2016).

Besides the activation of the peripheral immunity, a growing body of literature is focusing on microglia, the resident myeloid phagocytes of the CNS. Post-mortem neuropathological analysis showed evidences of microgliosis in the brain of depressed subjects (Steiner *et al.*, 2008; Torres-Platas *et al.*, 2014). In mice, several works have described a not clearly defined form of "microglia activation" in response to chronic-stress (Tynan *et al.*, 2010; Wohleb *et al.*, 2013; Kreisel *et al.*, 2014; Bisht *et al.*, 2016; Hellwig *et al.*, 2016; Milior *et al.*, 2016). Modelling a complex and multifaceted diseases as depression in rodents is a true challenge (Cryan and Mombereau, 2004). Our choice for the mouse model in issue was made upon the relatively short period of stress-exposure (only five days) necessary to observe stress-induced behavioral changes. In principle, this would allow to better monitor microglial alterations at early stages of the pathology.

2. A possible key role for CX3CR1 during chronic-stress: converging findings, yet differently interpreted

To date, at least five independent groups have described a stress-resistant phenotype in CX3CR1-deficient mice (Wohleb *et al.*, 2013; Rimmerman *et al.*, 2015; Hellwig *et al.*, 2016; Milior *et al.*, 2016; Winkler *et al.*, 2017), which is suggestive of a possible contribution of microglia in the physiopathology of chronic-stress. Multiple hypothesizes concerning gain or lack of microglial functions during chronic-stress have been suggested (Tay *et al.*, 2018). For example, *Kreisel and colleagues* suggested that microglia undergo a functional decline during chronic-stress, thus altering physiological brain's homeostasis (Kreisel *et al.*, 2014; Yirmiya *et al.*, 2015). However, in absence of CX3CR1 microglia acquire a mildly activated phenotype which makes them withstand the stress-induced atrophy (Rimmerman *et al.*, 2015). A complete different scenario was outlined by *Wohleb and colleagues*, who ascribed the observed phenotype to a reduced recruitment of inflammatory $CCR2^{+}Ly6C^{hi}$ monocytes to the brain (Wohleb *et al.*, 2013). Others hypothesized that *Cx3cr1* knock-out disrupts a crucial pathway of microglia-neuron communication, which would (directly or indirectly) mitigate the severity of the pathology (Hellwig *et al.*, 2016; Milior *et al.*, 2016). Different interpretations are not mutually exclusive and likely stem from specific features of each model.

At present, only one study compared the stress-induced transcriptomic changes in the hippocampus between wild-type and CX3CR1-deficient mice (Rimmerman *et al.*, 2017). Some genes were indeed differentially expressed in a genotype-dependent fashion. For example, $Cx3cr1^{-/-}$ mice exhibited a reduced activation of the Interferons (IFN)-related pathways, whereas an increased expression of estrogen-regulated genes was observed. Authors, however, analyzed the whole hippocampal tissue without sorting microglia, therefore this study could provide additional information about the gene-expression changes in microglia. A comprehensive analysis of the transcriptomic and epigenetic signature in sorted microglia from *Cx3cr1* knock-out mice may help shed more light on the real function of the CX3CR1 under chronic-stress. To my knowledge, a transcriptomic analysis on FACS sorted microglia after chronic-stress was performed just recently (Nie *et al.*, 2018; Weber *et al.*, 2018). Not surprisingly, an increased expression of cytokines (i.e. TNFα, IL1α, IL1β and IL6) and chemokines (i.e. CCL3, CCL4, CCL7 and CXCL10) was detected, thus indicating the acquisition of a pro-inflammatory phenotype. Nevertheless, the actual consequences of such phenotypic changes on brain physiology and function are still hypothetical.

3. Microglia hyper-ramification versus activation. What does "activation" really mean?

After application of the RFSS paradigm, I observed a morphological transition of microglia characterized by increased processes ramification. The most popular terminology tends to define a condition of "*microglia activation*" as processes retraction, increased cell body size, acquisition of a rounded (amoeboid) shape, and upregulation of pro-inflammatory genes. It should, however, be noted that the current nomenclature lacks well-defined categories to indicate the various microglial activation states. To avoid confusion, our group decided to use the term "*hyper-ramified microglia*", which was indeed more representative of the microglial phenotype observed in RFSS mice (Hellwig *et al.*, 2016). Here I want to remark that an increased ramification of microglial processes does not mean "reduced activity". This misconception may be suggested by the classical definition of "microglia activation" (as explained above). Amoeboid microglia can be observed during acute neuroinflammation, brain injury or neurodegeneration, which is not the case of stress-related disorders. Possibly, the hyper-ramified microglial phenotype acquires (or loses) biological functions which have very little to share with the prototypical inflammatory phenotype. For example, microglia may increase length and complexity of the processes to upscale the contacts with the surrounding cells, or to boost the immunological surveillance within the microenvironment. Another hypothesis is that microglia hyper-ramification is promoted by the altered neurochemical composition of the extracellular milieu during chronic-stress.

In the literature, hyper-ramified microglia were already described in rat after chronic-restraint stress (Hinwood *et al.*, 2013; Walker *et al.*, 2014) or upon administration of corticosteroids (Caetano *et al.*, 2016). Moreover, a study on a mouse model of RFSS (therefore analogue to the one here used) witnessed the presence of hyper-ramified microglia in the DG (Llorens-Martín *et al.*, 2016). Interestingly, microglia hyper-ramification was also observed in germ-free mice devoid of gut microbiota (Erny *et al.*, 2015). I describe here that hyper-ramified microglia were found in hippocampus, medial prefrontal cortex, amygdala and to a lesser extent in the nucleus accumbens. All these regions are known to be involved in the stress-response (Drevets *et al.*, 2008). By contrast, cerebellum, somatosensory cortex and caudate-putamen nucleus (which are not involved in stress-response) did not display obvious alterations in microglial morphology. This indicates that morphological change of microglia is region-specific and probably associated to the neurobiological response to chronic-stress. Further studies are now needed to better elucidate the possible implications of microglia hyper-ramification for brain physiology.

4. Microglia-deficient mice are partially resilient to RFSS-induced behavioral changes

To better understand whether and how microglia participate to RFSS-induced behavioral changes I wanted to assess depressive-like behavior in mice lacking microglia. Clodronate-filled liposomes do no cross the BBB (Steel *et al.*, 2010), therefore I excluded this type of treatment. Microglia can be efficiently depleted *in vivo* by feeding mice with the CSF1-receptor inhibitor (Elmore *et al.*, 2014). However, this approach has no specificity for microglia, as other tissue macrophages and bone marrow-derived monocytes are likely affected (MacDonald *et al.*, 2010; Hume and MacDonald, 2012).

To date, IL34 knock-out is the only transgenic mouse line exhibiting an important microglia deficiency, without obvious consequences on mice's viability (Wang *et al.*, 2012). I therefore chose this line as ideal candidate for my study. Interestingly, IL34 knock-out mice performed significantly better at the TST after 5 days of RFSS. This indicates that microglia-deficient mice are resilient to chronic-stress. I then conclude that the presence of microglia is indeed instrumental for RFSS-induced behavioral changes.

I hypothesized that under chronic-stress microglia release a plethora of factors affecting neuronal function, thus shaping behavioral changes. TNFα was often described to be increased in depressed patients and the use of TNFα-blockers was also proposed as a possible therapeutic strategy (Kappelmann *et al.*, 2016). Since microglia are the main source of TNFα in the brain, I decided to analyze depressive-like behavior in the $Cx3cr1^{Cre}$: $TNF\alpha^{flox/flox}$ line, lacking TNFα expression in microglia. Cre+ mice (with TNFα-deficient microglia) exhibited a very similar depressive-like behavior to Cre- mice (wild-type controls). This indicates that microglia-derived TNFα is not required for RFSS-induced behavioral changes (at least in our experimental conditions). Interestingly, neither IL34 knock-out mice nor $Cx3cr1^{Cre}$: $TNF\alpha^{flox/flox}$ mice exhibited altered performance at learning and memory tests, confirming previous evidences that microglia are not necessary for cognitive skills in mouse (Elmore *et al.*, 2014).

5. Hippocampal microglia upregulate expression of pro-inflammatory cytokines and CD11b in response to RFSS

Hyper-ramified microglia from the hippocampus upregulated classical pro-inflammatory cytokines, especially IL6 and IL1β. Interestingly, TNFα expression was unchanged in RFSS mice. This may explain why the deletion of TNFα in microglia did not produce detectable effects in this model of chronic-stress. As already described by others (Wohleb *et al.*, 2013; McKim *et al.*, 2017) I also detected increased CCL2 expression levels in microglia from RFSS mice. CCL2 is a chemokine involved in monocyte chemoattraction, however, as parenchymal microglia were the major focus of this work, I have not carefully investigated recruitment of blood monocytes to the brain. My FACS analysis showed no difference in the percentage of the $CD11b^{+}CD45^{hi}$ population between control and RFSS mice. Likewise, count of the Iba1+ cells in histology showed no difference between the two cohorts. These data suggest that this RFSS paradigm dos not cause monocytes infiltration into the brain. I have not investigated the $Ly6C^{lo}CX3CR1^{hi}CCR2^{-}$ and $Ly6C^{hi}CX3CR1^{lo}CCR2^{+}$ monocyte populations in the peripheral blood, which may indeed display important changes after RFSS. Nevertheless, I would exclude that the depressive-like behavior observed in this model may depend on the pro-inflammatory action of blood-derived monocytes. It is important to note that cytokines/chemokines expression in microglia from RFSS mice ranged between 2-3 folds higher than controls. This level of cytokines upregulation is rather mild, especially if compared to a prototypical neuroinflammatory disease. Altogether, I exclude that neuroinflammation be a main pathological hallmark in this model of chronic-stress.

In addition, hippocampal microglia exhibited increased CD11b expression in RFSS mice, while Iba1 and CX3CR1 were unchanged. In macrophages, CD11b (integrin-αM) together with CD18 (integrin-β2) forms the heterodimeric receptor complement-receptor-3 (CR3), which is required for the recognition of the complement component-3 (C3) (Stephan *et al.*, 2012). Microglial CR3 has been shown to play a role in the complement-mediated synaptic pruning during brain development (Stevens *et al.*, 2007; Schafer *et al.*, 2012), aging (Stephan *et al.*, 2013; Shi *et al.*, 2015) and neurodegeneration (Hong *et al.*, 2016; Shi *et al.*, 2017). It is thus possible that upregulated CD11b may stand for an increased engulfment of C3-tagged cargos, possibly synapses. Consistently, hyper-ramified microglia in the CA1 exhibited increased size of CD68+ lysosomes, which may suggest a skew towards a more phagocytic phenotype. At present, this only a theory and more convincing evidences are yet needed.

6. RFSS mice show reduced motility of microglial processes in the CA1

Microglia constantly survey neuronal environment under homeostasis through a continuous processes' extension and retraction (Hanisch and Kettenmann, 2007; Kettenmann *et al.,* 2013). It was also shown that microglial motility changes under pathological/inflammatory condition (Avignone *et al.*, 2008; Ohsawa and Kohsaka, 2011; Koizumi *et al.*, 2013; Krabbe *et al.*, 2013; Paris *et al.*, 2018), however, no studies have so far explored this phenomenon in the context of chronic-stress. To assess motility of hyper-ramified microglia I performed a time-lapse imaging in awake mice, before and after 5 days of RFSS. Although limited to the CA1, these data provide evidence of reduced motility of microglial terminal processes after RFSS, while motility of primary processes was essentially unaffected. Only one mouse exhibited a clear movement of a microglial cell body within the field of view (not shown). Therefore, I tend to exclude that RFSS may promote a significant migration microglial cells or infiltration of blood-derived monocytes. To sum up, I hypothesize that alterations in brain's physiology and/or hormonal imbalances due to chronic-stress may negatively affect microglial surveillance capacity. However, a possible interrelatedness between microglia hyper-ramification, upregulation of cytokines/CD11b and reduced motility remains elusive.

7. RFSS mice display loss of glutamatergic synapses in the CA1

In the present study I also described an important loss of glutamatergic synapses in the CA1 of RFSS mice. Glutamatergic disfunction in depression is well documented, however the underlying mechanisms are still matter of investigation (Duman and Aghajanian, 2012; Kang *et al.*, 2012; Ota *et al.*, 2014; Thompson *et al.*, 2015; Moriguchi *et al.*, 2018; Sekar *et al.*, 2018). Since the territory of hyper-ramified microglia overlapped with the area of synaptic loss, I could hypothesize that microglia were (at least partially) involved in this phenomenon, maybe through cytokines release or increased synaptic pruning (possibly via CR3). Indeed, in a FACS study I showed a higher VGLUT1 immunoreactivity in hippocampal microglia from RFSS mice, suggesting increased engulfment of VGLUT1-positive synapses. Alternatively, I can propose that CA1 synapses were lost through a cell-autonomous pathway and synaptic debris were rapidly scavenged by neighboring microglia. These two scenarios can equally explain the presence of VGLUT1 staining in the $CD11b^{+}CD45^{lo}$ population. Further studies are needed to determine whether microglia may indeed play an active role in synaptic loss during chronic-stress.

Discussion

8. RFSS-induced behavioral changes and synaptic loss are reduced in mice deficient of P2Y12-receptor

Mounting evidences indicate the involvement of the brain's purinergic system in the different neuropsychiatric diseases, including depression (Burnstock, 2008; Abbracchio *et al.*, 2009; Krügel, 2016; Cheffer *et al.*, 2018). It was previously shown that glutamatergic stimulation causes ATP release from either neurons or astrocytes (Pascual *et al.*, 2005, 2012; Lovatt *et al.*, 2012). ATP is also an important factor for microglial processes motility (Honda *et al.*, 2001; Davalos *et al.*, 2005; Ohsawa *et al.*, 2007; Eyo *et al.*, 2015). ATP gradients may then attract microglial processes towards excitatory synapses during glutamatergic firing (Fontainhas *et al.*, 2011; Li *et al.*, 2012; Dissing-Olesen *et al.*, 2014; Eyo *et al.*, 2015; Wu *et al.*, 2015). Microglia express a variety of purinergic receptors, but overall, P2Y12-receptor (P2Y12) was shown to be important for microglial processes motility and modulation of synaptic plasticity (Haynes *et al.*, 2006; Ohsawa *et al.*, 2010; Eyo *et al.*, 2014; Sipe *et al.*, 2016). Importantly, P2Y12 is exclusively expressed in microglia, in both the mouse and human brain (Sasaki *et al.*, 2003; Butovsky *et al.*, 2014; Mildner *et al.*, 2017). As discussed above, mice lacking *Cx3ct1* are resilient to chronic-stress (Wohleb *et al.*, 2013; Rimmerman *et al.*, 2015; Hellwig *et al.*, 2016; Milior *et al.*, 2016; Winkler *et al.*, 2017). At the same time, *Cx3ct1*-deficient microglia were described to be poorly responsive to ATP gradients (Pagani *et al.*, 2015). It is tempting to speculate whether ATP insensitivity of microglia may play a role in the stress-resilient phenotype of the *Cx3ct1* knock-out mice. To my knowledge, none has so far investigated P2Y12 in the context of chronic-stress. I therefore decided to assess RFSS-induced depressive-like behavior in mice lacking P2Y12. Remarkably, P2Y12 knock-out mice were resilient to the RFSS paradigm, providing comparable results to the IL34 knock-out line. Moreover, P2Y12-deficiency reduced both microglia hyper-ramification and loss of glutamatergic synapses in the CA1. Consistently, chronic treatment with the specific P2Y12 antagonist clopidogrel in wild-type mice could partially phenocopy the knock-out condition. These data suggest that P2Y12-dependent signaling triggers a microglia reaction in response to RFSS, hence promoting loss of glutamatergic synapses and depressive-like behavior.

9. LPS injection in RFSS mice improves RFSS-induced behavioral changes

A previous work showed that a single peripheral LPS challenge could improve depressive-like behaviors after chronic-stress (Kreisel *et al.*, 2014). Authors suggested that LPS boosted a trophic microglial phenotype, thus promoting the beneficial effects on mouse behavior (Yirmiya *et al.*, 2015). Intriguingly, a study from our clinic described rapid (although transient) anti-depressant effects of LPS administration in depressed patients (Bauer *et al.*, 1995). It is interesting to consider that LPS is known to promote sickness and ensuing depressive-like behaviors when injected in naïve mice (Dantzer *et al.*, 2008). I then wanted to test whether a single peripheral LPS injection could produce any behavioral change in the RFSS model used in the present study. In line with the findings from *Kreisel and colleagues*, I observed a significant improvement of depressive-like behavior in LPS injected mice compared to saline injected controls. As expected, microglia also exhibited a remarkable processes retraction.

It is now tempting to speculate whether processes retraction had any influence on the behavioral outcome. At present, I cannot provide robust evidences in support of this theory. Alternatively, I may hypothesize the LPS challenge induced the expression of neurotrophic factors from microglia and astrocytes, thus promoting recovery of depressive-like behavior. For example, microglia were shown to support synaptic remodeling by releasing BDNF (Parkhurst *et al.*, 2013). Moreover, LPS stimulation was also shown to increase BDNF production in microglia (Miwa *et al.*, 1997; Gomes *et al.*, 2013). Consistently, I also measured increased BDNF expression in microglia 24 hours after LPS injection in RFSS mice.

More studies are required to better understand this interesting phenomenon. The mechanism by which LPS improved depressive-like behavior is probably pleiotropic (involving both central and peripheral pathways). Although these findings are certainly noteworthy, this topic goes beyond the scope of the present work. For the future it will be interesting to better investigate the “therapeutic” potential of LPS endotoxin (or other Toll-like receptor agonists) in the context of depression.

Discussion

10. Conclusion

To conclude, with this study I provided important evidences that microglia contribute to shape depressive-like behavior under chronic-stress. Using the RFSS model, I could describe a series of phenotypical alterations in hippocampal microglia, including processes hyper-ramification, reduction of processes motility and increased cytokines/chemokines release. Furthermore, I showed that hyper-ramified microglia may promote loss of glutamatergic synapses in a P2Y12-dependent manner. I could hypothesize that chronic-stress promoted release of danger signals (such as ATP) from neurons and astrocytes, which may attract microglial processes towards the active excitatory synapses. Microglia may then promote displacement/removal or simply degradation/resorption of the synaptic inputs to dampen the glutamatergic tone. It was already shown that impairment of synaptic pruning in C1q knock-out mice was associated to spontaneous generation of epileptic seizures in the mouse cortex (Chu *et al.*, 2010). Moreover, it was repeatedly suggested that microglia-neuron communication may help protect neurons from excitotoxic damage (Vinet *et al.*, 2012; Eyo *et al.*, 2014; Kato *et al.*, 2016). Altogether, increased turnover of excitatory synapses under chronic-stress may be interpreted as a fine regulatory mechanism to prevent glutamatergic excitotoxicity with ensuing neuronal damage. Further studies are demanded to better clarify the biological significance of microglia hyper-ramification. Moreover, it will be important to clarify whether this microglial phenotype can be identified also in other models of depression. To this end, I performed a preliminary study on the Chronic Social Defeat model and I found evidences of microglia hyper-ramification in the nucleus accumbens (not sown).

Last, I also recommend caution with the expression “microglia activation”, which is often meant as a transition from a homeostatic towards a pro-inflammatory phenotype, characterized by processes retraction and increased cell body size. Relying strictly on this stereotype, an increased ramification would imply a lowered activation state compared to homeostasis, which sounds rather unlikely. Contrarily, as emerged in this study, microglia hyper-ramification represents an activated/reactive phenotype, probably characterized by a specific spectrum of functional properties. Interestingly, a recent systematic review from *Calcia and colleagues*, suggested that repeated stress-exposure causes a phenotypical change of microglia towards a hypertrophic morphology. This microglial phenotype is characterized by cytokines release and altered synaptic pruning (Calcia *et al.*, 2016). In the light of the emerging findings, the association between “microglia activation” and “processes shortening” appears rather inappropriate. I therefore encourage a careful reinterpretation of this classical terminology.

Future perspectives

A series of outstanding questions have not been addressed in the present study. Hereafter, I would like to outline some of the possible research goals for the near future.

Analysis of microglial transcriptome and epigenome

Gene-expression analysis may certainly help understand the transcriptomic change of microglia during chronic-stress. In particular, single-cells RNA sequencing (sc-RNA-Seq) will provide more insight into the phenotypical heterogeneity of microglia, possibly highlighting the presence of stress-associated sub-phenotypes. Moreover, techniques as the ChIP sequencing (ChIP-Seq) and ATAC-sequencing (ATAC-Seq) may help identify genomic regions undergoing histone modifications and changes in chromatin accessibility. However, this analysis requires a careful dissection of the brain regions of interest. Trivially, the sectioning procedure may produce unpredictable consequences on the quality of the microglial transcript. Second, such experiments will probably yield an enormous amount of data that will require years of work to be properly analyzed and interpreted. Nevertheless, transcriptomic and epigenetic changes in microglia during chronic-stress have never been investigated and I believe that this line of research should be pursued. Additionally, it will also be important to determine whether anti-depressant therapies can normalize such changes along with the recovery of the behavioral alterations. Ultimately, identification of the most dysregulated transcripts may uncover a list of key molecular players and potential therapeutic targets for stress-related diseases.

Moreover, some recent studies showed that optogenetic manipulation of mPFC and VTA circuits can either improve or worsen depressive-like behavior in mouse (Covington *et al.*, 2010; Chaudhury *et al.*, 2013; Tye *et al.*, 2013). It will be interesting to assess microglial response to such manipulations. Last, I am aware that disease models typically aim to replicate a single or a few features of a more complex pathology. Nevertheless, identification of key phenotypical changes in microglia, highly consistent and robust across different models, would represent a crucial step forward for this research. Ideally, stress-associated transcriptomic changes of microglia should be replicated using different mouse models of chronic-stress.

Future perspectives

What are the molecular signals driving the microglial reaction under chronic-stress?

In this study I suggested glutamate and ATP as possible upstream signals of a molecular cascade promoting microglia hyper-ramification and interaction with excitatory synapses. However, I could not provide strong evidences that these two molecules are necessarily required to evoke a stress-induced microglia reaction. Indeed, P2Y12R-deficient mice showed only a modest reduction of microglia hyper-ramification upon RFSS, suggesting the presence of additional (or compensatory) mechanisms promoting hyper-ramification. A careful analysis of the brain's secretome during psychological stress (including hormones, neurotransmitters, ATP and other synaptic molecules) may serve as a preliminary screening. Having identified a number of promising candidates, it would be interesting to block specific pathways and investigate stress-induced transcriptomic changes in microglia and effects on mouse behaviour. Although interesting, such a study may require a long-lasting and highly demanding research, with no guarantee of success. Indeed, it is unlikely that a single molecule could cover almost the 100% of the global effect. It is easier to imagine a complex network of signals working in concert and collectively shaping microglia reaction during chronic-stress.

Does microglial reaction occur prior or after the stress-related pathology begins?

A main question for this line of research is: "Do microglia play a role in the aetiology/onset of the pathology, or they only contribute to the disease progression/worsening at later stages?" As a matter of fact, depressive disorders imply a dysfunctional neuronal activity, especially among certain circuits involved in the regulation of the mood. What causes such a dysregulation is currently unknown, but chronic psychological stress may represent a major cause. Chronic-stress has several consequences both centrally and peripherally. For example, the neuroendocrine cascade of the hypothalamic-pituitary-adrenal (HPA) axis governs the hormonal stress-response, thus regulating blood pressure, heart beat, digestion, vigilance and immune system. I may then hypothesize that both microglial and neuronal alterations arise and develop (more or less) simultaneously in a stepwise and mutually regulated process. Alterations of brain's connectivity under chronic stress might be monitored in functional MRI, PET and electrophysiological recordings. Parallel multidimensional high-throughput RNA analysis (like MARS-Seq, CyTOF or Drop-Seq) may highlight the phenotypical changes of different brain cells across disease progression. A combination of behavioural analysis and RNA sequencing may then uncover the temporal checkpoints of the disease in neurons and microglia.

References

References

Abbracchio, M. P. et al. (2009) 'Purinergic signalling in the nervous system: an overview', *Trends in Neurosciences*, pp. 19–29. doi: 10.1016/j.tins.2008.10.001.

Abdallah, C. G. et al. (2015) 'Ketamine and Rapid-Acting Antidepressants: A Window into a New Neurobiology for Mood Disorder Therapeutics', *Annual Review of Medicine*, 66(1), pp. 509–523. doi: 10.1146/annurev-med-053013-062946.

Abiega, O. et al. (2016) 'Neuronal Hyperactivity Disturbs ATP Microgradients, Impairs Microglial Motility, and Reduces Phagocytic Receptor Expression Triggering Apoptosis/Microglial Phagocytosis Uncoupling', *PLoS Biology*, 14(5). doi: 10.1371/journal.pbio.1002466.

Abud, E. M. et al. (2017) 'iPSC-Derived Human Microglia-like Cells to Study Neurological Diseases', *Neuron*, 94(2), p. 278–293.e9. doi: 10.1016/j.neuron.2017.03.042.

Aguzzi, A., Barres, B. A. and Bennett, M. L. (2013) 'Microglia: Scapegoat, Saboteur, or Something Else?', *Science*, 339(6116), pp. 156–161. doi: 10.1126/science.1227901.

Agarwal, A. et al. (2012) 'In vivo imaging and noninvasive ablation of pyramidal neurons in adult NEX-CreERT2 mice.' *Cereb Cortex.* 22(7):1473-86. doi: 10.1093/cercor/bhr214.

Ajami, B. et al. (2011) 'Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool', *Nature Neuroscience*, 14(9), pp. 1142–1149. doi: 10.1038/nn.2887.

Ajami, B. et al. (2007) 'Local self-renewal can sustain CNS microglia maintenance and function throughout adult life.', *Nature neuroscience*, 10(12), pp. 1538–43. doi: 10.1038/nn2014.

Ajami, B. et al. (2018) 'Single-cell mass cytometry reveals distinct populations of brain myeloid cells in mouse neuroinflammation and neurodegeneration models', *Nature Neuroscience*, 21(4), pp. 541–551. doi: 10.1038/s41593-018-0100-x.

Akazawa, H. et al. (2004) 'Diphtheria toxin-induced autophagic cardiomyocyte death plays a pathogenic role in mouse model of heart failure', *Journal of Biological Chemistry*, 279(39), pp. 41095–41103. doi: 10.1074/jbc.M313084200.

Al-Aoukaty, A. et al. (1998) 'MIP-3alpha, MIP-3beta and fractalkine induce the locomotion and the mobilization of intracellular calcium, and activate the heterotrimeric G proteins in human natural killer cells.'*Immunology*,95(4),pp.618–624

Alliot, F., Godin, I. and Pessac, B. (1999) 'Microglia derive from progenitors, originating from the yolk sac, and which proliferate in the brain', *Developmental Brain Research*, 117(2), pp. 145–152. doi: 10.1016/S0165-3806(99)00113-3.

American Psychiatric Association (2013) 'Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-5)', *Diagnostic and Statistical Manual of Mental Disorders 4th edition TR.*, p. 280. doi: 10.1176/appi.books.9780890425596.744053.

Amit, I., Winter, D. R. and Jung, S. (2015) 'The role of the local environment and epigenetics in shaping macrophage identity and their effect on tissue homeostasis', *Nature Immunology*, 17(1), pp. 18–25. doi: 10.1038/ni.3325.

Ancuta, P. et al. (2003) 'Fractalkine preferentially mediates arrest and migration of CD16+ monocytes.', *The Journal of experimental medicine*, 197(12), pp. 1701–7. doi: 10.1084/jem.20022156.

Anderson, K.L. et al. (1998) 'Myeloid development is selectively disrupted in PU.1 null mice.'*Blood*, 91(10),pp.3702–3710.

References

Arnò, B. et al. (2014) 'Neural progenitor cells orchestrate microglia migration and positioning into the developing cortex.', *Nature communications*, 5, p. 5611. doi: 10.1038/ncomms6611.

Tay T.L. et al. (2018). Unique microglia recovery population revealed by single-cell RNAseq following neurodegeneration. Acta Neuropathologica Communication 5;6(1):87. doi: 10.1186/s40478-018-0584-3.

Askew, K. et al. (2017) 'Coupled Proliferation and Apoptosis Maintain the Rapid Turnover of Microglia in the Adult Brain', *Cell Reports*, 18(2), pp. 391–405. doi: 10.1016/j.celrep.2016.12.041.

Avignone, E. et al. (2008) 'Status Epilepticus Induces a Particular Microglial Activation State Characterized by Enhanced Purinergic Signaling', *Journal of Neuroscience*, 28(37), pp. 9133–9144. doi: 10.1523/JNEUROSCI.1820-08.2008.

Baker, M. et al. (2006) 'Mutations in progranulin cause tau-negative frontotemporal dementia linked to chromosome 17', *Nature*, 442(7105), pp. 916–919. doi: 10.1038/nature05016.

Barres, B. A. (2008) 'The Mystery and Magic of Glia: A Perspective on Their Roles in Health and Disease', *Neuron*, pp. 430–440. doi: 10.1016/j.neuron.2008.10.013.

Basilico, B. et al. (2018), 'Microglia shape presynaptic properties at developing glutamatergic synapses', Glia. doi: 10.1002/glia.23508.

Bauer, J. et al. (1995) 'Induction of cytokine synthesis and fever suppresses REM sleep and improves mood in patients with major depression', *Biological Psychiatry*, 38(9), pp. 611–621. doi: 10.1016/0006-3223(95)00374-X.

Bayramgürler, D. et al. (2013) 'Effects of long-term etanercept treatment on anxiety- and depression-like neurobehaviors in rats', *Physiology and Behavior*, 119, pp. 145–148. doi: 10.1016/j.physbeh.2013.06.010.

Bazan, J. F. et al. (1997) 'A new class of membrane-bound chemokine with a CX3C motif.', *Nature*, pp. 640–4. doi: 10.1038/385640a0.

Beattie, E. C. et al. (2002) 'Control of synaptic strength by glial TNFα', *Science*, 295(5563), pp. 2282–2285. doi: 10.1126/science.1067859.

Beers, D. R. et al. (2006) 'Wild-type microglia extend survival in PU.1 knockout mice with familial amyotrophic lateral sclerosis.', *Proceedings of the National Academy of Sciences of the United States of America*, 103(43), pp. 16021–6. doi: 10.1073/pnas.0607423103.

Bennett, F. C. et al. (2018) 'A Combination of Ontogeny and CNS Environment Establishes Microglial Identity', *Neuron*. doi: 10.1016/j.neuron.2018.05.014.

Bennett, J. S. (2001) 'Novel Platelet Inhibitors', *Annu Rev Med*, pp. 52, 161–184. doi: 10.1146/annurev.med.52.1.161.

Bennett, M. L. et al. (2016) 'New tools for studying microglia in the mouse and human CNS', *Pnas*, p. 1525528113-. doi: 10.1073/pnas.1525528113.

Berman, R. M. et al. (2000) 'Antidepressant effects of ketamine in depressed patients', *Biological Psychiatry*, 47(4), pp. 351–354. doi: 10.1016/S0006-3223(99)00230-9.

Bertrand, J. Y. et al. (2005) 'Three pathways to mature macrophages in the early mouse yolk sac', *Blood*, 106(9), pp. 3004–3011. doi: 10.1182/blood-2005-02-0461.

Bialas, A. R. et al. (2017) 'Microglia-dependent synapse loss in type I interferon-mediated lupus', *Nature*. doi: 10.1038/nature22821.

References

Bialas, A. R. and Stevens, B. (2013) 'TGF-β signaling regulates neuronal C1q expression and developmental synaptic refinement', *Nature Neuroscience*, 16(12), pp. 1773–1782. doi: 10.1038/nn.3560.

De Biase, L. M. et al. (2017) 'Local Cues Establish and Maintain Region-Specific Phenotypes of Basal Ganglia Microglia', *Neuron*, 95(2), p. 341–356.e6. doi: 10.1016/j.neuron.2017.06.020.

Biber, K. et al. (2016) 'Central nervous system myeloid cells as drug targets: current status and translational challenges.', *Nature reviews. Drug discovery*, 15(2), pp. 110–24. doi: 10.1038/nrd.2015.14.

Biber, K. et al. (2007) 'Neuronal "On" and "Off" signals control microglia', *Trends in Neurosciences*, pp. 596–602. doi: 10.1016/j.tins.2007.08.007.

Biber, K., Owens, T. and Boddeke, E. (2014) 'What is microglia neurotoxicity (Not)?', *GLIA*, pp. 841–854. doi: 10.1002/glia.22654.

Bilimoria, P. M. and Stevens, B. (2015) 'Microglia function during brain development: New insights from animal models', *Brain Research*, 1617, pp. 7–17. doi: 10.1016/j.brainres.2014.11.032.

Bisht, K. et al. (2016) 'Dark microglia: A new phenotype predominantly associated with pathological states', *GLIA*, 64(5), pp. 826–839. doi: 10.1002/glia.22966.

Blank, T. and Prinz, M. (2016) 'CatacLysMic specificity when targeting myeloid cells?' *Eur J Immunol.* 46(6):1340-2. doi: 10.1002/eji.201646437.

Blinzinger, K. and Kreutzberg, G. (1968) 'Displacement of synaptic terminals from regenerating motoneurons by microglial cells', *Zeitschrift fuer Zellforschung und Mikroskopische Anatomie*, 85(2), pp. 145–157. doi: 10.1007/BF00325030.

Boddeke, E. W. G. M. et al. (1999) 'Functional expression of the fractalkine (CX3C) receptor and its regulation by lipopolysaccharide in rat microglia', *European Journal of Pharmacology*, 374(2), pp. 309–313. doi: 10.1016/S0014-2999(99)00307-6.

Bohlen, C. J. et al. (2017) 'Diverse Requirements for Microglial Survival, Specification, and Function Revealed by Defined-Medium Cultures', *Neuron*, 94(4), p. 759–773.e8. doi: 10.1016/j.neuron.2017.04.043.

Bolós, M. et al. (2017) 'Absence of microglial CX3CR1 impairs the synaptic integration of adult-born hippocampal granule neurons', *Brain, Behavior, and Immunity*. doi: 10.1016/j.bbi.2017.10.002.

Bonnardel, J. and Guilliams, M. (2018) 'Developmental control of macrophage function', Current Opinion in Immunology, pp. 64–74. doi: 10.1016/j.coi.2017.12.001.

Böttcher, C. (2018), 'Human microglia regional heterogeneity and phenotypes determined by multiplexed single-cell mass cytometry', Nat Neurosci. 22(1):78-90. doi: 10.1038/s41593-018-0290-2.

Boucsein, C., Kettenmann, H. and Nolte, C. (2000) 'Electrophysiological properties of microglial cells in normal and pathologic rat brain slices', *Eur.J.Neurosci.*, 12(6), pp. 2049–2058. doi: ejn100 [pii].

Boucsein, C. et al. (2003) 'Purinergic receptors on microglial cells: functional expression in acute brain slices and modulation of microglial activation in vitro.', *The European journal of neuroscience*, 17(11), pp. 2267–2276. doi: 10.1046/j.1460-9568.2003.02663.x.

Brendecke, S. M. and Prinz, M. (2015) 'Do not judge a cell by its cover—diversity of CNS resident, adjoining and infiltrating myeloid cells in inflammation', *Seminars in Immunopathology*, pp.591–605.doi:10.1007/s00281-015-0520-6.

References

Brockhaus, J. and Iischner, S. (1993) 'Membrane Properties of Ameboid Microglial Callosum Slice from Early Postnatal Mice Cells in the Corpus', *The Journal of neuroscience :13*, 13(10), pp. 4412–4421.

Brockhaus, J., Möller, T. and Kettenmann, H. (1996) 'Phagocytozing ameboid microglial cells studied in a mouse corpus callosum slice preparation', *GLIA*, 16(1), pp. 81–90. doi: 10.1002/(SICI)1098-1136(199601)16:1<81::AID-GLIA9>3.0.CO;2-E.

Brown, G. C. and Neher, J. J. (2012) 'Eaten alive! Cell death by primary phagocytosis: "Phagoptosis"', *Trends in Biochemical Sciences*, pp. 325–332. doi: 10.1016/j.tibs.2012.05.002.

Bruttger, J. et al. (2015) 'Genetic Cell Ablation Reveals Clusters of Local Self-Renewing Microglia in the Mammalian Central Nervous System', *Immunity*, 43(1), pp. 92–107. doi: 10.1016/j.immuni.2015.06.012.

Burnstock, G. (2008) 'Purinergic signalling and disorders of the central nervous system', *Nature Reviews Drug Discovery*, pp. 575–590. doi: 10.1038/nrd2605.

Butovsky, O. et al. (2014) 'Identification of a unique TGF-β-dependent molecular and functional signature in microglia.', *Nature neuroscience*, 17(1), pp. 131–43. doi: 10.1038/nn.3599.

Buttgereit, A. et al. (2016) 'Sall1 is a transcriptional regulator defining microglia identity and function', *Nature Immunology*, 17(12), pp. 1397–1406. doi: 10.1038/ni.3585.

Caetano, L. et al. (2016) 'Adenosine A2A receptor regulation of microglia morphological remodeling-gender bias in physiology and in a model of chronic anxiety', *Molecular Psychiatry*. doi: 10.1038/mp.2016.173.

Cahoy, J. D. et al. (2008) 'A Transcriptome Database for Astrocytes, Neurons, and Oligodendrocytes: A New Resource for Understanding Brain Development and Function', *Journal of Neuroscience*, 28(1), pp. 264–278. doi: 10.1523/JNEUROSCI.4178-07.2008.

Calcia, M. A. et al. (2016) 'Stress and neuroinflammation: A systematic review of the effects of stress on microglia and the implications for mental illness', *Psychopharmacology*, pp. 1637–1650. doi: 10.1007/s00213-016-4218-9.

van Calker D et al. (2018) 'Recent insights into antidepressant therapy: Distinct pathways and potential common mechanisms in the treatment of depressive syndromes', *Neurosci Biobehav Rev.*, 88(March), pp. 63–72.

Campbell, S. et al. (2004) 'Lower hippocampal volume in patients suffering from depression: a meta-analysis.', *The American journal of psychiatry*, 161(4), pp. 598–607. doi: 10.1176/appi.ajp.161.4.598.

Carbonell, W. S. (2005) 'Migration of Perilesional Microglia after Focal Brain Injury and Modulation by CC Chemokine Receptor 5: An In Situ Time-Lapse Confocal Imaging Study', *Journal of Neuroscience*, 25(30), pp. 7040–7047. doi: 10.1523/JNEUROSCI.5171-04.2005.

Cardona, A. E. et al. (2006) 'Control of microglial neurotoxicity by the fractalkine receptor.', *Nature neuroscience*, 9(7), pp. 917–924. doi: 10.1038/nn1715.

Casano, A. M. and Peri, F. (2015) 'Microglia: Multitasking specialists of the brain', *Developmental Cell*, pp. 469–477. doi: 10.1016/j.devcel.2015.01.018.

Cavaliere, F., Dinkel, K. and Reymann, K. (2005) 'Microglia response and P2 receptor participation in oxygen/glucose deprivation-induced cortical damage', *Neuroscience*, 136(3), pp. 615–623. doi: 10.1016/j.neuroscience.2005.04.038.

Chakfe, Y. et al. (2002) 'ADP and AMP Induce Interleukin-1beta Release from Microglial Cells through Activation of ATP-Primed P2X7 Receptor Channels', *Journal of neuroscience*, 22(8), pp. 3061–3069. doi: 20026250.

References

Chaudhury, D. et al. (2013) 'Rapid regulation of depression-related behaviours by control of midbrain dopamine neurons.' *Nature* 24;493(7433):532-6. doi: 10.1038/nature11713.

Cheffer, A. et al. (2018) 'Purinergic system in psychiatric diseases', *Molecular Psychiatry*, pp. 94–106. doi: 10.1038/mp.2017.188.

Chen-Plotkin, A. S. et al. (2010) 'Brain progranulin expression in GRN-associated frontotemporal lobar degeneration', *Acta Neuropathologica*, 119(1), pp. 111–122. doi: 10.1007/s00401-009-0576-2.

Chen, M. J. et al. (2011) 'Erythroid/myeloid progenitors and hematopoietic stem cells originate from distinct populations of endothelial cells', *Cell Stem Cell*, 9(6), pp. 541–552. doi: 10.1016/j.stem.2011.10.003.

Chen, S. K. et al. (2010) 'Hematopoietic origin of pathological grooming in Hoxb8 mutant mice', *Cell*, 141(5), pp. 775–785. doi: 10.1016/j.cell.2010.03.055.

Chen, Z. et al. (2014) 'Microglial displacement of inhibitory synapses provides neuroprotection in the adult brain', *Nature Communications*, 5. doi: 10.1038/ncomms5486.

Chen, Z. et al. (2012) 'Lipopolysaccharide-Induced Microglial Activation and Neuroprotection against Experimental Brain Injury Is Independent of Hematogenous TLR4', *Journal of Neuroscience*, 32(34), pp. 11706–11715. doi: 10.1523/JNEUROSCI.0730-12.2012.

Chitu, V. et al. (2016) 'Emerging Roles for CSF-1 Receptor and its Ligands in the Nervous System', *Trends in Neurosciences*, pp. 378–393. doi: 10.1016/j.tins.2016.03.005.

Chiu, I. M. et al. (2013) 'A neurodegeneration-specific gene-expression signature of acutely isolated microglia from an amyotrophic lateral sclerosis mouse model', *Cell Reports*, 4(2), pp. 385–401. doi: 10.1016/j.celrep.2013.06.018.

Choi, H. B. et al. (2007) 'Modulation of the purinergic P2X7 receptor attenuates lipopolysaccharide-mediated microglial activation and neuronal damage in inflamed brain.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 27(18), pp. 4957–4968. doi: 10.1523/JNEUROSCI.5417-06.2007.

Christoffel, D. J., Golden, S. A. and Russo, S. J. (2011) 'Structural and synaptic plasticity in stress-related disorders', *Reviews in the Neurosciences*, 22(5), pp. 535–549. doi: 10.1515/RNS.2011.044.

Chu, Y. et al. (2010) 'Enhanced synaptic connectivity and epilepsy in C1q knockout mice', *Proceedings of the National Academy of Sciences*, 107(17), pp. 7975–7980. doi: 10.1073/pnas.0913449107.

Chung W. S. et al. (2015). 'Do glia drive synaptic and cognitive impairment in disease?' Nat Neurosci. 18(11):1539-1545. doi: 10.1038/nn.4142.

Chung, W. S. et al. (2013) 'Astrocytes mediate synapse elimination through MEGF10 and MERTK pathways', *Nature*, 504(7480), pp. 394–400. doi: 10.1038/nature12776.

Clausen, B. E. et al. (1999) 'Conditional gene targeting in macrophages and granulocytes using LysMcre mice', *Transgenic Research*, 8(4), pp. 265–277. doi: 10.1023/A:1008942828960.

Colonna, M. and Butovsky, O. (2017) 'Microglia Function in the Central Nervous System During Health and Neurodegeneration', *Annual Review of Immunology*, 35(1), p. annurev-immunol-051116-052358. doi: 10.1146/annurev-immunol-051116-052358.

Colonna, M. and Wang, Y. (2016) 'TREM2 variants: new keys to decipher Alzheimer disease pathogenesis', *Nature Reviews Neuroscience*, 17(4), pp. 201–207. doi: 10.1038/nrn.2016.7.

References

Corona, A. W. et al. (2010) 'Fractalkine receptor (CX3CR1) deficiency sensitizes mice to the behavioral changes induced by lipopolysaccharide.', *Journal of neuroinflammation*, 7(1), p. 93. doi: 10.1186/1742-2094-7-93.

Corona, A. W. et al. (2013) 'Indoleamine 2,3-dioxygenase inhibition attenuates lipopolysaccharide induced persistent microglial activation and depressive-like complications in fractalkine receptor (CX3CR1)-deficient mice', *Brain, Behavior, and Immunity*, 31, pp. 134–142. doi: 10.1016/j.bbi.2012.08.008.

Covington, H. E. et al. (2010) 'Antidepressant effect of optogenetic stimulation of the medial prefrontal cortex', *J Neurosci.* 1;30(48):16082-90. doi: 10.1523/JNEUROSCI.1731-10.2010.

Cronk, J. C. et al. (2015) 'Methyl-CpG Binding Protein 2 Regulates Microglia and Macrophage Gene Expression in Response to Inflammatory Stimuli', *Immunity*, 42(4), pp. 679–691. doi: 10.1016/j.immuni.2015.03.013.

Cronk, J. C. et al. (2018) 'Peripherally derived macrophages can engraft the brain independent of irradiation and maintain an identity distinct from microglia.', *The Journal of experimental medicine*, p. jem.20180247. doi: 10.1084/jem.20180247.

Crotti, A. and Ransohoff, R. M. (2016) 'Microglial Physiology and Pathophysiology: Insights from Genome-wide Transcriptional Profiling', *Immunity*, pp. 505–515. doi: 10.1016/j.immuni.2016.02.013.

Cryan, J. F. and Mombereau, C. (2004) 'In search of a depressed mouse: utility of models for studying depression-related behavior in genetically modified mice.', *Molecular psychiatry*, 9(4), pp. 326–57. doi: 10.1038/sj.mp.4001457.

Cryan, J. F., Mombereau, C. and Vassout, A. (2005) 'The tail suspension test as a model for assessing antidepressant activity: review of pharmacological and genetic studies in mice', *Neuroscience and biobehavioral reviews*, 29(4–5), pp. 571–625. doi: 10.1016/j.neubiorev.2005.03.009.

Cullheim, S. and Thams, S. (2007) 'The microglial networks of the brain and their role in neuronal network plasticity after lesion', *Brain Research Reviews*, pp. 89–96. doi: 10.1016/j.brainresrev.2007.03.012.

Cumano, A. and Godin, I. (2007). 'Ontogeny of the hematopoietic system.' Annu Rev Immunol. 2007;25:745-85.

Cunningham, C. L., Martinez-Cerdeno, V. and Noctor, S. C. (2013) 'Microglia Regulate the Number of Neural Precursor Cells in the Developing Cerebral Cortex', *Journal of Neuroscience*, 33(10), pp. 4216–4233. doi: 10.1523/JNEUROSCI.3441-12.2013.

Dai, X. M. et al. (2002) 'Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects', *Blood*, 99(1), pp. 111–120. doi: 10.1182/blood.V99.1.111.

Daneman, R. et al. (2010) 'Pericytes are required for blood-brain barrier integrity during embryogenesis', *Nature*, 468(7323), pp. 562–566. doi: 10.1038/nature09513.

Dantzer, R. et al. (2008) 'From inflammation to sickness and depression: when the immune system subjugates the brain', *Nature Reviews Neuroscience*, 9(1), pp. 46–56. doi: 10.1038/nrn2297.

Datta, M. et al. (2018) 'Histone Deacetylases 1 and 2 Regulate Microglia Function during Development, Homeostasis, and Neurodegeneration in a Context-Dependent Manner', *Immunity*, 48(3), p. 514–529.e6. doi: 10.1016/j.immuni.2018.02.016.

Davalos, D. et al. (2005) 'ATP mediates rapid microglial response to local brain injury in vivo.', *Nature neuroscience*, 8(6), pp. 752–8. doi: 10.1038/nn1472.

Davalos, D. et al. (2012) 'Fibrinogen-induced perivascular microglial clustering is required for the development of axonal damage in neuroinflammation', *Nature Communications*, 3, p. 1227. doi: 10.1038/ncomms2230.

References

De, I. et al. (2014) 'CSF1 overexpression has pleiotropic effects on microglia in vivo', *GLIA*, 62(12), pp. 1955–1967. doi: 10.1002/glia.22717.

Delpech, J. C. et al. (2015) 'Microglia in neuronal plasticity: Influence of stress', *Neuropharmacology*, (Part A), pp. 19–28. doi: 10.1016/j.neuropharm.2014.12.034.

Derecki, N. C. et al. (2012) 'Wild-type microglia arrest pathology in a mouse model of Rett syndrome', *Nature*, 484(7392), pp. 105–109. doi: 10.1038/nature10907.

Derecki, N. C. et al. (2014) 'Microglia as a critical player in both developmental and late-life CNS pathologies', *Acta Neuropathologica*, pp. 333–345. doi: 10.1007/s00401-014-1321-z.

Dissing-Olesen, L. et al. (2014) 'Activation of Neuronal NMDA Receptors Triggers Transient ATP-Mediated Microglial Process Outgrowth', *Journal of Neuroscience*, 34(32), pp. 10511–10527. doi: 10.1523/JNEUROSCI.0405-14.2014.

Djukic, M. et al. (2006) 'Circulating monocytes engraft in the brain, differentiate into microglia and contribute to the pathology following meningitis in mice', *Brain*, 129(9), pp. 2394–2403. doi: 10.1093/brain/awl206.

Dombeck, D. A. et al. (2007) 'Imaging Large-Scale Neural Activity with Cellular Resolution in Awake, Mobile Mice', *Neuron*, 56(1), pp. 43–57. doi: 10.1016/j.neuron.2007.08.003.

Dombeck, D. and Tank, D. (2014) 'Two-photon imaging of neural activity in awake mobile mice', *Cold Spring Harbor Protocols*, 2014(7), pp. 726–736. doi: 10.1101/pdb.top081810.

Douvaras, P. et al. (2017) 'Directed Differentiation of Human Pluripotent Stem Cells to Microglia', *Stem Cell Reports*, 8(6), pp. 1516–1524. doi: 10.1016/j.stemcr.2017.04.023.

Dowlati, Y. et al. (2010) 'A Meta-Analysis of Cytokines in Major Depression', *Biological Psychiatry*, 67(5), pp. 446–457. doi: 10.1016/j.biopsych.2009.09.033.

Draheim, H. J. et al. (1999) 'Induction of potassium channels in mouse brain microglia: Cells acquire responsiveness to pneumococcal cell wall components during late development', *Neuroscience*, 89(4), pp. 1379–1390. doi: 10.1016/S0306-4522(98)00407-2.

Drevets, W. C., Price, J. L. and Furey, M. L. (2008) 'Brain structural and functional abnormalities in mood disorders: implications for neurocircuitry models of depression.', *Brain structure & function*, 213(1–2), pp. 93–118. doi: 10.1007/s00429-008-0189-x.

Dreymueller, D. et al. (2012) 'The role of ADAM-mediated shedding in vascular biology', *European Journal of Cell Biology*, pp. 472–485. doi: 10.1016/j.ejcb.2011.09.003.

Drysdale, A. T. et al. (2017). 'Resting-state connectivity biomarkers define neurophysiological subtypes of depression', Nat Med. 2017 Jan;23(1):28-38. doi: 10.1038/nm.4246.

Duman, R. S. and Aghajanian, G. K. (2012) 'Synaptic dysfunction in depression: potential therapeutic targets.', *Science (New York, N.Y.)*, 338(6103), pp. 68–72. doi: 10.1126/science.1222939.

Elmore, M. R. P. et al. (2014) 'Colony-stimulating factor 1 receptor signaling is necessary for microglia viability, unmasking a microglia progenitor cell in the adult brain', *Neuron*, 82(2), pp. 380–397. doi: 10.1016/j.neuron.2014.02.040.

Epelman, S., Lavine, K. J. and Randolph, G. J. (2014) 'Origin and Functions of Tissue Macrophages', *Immunity*, pp. 21–35. doi: 10.1016/j.immuni.2014.06.013.

References

Erblich, B. et al. (2011) 'Absence of colony stimulation factor-1 receptor results in loss of microglia, disrupted brain development and olfactory deficits', *PLoS ONE*, 6(10). doi: 10.1371/journal.pone.0026317.

Erny, D. et al. (2015) 'Host microbiota constantly control maturation and function of microglia in the CNS', *Nature Neuroscience*, 18(7), pp. 965–977. doi: 10.1038/nn.4030.

Eyo, U. B. et al. (2015) 'Modulation of Microglial Process Convergence Toward Neuronal Dendrites by Extracellular Calcium', *Journal of Neuroscience*, 35(6), pp. 2417–2422. doi: 10.1523/JNEUROSCI.3279-14.2015.

Eyo, U. B. et al. (2014) 'Neuronal Hyperactivity Recruits Microglial Processes via Neuronal NMDA Receptors and Microglial P2Y12 Receptors after Status Epilepticus', *Journal of Neuroscience*, 34(32), pp. 10528–10540. doi: 10.1523/JNEUROSCI.0416-14.2014.

Färber, K. and Kettenmann, H. (2006) 'Functional role of calcium signals for microglial function', *GLIA*, pp. 656–665. doi: 10.1002/glia.20412.

Färber, K. and Kettenmann, H. (2005) 'Physiology of microglial cells', in *Brain Research Reviews*, pp. 133–143. doi: 10.1016/j.brainresrev.2004.12.003.

Fernández de Cossío, L. et al. (2016) 'Prenatal infection leads to ASD-like behavior and altered synaptic pruning in the mouse offspring', *Brain, Behavior, and Immunity*. doi: 10.1016/j.bbi.2016.09.028.

Ferrero, G. et al. (2018). 'Embryonic Microglia Derive from Primitive Macrophages and Are Replaced by cmyb-Dependent Definitive Microglia in Zebrafish'. Cell Rep. 3;24(1):130-141. doi: 10.1016/j.celrep.2018.05.066.

Filipello, F. et al. (2018) 'The Microglial Innate Immune Receptor TREM2 Is Required for Synapse Elimination and Normal Brain Connectivity', *Immunity*, 48(5), p. 979–991.e8. doi: 10.1016/j.immuni.2018.04.016.

Flügel, A. et al. (2001) 'Migratory Activity and Functional Changes of Green Fluorescent Effector Cells before and during Experimental Autoimmune Encephalomyelitis', *Immunity*, 14(5), pp. 547–560. doi: 10.1016/S1074-7613(01)00143-1.

Fong, M. et al. (1998) 'Fractalkine and CX3CR1 mediate a novel mechanism of leukocyte capture, firm adhesion, and activation under physiologic flow.', *The Journal of experimental medicine*, 188(8), pp. 1413–1419. doi: 10.1084/jem.188.8.1413.

Fonseca, M. I. et al. (2017) 'Cell-specific deletion of C1qa identifies microglia as the dominant source of C1q in mouse brain', *Journal of Neuroinflammation*, 14(1). doi: 10.1186/s12974-017-0814-9.

Fontainhas, A. M. et al. (2011) 'Microglial morphology and dynamic behavior is regulated by ionotropic glutamatergic and GABAergic neurotransmission', *PLoS ONE*, 6(1). doi: 10.1371/journal.pone.0015973.

Fourgeaud, L. et al. (2016) 'TAM receptors regulate multiple features of microglial physiology.', *Nature*, 532(7598), pp. 1–15. doi: 10.1038/nature17630.

Friedman, BA. et al. (2018) 'Diverse Brain Myeloid Expression Profiles Reveal Distinct Microglial Activation States and Aspects of Alzheimer's Disease Not Evident in Mouse Models.' Cell Rep. 16;22(3):832-847. doi: 10.1016/j.celrep.2017.12.066.

Füger, P. et al. (2017) 'Microglia turnover with aging and in an Alzheimer's model via long-term in vivo single-cell imaging', *Nature Neuroscience*, 20(10), pp. 1371–1376. doi: 10.1038/nn.4631.

Fuhrmann, M. et al. (2010) 'Microglial Cx3cr1 knockout prevents neuron loss in a mouse model of Alzheimer's disease', *Nature Neuroscience*, 13(4), pp. 411–413. doi: 10.1038/nn.2511.

References

Galatro, TF et al. (2017). 'Transcriptomic analysis of purified human cortical microglia reveals age-associated changes.' Nat Neurosci. 20(8):1162-1171. doi: 10.1038/nn.4597.

Garcia, J. et al. (2013) 'Regulation of adaptive immunity by the fractalkine receptor during autoimmune inflammation.', *Journal of immunology (Baltimore, Md. : 1950)*, 191(3), pp. 1063–72. doi: 10.4049/jimmunol.1300040.

Garré, J. M. et al. (2017) 'CX3CR1+monocytes modulate learning and learning-dependent dendritic spine remodeling via TNF-α', *Nature Medicine*, 23(6), pp. 714–722. doi: 10.1038/nm.4340.

Garton, K. J. et al. (2001) 'Tumor Necrosis Factor-??-converting Enzyme (ADAM17) Mediates the Cleavage and Shedding of Fractalkine (CX3CL1)', *Journal of Biological Chemistry*, 276(41), pp. 37993–38001. doi: 10.1074/jbc.M106434200.

Gautier, E. L. et al. (2012) 'Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages.', *Nature immunology*, 13(11), pp. 1118–28. doi: 10.1038/ni.2419.

Geissmann, F., Jung, S. and Littman, D. R. (2003) 'Blood monocytes consist of two principal subsets with distinct migratory properties', *Immunity*, 19(1), pp. 71–82. doi: 10.1016/S1074-7613(03)00174-2.

Geissmann, F. et al. (2010) 'Development of monocytes, macrophages, and dendritic cells', *Science*, 327(5966), pp. 656–661. doi: 10.1126/science.1178331.

Ginhoux, F. et al. (2010) 'Fate mapping analysis reveals that adult microglia derive from primitive macrophages.', *Science*, 330(6005), pp. 841–5. doi: 10.1126/science.1194637.

Ginhoux, F. and Guilliams, M. (2016) 'Tissue-Resident Macrophage Ontogeny and Homeostasis', *Immunity*, pp. 439–449. doi: 10.1016/j.immuni.2016.02.024.

Ginhoux, F. and Jung, S. (2014) 'Monocytes and macrophages: developmental pathways and tissue homeostasis.', *Nature reviews. Immunology*, 14(6), pp. 392–404. doi: 10.1038/nri3671.

Ginhoux, F. and Prinz, M. (2015) 'Origin of microglia: Current concepts and past controversies', *Cold Spring Harbor Perspectives in Biology*, 7(8). doi: 10.1101/cshperspect.a020537.

Ginhoux, F. et al. (2015) 'New insights into the multidimensional concept of macrophage ontogeny, activation and function.', *Nature immunology*, 17(1), pp. 34–40. doi: 10.1038/ni.3324.

Gold, P. W. (2015) 'The organization of the stress system and its dysregulation in depressive illness', *Molecular Psychiatry*, pp. 32–47. doi: 10.1038/mp.2014.163.

Goldmann, T. et al. (2016) 'Origin, fate and dynamics of macrophages at central nervous system interfaces.', *Nature immunology*, 17(7), pp. 797–805. doi: 10.1038/ni.3423.

Goldmann, T. et al. (2013) 'A new type of microglia gene targeting shows TAK1 to be pivotal in CNS autoimmune inflammation', *Nat Neurosci*, 16(11), pp. 1618–1626. doi: 10.1038/nn.3531.

Gomes, C. et al. (2013) 'Activation of microglial cells triggers a release of brain-derived neurotrophic factor (BDNF) inducing their proliferation in an adenosine A2A receptor-dependent manner: A2A receptor blockade prevents BDNF release and proliferation of microglia', *Journal of Neuroinflammation*, 10. doi: 10.1186/1742-2094-10-16.

Gomez Perdiguero, E. et al. (2014) 'Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors', *Nature*, 518(7540), pp. 547–551. doi: 10.1038/nature13989.

References

Gosselin, D. et al. (2014) 'Environment drives selection and function of enhancers controlling tissue-specific macrophage identities', *Cell*, 159(6), pp. 1327–1340. doi: 10.1016/j.cell.2014.11.023.

Gosselin, D. et al. (2017) 'An environment-dependent transcriptional network specifies human microglia identity', *Science*, 356(6344), pp. 1248–1259. doi: 10.1126/science.aal3222.

Grabert, K. et al. (2016) 'Microglial brain region-dependent diversity and selective regional sensitivities to aging.', *Nature neuroscience*, 19(3), pp. 504–516. doi: 10.1038/nn.4222.

Graeber, M. B., Bise, K. and Mehraein, P. (1993) 'Synaptic stripping in the human facial nucleus', *Acta Neuropathol*, 86(2), pp. 179–181.

Grathwohl, S.A., (2009) 'Formation and maintenance of Alzheimer's disease beta-amyloid plaques in the absence of microglia', *Nature Neuroscience*,12(11):1361-3. doi: 10.1038/nn.2432.

Greter, M., Lelios, I. and Croxford, A. L. (2015) 'Microglia versus myeloid cell nomenclature during brain inflammation', *Frontiers in Immunology*. doi: 10.3389/fimmu.2015.00249.

Greter, M. et al. (2012) 'Stroma-Derived Interleukin-34 Controls the Development and Maintenance of Langerhans Cells and the Maintenance of Microglia', *Immunity*, 37(6), pp. 1050–1060. doi: 10.1016/j.immuni.2012.11.001.

Gu, L. et al. (2014) 'Long-Term In Vivo Imaging of Dendritic Spines in the Hippocampus Reveals Structural Plasticity', *Journal of Neuroscience*, 34(42), pp. 13948–13953. doi: 10.1523/JNEUROSCI.1464-14.2014.

Guerreiro, R. et al. (2013) 'TREM2 variants in Alzheimer's disease.' N Engl J Med. 10;368(2):117-27. doi: 10.1056/NEJMoa1211851. Epub 2012 Nov 14.

Guilliams, M. and Scott, CL. (2017) 'Does niche competition determine the origin of tissue-resident macrophages?' Nat Rev Immunol. 17(7):451-460. doi: 10.1038/nri.2017.42.

Gundra, U. M. et al. (2017) 'Vitamin A mediates conversion of monocyte-derived macrophages into tissue-resident macrophages during alternative activation', *Nature Immunology*. doi: 10.1038/ni.3734.

Gyoneva, S. et al. (2014) 'Systemic inflammation regulates microglial responses to tissue damage in vivo', *GLIA*, 62(8), pp. 1345–1360. doi: 10.1002/glia.22686.

Haas, S. et al. (1996) 'ATP-induced membrane currents in ameboid microglia acutely isolated from mouse brain slices', *Neuroscience*, 75(1), pp. 257–261. doi: 10.1016/0306-4522(96)00270-9.

Hagemeyer, N. et al. (2017) 'Microglia contribute to normal myelinogenesis and to oligodendrocyte progenitor maintenance during adulthood', *Acta Neuropathologica*, 134(3), pp. 441–458. doi: 10.1007/s00401-017-1747-1.

Hagemeyer, N. et al. (2016) 'Transcriptome-based profiling of yolk sac-derived macrophages reveals a role for Irf8 in macrophage maturation', *The EMBO Journal*, p. e201693801. doi: 10.15252/embj.201693801.

Haimon, Z. et al. (2018) 'Re-evaluating microglia expression profiles using RiboTag and cell isolation strategies /631/1647/2017 /631/1647/2017/2079 technical-report', *Nature Immunology*, 19(6), pp. 636–644. doi: 10.1038/s41590-018-0110-6.

Hammond, T. R. et al., (2018) 'Single-Cell RNA Sequencing of Microglia throughout the Mouse Lifespan and in the Injured Brain Reveals Complex Cell-State Changes', *Immunity* 2018, doi: 10.1016/j.immuni.2018.11.004.

References

Hanisch, U-K. and Kettenmann, H. (2007) 'Microglia: active sensor and versatile effector cells in the normal and pathologic brain', *Nature Neuroscience*, 10(11), pp. 1387–1394. doi: 10.1038/nn1997.

Harrison, J. K. et al. (1998) 'Role for neuronally derived fractalkine in mediating interactions between neurons and CX3CR1-expressing microglia.', *Proceedings of the National Academy of Sciences of the United States of America*, 95(18), pp. 10896–10901. doi: 10.1073/pnas.95.18.10896.

Hashimoto, D. et al. (2013) 'Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes', *Immunity*, 38(4), pp. 792–804. doi: 10.1016/j.immuni.2013.04.004.

Haskell, C. A., Cleary, M. D. and Charo, I. F. (1999) 'Molecular uncoupling of fractalkine-mediated cell adhesion and signal transduction: Rapid flow arrest of CX3CR1-expressing cells is independent of G-protein activation', *Journal of Biological Chemistry*, 274(15), pp. 10053–10058. doi: 10.1074/jbc.274.15.10053.

Hasko, G. et al. (2005) 'Adenosine receptor signaling in the brain immune system', *Trends in Pharmacological Sciences*, 26(10), pp. 511–516. doi: 10.1016/j.tips.2005.08.004.

Haynes, S. E. et al. (2006) 'The P2Y12 receptor regulates microglial activation by extracellular nucleotides.', *Nature neuroscience*, 9(12), pp. 1512–1519. doi: 10.1038/nn1805.

Hellwig, S. et al. (2016) 'Altered microglia morphology and higher resilience to stress-induced depression-like behavior in CX3CR1-deficient mice', *Brain, Behavior, and Immunity*, 55, pp. 126–137. doi: 10.1016/j.bbi.2015.11.008.

Hellwig, S., Heinrich, A. and Biber, K. (2013) 'The brain's best friend: microglial neurotoxicity revisited.', *Frontiers in cellular neuroscience*, 7(May), p. 71. doi: 10.3389/fncel.2013.00071.

Hickman, S. E. et al. (2013) 'The microglial sensome revealed by direct RNA sequencing.', *Nature neuroscience*, 16(12), pp. 1896–905. doi: 10.1038/nn.3554.

Hinwood, M. et al. (2012) 'Evidence that microglia mediate the neurobiological effects of chronic psychological stress on the medial prefrontal cortex', *Cerebral Cortex*, 22(6), pp. 1442–1454. doi: 10.1093/cercor/bhr229.

Hinwood, M. et al. (2013) 'Chronic stress induced remodeling of the prefrontal cortex: Structural re-organization of microglia and the inhibitory effect of minocycline', *Cerebral Cortex*, 23(8), pp. 1784–1797. doi: 10.1093/cercor/bhs151.

Hirasawa, T. et al. (2005) 'Visualization of microglia in living tissues using Iba1-EGFP transgenic mice', *Journal of Neuroscience Research*, 81(3), pp. 357–362. doi: 10.1002/jnr.20480.

Hodes, G. E. et al. (2015) 'Neuroimmune mechanisms of depression', *Nature Neuroscience*, pp. 1386–1393. doi: 10.1038/nn.4113.

Hoeffel, G. et al. (2015) 'C-Myb^{+} Erythro-Myeloid Progenitor-Derived Fetal Monocytes Give Rise to Adult Tissue-Resident Macrophages', *Immunity*, 42(4), pp. 665–678. doi: 10.1016/j.immuni.2015.03.011.

Hoeffel, G. and Ginhoux, F. (2018) 'Fetal monocytes and the origins of tissue-resident macrophages', Cellular Immunology. doi: 10.1016/j.cellimm.2018.01.001.

Hoffmann, A. et al. (2003) 'Elevation of basal intracellular calcium as a central element in the activation of brain macrophages (microglia): suppression of receptor-evoked calcium signaling and control of release function.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 23(11), pp. 4410–9. doi: 23/11/4410 [pii].

Holmes, S. et al. (2017) 'Elevated translocator protein in anterior cingulate in major depression and a role for inflammation in suicidal thinking: a positron emission tomography study', *Biological Psychiatry*. doi: 10.1016/j.biopsych.2017.08.005.

References

Holtman, I. R. et al. (2015) 'Induction of a common microglia gene expression signature by aging and neurodegenerative conditions: a co-expression meta-analysis', *Acta Neuropathologica Communications*, 3(1), p. 31. doi: 10.1186/s40478-015-0203-5.

Honda, S. et al. (2001) 'Extracellular ATP or ADP induce chemotaxis of cultured microglia through Gi/o-coupled P2Y receptors.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 21(6), pp. 1975–82. doi: 21/6/1975 [pii].

Hong, S. et al. (2016) 'Complement and microglia mediate early synapse loss in Alzheimer mouse models', *Science*, 352(6286), pp. 712–716. doi: 10.1126/science.aad8373.

Hong, S. and Stevens, B. (2016) 'Microglia: Phagocytosing to Clear, Sculpt, and Eliminate', *Developmental Cell*, pp. 126–128. doi: 10.1016/j.devcel.2016.07.006.

Hoshiko, M. et al. (2012) 'Deficiency of the microglial receptor CX3CR1 impairs postnatal functional development of thalamocortical synapses in the barrel cortex', *J Neurosci*, 32(43),pp.15106–15111.doi:10.1523/JNEUROSCI.1167-12.2012

Huang, Y. et al. (2018) 'Repopulated microglia are solely derived from the proliferation of residual microglia after acute depletion', *Nature Neuroscience*, 21(4), pp. 530–540. doi: 10.1038/s41593-018-0090-8.

Hughes, E. G. et al. (2013) 'Oligodendrocyte progenitors balance growth with self-repulsion to achieve homeostasis in the adult brain', *Nature Neuroscience*, 16(6), pp. 668–676. doi: 10.1038/nn.3390.

Huh, G. S. et al. (2000) 'Functional Requirement for Class I MHC in CNS Development and Plasticity', *Science*, 290(5499), pp. 2155–2159. doi: 10.1126/science.290.5499.2155.

Hultman, R. et al. (2018) 'Brain-wide Electrical Spatiotemporal Dynamics Encode Depression Vulnerability', *Cell*, 173(1), p. 166–180.e14. doi: 10.1016/j.cell.2018.02.012.

Hume, D. A. and MacDonald, K. P. A. (2012) 'Therapeutic applications of macrophage colony-stimulating factor-1 (CSF-1) and antagonists of CSF-1 receptor (CSF-1R) signaling', *Blood*, pp. 1810–1820. doi: 10.1182/blood-2011-09-379214.

Hundhausen, C. et al. (2003) 'The disintegrin-like metalloproteinase ADAM10 is involved in constitutive cleavage of CX3CL1 (fractalkine) and regulates CX3CL1-mediated cell-cell adhesion', *Blood*, 102(4), pp. 1186–1195. doi: 10.1182/blood-2002-12-3775.

Illes, P., Norenberg, W. and Gebicke-Haerter, P. J. (1996) 'Molecular mechanisms of microglial activation. B. Voltage- and purinoceptor-operated channels in microglia', *Neurochemistry International*, pp.13–24.doi:10.1016/0197-0186(95)00133-6.

Ilschner, S. et al. (1995) 'Modulation of potassium currents in cultured murine microglial cells by receptor activation and intracellular pathways', *Neuroscience*, 66(4), pp. 983–1000. doi: 10.1016/0306-4522(95)00030-M.

Imai, T. et al. (1997) 'Identification and Molecular Characterization of Fractalkine Receptor CX3CR1, which Mediates Both Leukocyte Migration and Adhesion', *Cell*, 91(4), pp. 521–530. doi: 10.1016/S0092-8674(00)80438-9.

Imai, Y. et al. (1996) 'A novel gene iba1 in the major histocompatibility complex class III region encoding an EF hand protein expressed in a monocytic lineage.', *Biochemical and biophysical research communications*, 224(3), pp. 855–62. doi: 10.1006/bbrc.1996.1112.

Irino, Y. et al. (2008) 'Akt activation is involved in P2Y12 receptor-mediated chemotaxis of microglia', *Journal of Neuroscience Research*, 86(7), pp. 1511–1519. doi: 10.1002/jnr.21610.

References

Ito, D. et al. (1998) 'Microglia-specific localisation of a novel calcium binding protein, Iba1', *Molecular Brain Research*, 57(1), pp. 1–9. doi: 10.1016/S0169-328X(98)00040-0.

Ito, D. et a. (2001) 'Enhanced expression of Iba1, ionized calcium-binding adapter molecule 1, after transient focal cerebral ischemia in rat brain', *Stroke*, 32(5), pp. 1208–1215. doi: 10.1161/01.STR.32.5.1208.

Iwasaki, H. et al. (2005) 'Distinctive and indispensable roles of PU.1 in maintenance of hematopoietic stem cells and their differentiation', *Blood*, 106(5), pp. 1590–1600. doi: 10.1182/blood-2005-03-0860.

Iwata, M. et al. (2016) 'Psychological stress activates the inflammasome via release of adenosine triphosphate and stimulation of the purinergic type 2X7 receptor', *Biological Psychiatry*, 80(1), pp. 12–22. doi: 10.1016/j.biopsych.2015.11.026.

Jagannathan-Bogdan, M. and Zon, L. I. (2013) 'Hematopoiesis.', *Development (Cambridge, England)*, 140(12), pp. 2463–7. doi: 10.1242/dev.083147.

Janova, H. et al. (2018) 'Microglia ablation alleviates myelin-associated catatonic signs in mice', *Journal of Clinical Investigation*, 128(2), pp. 734–745. doi: 10.1172/JCI97032.

Ji, K. et al. (2013) 'Microglia Actively Regulate the Number of Functional Synapses', *PLoS ONE*, 8(2). doi: 10.1371/journal.pone.0056293.

Jonsson, T. et al. (2013) 'Variant of TREM2 associated with the risk of Alzheimer's disease'. *N Engl J Med.* 10;368(2):107-16. doi: 10.1056/NEJMoa1211103.

Jordão, MJC et al. (2019) 'Single-cell profiling identifies myeloid cell subsets with distinct fates during neuroinflammation', *Science* 25;363(6425).

Jung, S. et al. (2000) 'Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion', *Mol Cell Biol*, 20(11), pp. 4106–4114. doi: 10.1128/MCB.20.11.4106-4114.2000.Updated.

Kaifu, T. et al. (2003) 'Osteopetrosis and thalamic hypomyelinosis with synaptic degeneration in DAP12-deficient mice', *Journal of Clinical Investigation*, 111(3), pp. 323–332. doi: 10.1172/JCI200316923.

Kaneko, M. et al. (2008) 'Tumor Necrosis Factor-alpha Mediates One Component of Competitive, Experience-Dependent Plasticity in Developing Visual Cortex', *Neuron*, 58(5), pp. 673–680. doi: 10.1016/j.neuron.2008.04.023.

Kang, H. J. et al. (2012) 'Decreased expression of synapse-related genes and loss of synapses in major depressive disorder', *Nature Medicine*, 18(9), pp. 1413–1417. doi: 10.1038/nm.2886.

Kao, A. W. et al. (2017) 'Progranulin, lysosomal regulation and neurodegenerative disease', *Nature Reviews Neuroscience*, pp. 1–9. doi: 10.1038/nrn.2017.36.

Kappelmann, N. et al. (2016) 'Antidepressant activity of anti-cytokine treatment: a systematic review and meta-analysis of clinical trials of chronic inflammatory conditions', *Nature Publishing Group*, doi(10), pp. 1–9. doi: 10.1038/mp.2016.167.

Kato, G. et al. (2016) 'Microglial Contact Prevents Excess Depolarization and Rescues Neurons from Excitotoxicity', *eNeuro*, 3(3). doi: 10.1523/ENEURO.0004-16.2016.

Kendler, K. S., Karkowski, L. M. and Prescott, C. A. (1999) 'Causal Relationship Between Stressful Life Events and the Onset of Major Depression', *Psychiatry Interpersonal and Biological Processes*, 156(June), pp. 837–841. doi: 10.1176/ajp.156.6.837.

References

Kennedy, D. W. and Abkowitz, J. L. (1997) 'Kinetics of central nervous system microglial and macrophage engraftment: analysis using a transgenic bone marrow transplantation model.', *Blood*, 90(3), pp. 986–93. doi: 10.1002/ajmg.1320490212.

Keren-Shaul, H. et al. (2017) 'A Unique Microglia Type Associated with Restricting Development of Alzheimer's Disease', *Cell*, 169(7), p. 1276–1290.e17. doi: 10.1016/j.cell.2017.05.018.

Kessler, R. C. and Bromet, E. J. (2013) 'The Epidemiology of Depression Across Cultures', *Annual Review of Public Health*, 34(1), pp. 119–138. doi: 10.1146/annurev-publhealth-031912-114409.

Kettenmann, H. (2007) 'The brain's garbage men', *Nature*, 446(26), pp. 989–991. doi: 10.1029/2005GB002558.

Kettenmann, H. et al. (2011) 'Physiology of microglia.', *Physiological reviews*, 91(2), pp. 461–553. doi: 10.1152/physrev.00011.2010.

Kettenmann, H. et al. (1990) 'Cultured microglial cells have a distinct pattern of membrane channels different from peritoneal macrophages', *Journal of Neuroscience Research*, 26(3), pp. 278–287. doi: 10.1002/jnr.490260303.

Kettenmann, H., Kirchhoff, F. and Verkhratsky, A. (2013) 'Microglia: New Roles for the Synaptic Stripper', *Neuron*, pp. 10–18. doi: 10.1016/j.neuron.2012.12.023.

Kheirbek, M.A. et al. (2013) 'Differential control of learning and anxiety along the dorsoventral axis of the dentate gyrus', *Neuron* 77(5):955-68. doi: 10.1016/j.neuron.2012.12.038.

El Khoury, J. et al. (2007) 'Ccr2 deficiency impairs microglial accumulation and accelerates progression of Alzheimer-like disease.', *Nature medicine*, 13(4), pp. 432–8. doi: 10.1038/nm1555.

Kierdorf, K. et al. (2013) 'Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8-dependent pathways', *Nature Neuroscience*, 16, pp. 273–280. doi: 10.1038/nn.3318.

Kierdorf, K. et al. (2016) 'Development and function of tissue resident macrophages in mice', *Seminars in Immunology*. doi: 10.1016/j.smim.2016.03.017.

Kierdorf, K. and Prinz, M. (2017) 'Microglia in steady state.' J Clin Invest. 1;127(9):3201-3209. doi: 10.1172/JCI90602.

Kim, H.-J. et al. (2016) 'Deficient autophagy in microglia impairs synaptic pruning and causes social behavioral defects', *Molecular Psychiatry*. doi: 10.1038/mp.2016.103.

Kim, K. et al. (2011) 'In vivo structure / function and expression analysis of the CX3C chemokine fractalkine', *Blood*, 118(22), pp. 156–167. doi: 10.1182/blood-2011-04-348946.This.

Knowland, D. and Lim, B. K. (2018) 'Circuit-based frameworks of depressive behaviors: The role of reward circuitry and beyond', *Pharmacology Biochemistry and Behavior*. doi: 10.1016/j.pbb.2017.12.010.

Köhler, C. A. et al., (2017) 'Peripheral cytokine and chemokine alterations in depression: a meta-analysis of 82 studies', Acta Psychiatr Scand. 2017 May;135(5):373-387. doi: 10.1111/acps.12698.

Koizumi, S. et al. (2013) 'Purinergic receptors in microglia: Functional modal shifts of microglia mediated by P2 and P1 receptors', *GLIA*, 61(1), pp. 47–54. doi: 10.1002/glia.22358.

Koizumi, S. et al. (2007) 'UDP acting at P2Y6 receptors is a mediator of microglial phagocytosis', *Nature*, 446(7139), pp. 1091–1095. doi: 10.1038/nature05704.

References

Koronyo, Y. et al. (2015) 'Therapeutic effects of glatiramer acetate and grafted CD115(+) monocytes in a mouse model of Alzheimer's disease', *Brain*, 138, pp. 2399–2422. doi: 10.1093/brain/awv150.

Krabbe, G. et al. (2013) 'Functional Impairment of Microglia Coincides with Beta-Amyloid Deposition in Mice with Alzheimer-Like Pathology', *PLoS ONE*, 8(4). doi: 10.1371/journal.pone.0060921.

Krabbe, G. et al. (2017) 'Microglial NFκB-TNFα hyperactivation induces obsessive–compulsive behavior in mouse models of progranulin-deficient frontotemporal dementia', *Proceedings of the National Academy of Sciences*, p. 201700477. doi: 10.1073/pnas.1700477114.

Krasemann, S. et al. (2017) 'The TREM2-APOE Pathway Drives the Transcriptional Phenotype of Dysfunctional Microglia in Neurodegenerative Diseases', *Immunity*, 47(3), p. 566–581.e9. doi: 10.1016/j.immuni.2017.08.008.

Kreisel, T. et al. (2014) 'Dynamic microglial alterations underlie stress-induced depressive-like behavior and suppressed neurogenesis', *Molecular Psychiatry*, 19(6), pp. 699–709. doi: 10.1038/mp.2013.155.

Kress, H. et al. (2007) 'Filopodia act as phagocytic tentacles and pull with discrete steps and a load-dependent velocity.', *Proceedings of the National Academy of Sciences of the United States of America*, 104(28), pp. 11633–11638. doi: 10.1073/pnas.0702449104.

Kreutzberg, G. W. (1996) 'Microglia: A sensor for pathological events in the CNS', *Trends in Neurosciences*, pp. 312–318. doi: 10.1016/0166-2236(96)10049-7.

Krishnan, V. and Nestler, E. J. (2008) 'The molecular neurobiology of depression', *Nature*, pp. 894–902. doi: 10.1038/nature07455.

Krügel, U. (2016) 'Purinergic receptors in psychiatric disorders', *Neuropharmacology*, pp. 212–225. doi: 10.1016/j.neuropharm.2015.10.032.

Krügel, U. et al. (2013) 'Antidepressant effects of TNF-α blockade in an animal model of depression', *Journal of Psychiatric Research*, 47(5), pp. 611–616. doi: 10.1016/j.jpsychires.2013.01.007.

Kurpius, D., Nolley, E. P. and Dailey, M. E. (2007) 'Purines induce directed migration and rapid homing of microglia to injured pyramidal neurons in developing hippocampus', *GLIA*, 55(8), pp. 873–884. doi: 10.1002/glia.20509.

van de Laar, L. et al. (2016) 'Yolk Sac Macrophages, Fetal Liver, and Adult Monocytes Can Colonize an Empty Niche and Develop into Functional Tissue-Resident Macrophages', *Immunity*, 44(4), pp.755–768. doi:10.1016/j.immuni.2016

Landsman, L. et al. (2009) 'CX3CR1 is required for monocyte homeostasis and atherogenesis by promoting cell survival', *Blood*, 113(4), pp. 963–972. doi: 10.1182/blood-2008-07-170787.

Lavin, Y. et al. (2015) 'Regulation of macrophage development and function in peripheral tissues.', *Nature reviews. Immunology*, 15(12), pp. 731–44. doi: 10.1038/nri3920.

Lavin, Y. et al. (2014) 'Tissue-resident macrophage enhancer landscapes are shaped by the local microenvironment', *Cell*, 159(6), pp. 1312–1326. doi: 10.1016/j.cell.2014.11.018.

Lawson, L. J., Perry, V. H. and Gordon, S. (1992) 'Turnover of resident microglia in the normal adult mouse brain', *Neuroscience*, 48(2), pp. 405–415. doi: 10.1016/0306-4522(92)90500-2.

Lebson, L. A. (2010) 'Monocytes as gene therapy vectors for the treatment of Alzheimer's disease'. J Neurosci. 21; 30(29): 9651–9658. doi: 10.1523/JNEUROSCI.0329-10.2010

References

Leday, G. G. R. et al. (2017) 'Replicable and Coupled Changes in Innate and Adaptive Immune Gene Expression in Two Case-Control Studies of Blood Microarrays in Major Depressive Disorder', *Biological Psychiatry*. doi: 10.1016/j.biopsych.2017.01.021.

Lee, S. et al. (2010) 'CX3CR1 deficiency alters microglial activation and reduces beta-amyloid deposition in two Alzheimer's disease mouse models.', *The American journal of pathology*, 177(5), pp. 2549–62. doi: 10.2353/ajpath.2010.100265.

Lee, H. et al. (2014), 'Synapse elimination and learning rules co-regulated by MHC class I H2-Db', Nature, 509(7499):195-200. doi: 10.1038/nature13154.

Lehrman, E. K. et al. (2018) 'CD47 Protects Synapses from Excess Microglia-Mediated Pruning during Development', *Neuron* 10;100(1):120-134.e6. doi: 10.1016/j.neuron.2018.09.017.

Leighton, S. P. et al. (2017) 'Chemokines in depression in health and in inflammatory illness: a systematic review and meta-analysis', *Molecular Psychiatry*. doi: 10.1038/mp.2017.205.

Lewitus, G. M. et al. (2016) 'Microglial TNF-α Suppresses Cocaine-Induced Plasticity and Behavioral Sensitization', *Neuron*, 90(3), pp. 483–491. doi: 10.1016/j.neuron.2016.03.030.

Li, Q. et al. (2019) 'Developmental Heterogeneity of Microglia and Brain Myeloid Cells Revealed by Deep Single-Cell RNA Sequencing', *Neuron* 16;101(2):207-223.e10. doi: 10.1016/j.neuron.2018.12.006.

Li, Q. and Barres, B. A. (2017) 'Microglia and macrophages in brain homeostasis and disease', *Nature Reviews Immunology*. doi: 10.1038/nri.2017.125.

Li, Y. et al. (2012) 'Reciprocal Regulation between Resting Microglial Dynamics and Neuronal Activity In Vivo', *Developmental Cell*, 23(6), pp. 1189–1202. doi: 10.1016/j.devcel.2012.10.027.

Liddelow, S. A. et al. (2017) 'Neurotoxic reactive astrocytes are induced by activated microglia', *Nature*, 541(7638), pp. 481–487. doi: 10.1038/nature21029.

Lim, S. H. et al. (2013) 'Neuronal synapse formation induced by microglia and interleukin 10', *PLoS ONE*, 8(11). doi: 10.1371/journal.pone.0081218.

Limatola, C. and Ransohoff, R. M. (2014) 'Modulating neurotoxicity through CX3CL1/CX3CR1 signaling', *Frontiers in Cellular Neuroscience*, 8. doi: 10.3389/fncel.2014.00229.

Lin, H. et al. (2008) 'Discovery of a Cytokine and Its Receptor by Functional Screening of the Extracellular Proteome', *Science*, 320(5877), pp. 807–811. doi: 10.1126/science.1154370.

Liu, G. J. et al. (2009) 'Glutamate induces directed chemotaxis of microglia', *European Journal of Neuroscience*, 29(6), pp. 1108–1118. doi: 10.1111/j.1460-9568.2009.06659.x.

Liu, Z. et al. (2010) 'CX3CR1 in microglia regulates brain amyloid deposition through selective protofibrillar amyloid-β phagocytosis.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 30(50), pp. 17091–101. doi: 10.1523/JNEUROSCI.4403-10.2010.

Llorens-Martín, M. et al. (2016) 'Forced swimming sabotages the morphological and synaptic maturation of newborn granule neurons and triggers a unique pro-inflammatory milieu in the hippocampus', *Brain, Behavior, and Immunity*, 53, pp. 242–254. doi: 10.1016/j.bbi.2015.12.019.

References

Lovatt, D. et al. (2012) 'Neuronal adenosine release, and not astrocytic ATP release, mediates feedback inhibition of excitatory activity', *Proceedings of the National Academy of Sciences*, 109(16), pp. 6265–6270. doi: 10.1073/pnas.1120997109.

Lui, H. et al. (2016) 'Progranulin Deficiency Promotes Circuit-Specific Synaptic Pruning by Microglia via Complement Activation', *Cell*, 165(4), pp. 921–935. doi: 10.1016/j.cell.2016.04.001.

Lund, H. et al. (2018) 'Fatal demyelinating disease is induced by monocyte-derived macrophages in the absence of TGF-β signaling', *Nature Immunology*. doi: 10.1038/s41590-018-0091-5.

Lund, H. (2018b) 'Competitive repopulation of an empty microglial niche yields functionally distinct subsets of microglia-like cells', Nat Commun. 9(1):4845. doi: 10.1038/s41467-018-07295-7.

Luo, J. et al. (2013) 'Colony-stimulating factor 1 receptor (CSF1R) signaling in injured neurons facilitates protection and survival.', *The Journal of experimental medicine*, 210(1), pp. 157–72. doi: 10.1084/jem.20120412.

Lyons, A. et al. (2009) 'Fractalkine-induced activation of the phosphatidylinositol-3 kinase pathway attentuates microglial activation in vivo and in vitro', *Journal of Neurochemistry*, 110(5), pp. 1547–1556. doi: 10.1111/j.1471-4159.2009.06253.x.

MacDonald, K. P. A. et al. (2010) 'An antibody against the colony-stimulating factor 1 receptor depletes the resident subset of monocytes and tissue- and tumor-associated macrophages but does not inhibit inflammation', *Blood*, 116(19), pp. 3955–3963. doi: 10.1182/blood-2010-02-266296.

MacQueen, G. and Frodl, T. (2011) 'The hippocampus in major depression: Evidence for the convergence of the bench and bedside in psychiatric research', *Molecular Psychiatry*, 16(3), pp. 252–264. doi: 10.1038/mp.2010.80.

Madry, C. et al. (2018) 'Microglial Ramification, Surveillance, and Interleukin-1β Release Are Regulated by the Two-Pore Domain K + Channel THIK-1', *Neuron*, 97(2), p. 299–312.e6. doi: 10.1016/j.neuron.2017.12.002.

Madry, C. et al. (2018b) 'Effects of the ecto-ATPase apyrase on microglial ramification and surveillance reflect cell depolarization, not ATP depletion', *Proceedings of the National Academy of Sciences*, p. 201715354. doi: 10.1073/pnas.1715354115.

Maggi, L. et al. (2011) 'CX(3)CR1 deficiency alters hippocampal-dependent plasticity phenomena blunting the effects of enriched environment', *Front Cell Neurosci.*, 5(1662–5102 (Electronic)), p. 22. doi: 10.3389/fncel.2011.00022.

Marín-Teva, J. L. et al. (2004) 'Microglia Promote the Death of Developing Purkinje Cells', *Neuron*, 41(4), pp. 535–547. doi: 10.1016/S0896-6273(04)00069-8.

Masgrau, R. et al. (2017) 'Should We Stop Saying "Glia" and "Neuroinflammation"?', *Trends in Molecular Medicine*, pp. 486–500. doi: 10.1016/j.molmed.2017.04.005.

Mass, E. et al. (2016) 'Specification of tissue-resident macrophages during organogenesis', *Science*, 9;353(6304). pii: aaf4238. doi: 10.1126/science.aaf4238.

Mass, E. et al. (2017) 'A somatic mutation in erythro-myeloid progenitors causes neurodegenerative disease', *Nature*, 549(7672), pp. 389–393. doi: 10.1038/nature23672.

Masuch, A. et al. (2016) 'Mechanism of microglia neuroprotection: Involvement of P2X7, TNFα, and valproic acid', *GLIA*, 64(1), pp. 76–89. doi: 10.1002/glia.22904.

Matcovitch-Natan, O. et al. (2016) 'Microglia development follows a stepwise program to regulate brain homeostasis', *Science*, 353(6301), pp. aad8670-aad8670. doi: 10.1126/science.aad8670.

References

Mathys, H. et al. (2017) 'Temporal Tracking of Microglia Activation in Neurodegeneration at Single-Cell Resolution', *Cell Reports*, 21(2), pp. 366–380. doi: 10.1016/j.celrep.2017.09.039.

Mazaheri, F. et al. (2014) 'Distinct roles for BAI1 and TIM-4 in the engulfment of dying neurons by microglia', *Nature Communications*, 5. doi: 10.1038/ncomms5046.

McGrath, K. E. et al. (2015) 'Distinct Sources of Hematopoietic Progenitors Emerge before HSCs and Provide Functional Blood Cells in the Mammalian Embryo', *Cell Reports*, 11(12), pp. 1892–1904. doi: 10.1016/j.celrep.2015.05.036.

McGrath, K. E. et al. (2003) 'Circulation is established in a stepwise pattern in the mammalian embryo', *Blood*, 101(5), pp. 1669–1676. doi: 10.1182/blood-2002-08-2531.

McKercher, S. R. et al. (1996) 'Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities.', *The EMBO journal*, 15(20), pp. 5647–5658.

McKim, D. B. et al. (2017) 'Microglial recruitment of IL-1β-producing monocytes to brain endothelium causes stress-induced anxiety', *Molecular Psychiatry*. doi: 10.1038/mp.2017.64.

McVicar, D. W. and Trinchieri, G. (2009) 'CSF-1R, DAP12 and beta-catenin: a menage a trois', *Nat Immunol*, 10(7), pp. 681–683. doi: 10.1038/ni0709-681.

Menard, C. et al. (2017) 'Social stress induces neurovascular pathology promoting depression', *Nature Neuroscience*, 20(12), pp. 1752–1760. doi: 10.1038/s41593-017-0010-3.

Meyer-Luehmann, M. and Prinz, M. (2015) 'Myeloid Cells in Alzheimer's Disease: Culprits, Victims or Innocent Bystanders?', *Trends in Neurosciences*, pp. 659–668. doi: 10.1016/j.tins.2015.08.011.

Mildner, A. et al. (2017) 'P2Y12 receptor is expressed on human microglia under physiological conditions throughout development and is sensitive to neuroinflammatory diseases', *Glia*, 65(2), pp. 375–387. doi: 10.1002/glia.23097.

Mildner, A. et al. (2011) 'Distinct and non-redundant roles of microglia and myeloid subsets in mouse models of Alzheimer's disease.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 31(31), pp. 11159–71. doi: 10.1523/JNEUROSCI.6209-10.2011.

Mildner, A. et al. (2007) 'Microglia in the adult brain arise from Ly-6ChiCCR2+ monocytes only under defined host conditions.', *Nature neuroscience*, 10(12), pp. 1544–53. doi: 10.1038/nn2015.

Milior, G. et al. (2016) 'Fractalkine receptor deficiency impairs microglial and neuronal responsiveness to chronic stress', *Brain, Behavior, and Immunity*, 55, pp. 114–125. doi: 10.1016/j.bbi.2015.07.024.

Miller, A. H., Maletic, V. and Raison, C. L. (2009) 'Inflammation and Its Discontents: The Role of Cytokines in the Pathophysiology of Major Depression', *Biological Psychiatry*, pp. 732–741. doi: 10.1016/j.biopsych.2008.11.029.

Miller, A. H. and Raison, C. L. (2016) 'The role of inflammation in depression: From evolutionary imperative to modern treatment target', *Nature Reviews Immunology*, pp. 22–34. doi: 10.1038/nri.2015.5.

Ming, G. li and Song, H. (2011) 'Adult Neurogenesis in the Mammalian Brain: Significant Answers and Significant Questions', *Neuron*, pp. 687–702. doi: 10.1016/j.neuron.2011.05.001.

Miwa, T. et al. (1997) 'Lipopolysaccharide enhances synthesis of brain-derived neurotrophic factor in cultured rat microglia.', *Journal of neuroscience research*, 50(6), pp. 1023–9. doi: 10.1002/(sici)1097-4547(19971215)50:6<1023::aid-jnr13>3.3.co;2-s.

References

Miyamoto, A. et al. (2016) 'Microglia contact induces synapse formation in developing somatosensory cortex.', *Nature communications*, 7, p. 12540. doi: 10.1038/ncomms12540.

Miyamoto, A. et al. (2013) 'Microglia and synapse interactions: fine tuning neural circuits and candidate molecules', *Frontiers in Cellular Neuroscience*, 7. doi: 10.3389/fncel.2013.00070.

Mizuno, T. et al. (2003) 'Production and neuroprotective functions of fractalkine in the central nervous system', *Brain Research*, 979(1–2), pp. 65–70. doi: 10.1016/S0006-8993(03)02867-1.

Mizutani, M. et al. (2012) 'The Fractalkine Receptor but Not CCR2 Is Present on Microglia from Embryonic Development throughout Adulthood', *The Journal of Immunology*, 188(1), pp. 29–36. doi: 10.4049/jimmunol.1100421.

Moghaddam, B. (2002) 'Stress activation of glutamate neurotransmission in the prefrontal cortex: Implications for dopamine-associated psychiatric disorders', *Biological Psychiatry*, pp. 775–787. doi: 10.1016/S0006-3223(01)01362-2.

Möller, T. et al. (2016) 'Critical data-based re-evaluation of minocycline as a putative specific microglia inhibitor', *GLIA*, pp. 1788–1794. doi: 10.1002/glia.23007.

Möller, T. et al. (2000) 'Activation of mouse microglial cells affects P2 receptor signaling', *Brain Research*, 853(1), pp. 49–59. doi: 10.1016/S0006-8993(99)02244-1.

Mondelli, V. et al. (2017) 'Brain microglia in psychiatric disorders', *The Lancet Psychiatry*, pp. 563–572. doi: 10.1016/S2215-0366(17)30101-3.

Monif, M. et al. (2009) 'The P2X7 Receptor Drives Microglial Activation and Proliferation: A Trophic Role for P2X7R Pore', *J. Neurosci.*, 29(12), pp. 3781–3791. doi: 10.1523/JNEUROSCI.5512-08.2009.

Moran, L. B. and Graeber, M. B. (2004) 'The facial nerve axotomy model', *Brain Research Reviews*, pp. 154–178. doi: 10.1016/j.brainresrev.2003.11.004.

Moriguchi, S. et al. (2018), 'Glutamatergic neurometabolite levels in major depressive disorder: a systematic review and meta-analysis of proton magnetic resonance spectroscopy studies, Mol Psychiatry. doi: 10.1038/s41380-018-0252-9.

Mrdjen, D. et al. (2018) 'High-Dimensional Single-Cell Mapping of Central Nervous System Immune Cells Reveals Distinct Myeloid Subsets in Health, Aging, and Disease', *Immunity*, 48(2), p. 380–395.e6. doi: 10.1016/j.immuni.2018.01.011.

Mucenski, M. L. et al. (1991) 'A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis', *Cell*, 65(4), pp. 677–689. doi: 10.1016/0092-8674(91)90099-K.

Muffat, J. et al. (2016) 'Efficient derivation of microglia-like cells from human pluripotent stem cells', *Nat Med*, 22(11), pp. 1358–1367. doi: 10.1038/nm.4189.

Mul, J. D., Zheng, J. and Goodyear, L. J. (2016) 'Validity Assessment of 5 Day Repeated Forced-Swim Stress to Model Human Depression in Young-Adult C57BL/6J and BALB/cJ Mice', *eneuro*, 3(6), p. ENEURO.0201-16.2016.

Musazzi, L. et al. (2013) 'The action of antidepressants on the glutamate system: Regulation of glutamate release and glutamate receptors', *Biological Psychiatry*, pp. 1180–1188. doi: 10.1016/j.biopsych.2012.11.009.

Musazzi, L., Treccani, G. and Popoli, M. (2015) 'Functional and structural remodeling of glutamate synapses in prefrontal and frontal cortex induced by behavioral stress', *Frontiers in Psychiatry*. doi: 10.3389/fpsyt.2015.00060.

References

Nagarajan, N. et al. (2017) 'Corticostriatal circuit defects in Hoxb8 mutant mice.' *Mol Psychiatry*. doi: 10.1038/mp.2017.251.

Nandi, S. et al. (2013) 'Receptor-type protein-tyrosine phosphatase ζ is a functional receptor for interleukin-34', *Journal of Biological Chemistry*, 288(30), pp. 21972–21986. doi: 10.1074/jbc.M112.442731.

Nandi, S. et al. (2012) 'The CSF-1 receptor ligands IL-34 and CSF-1 exhibit distinct developmental brain expression patterns and regulate neural progenitor cell maintenance and maturation', *Developmental Biology*, 367(2), pp. 100–113. doi: 10.1016/j.ydbio.2012.03.026.

Nayak, D., Roth, T. L. and McGavern, D. B. (2014) 'Microglia Development and Function', *Annual Review of Immunology*, 32(1), pp. 367–402. doi: 10.1146/annurev-immunol-032713-120240.

Neniskyte, U. and Gross, C. T. (2017) 'Errant gardeners: Glial-cell-dependent synaptic pruning and neurodevelopmental disorders', *Nature Reviews Neuroscience*, pp. 658–670. doi: 10.1038/nrn.2017.110.

Nie, X. et al. (2018). The Innate Immune Receptors TLR2/4 Mediate Repeated Social Defeat Stress-Induced Social Avoidance through Prefrontal Microglial Activation. *Neuron*, 8;99(3):464-479.e7. doi: 10.1016/j.neuron.2018.06.035.

Nimmerjahn, A., Kirchhoff, F. and Helmchen, F. (2005) 'Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo', *Neuroforum*, 11(3), pp. 95–96. doi: 10.1126/science.1110647.

Nishiyori, A. et al. (1998) 'Localization of fractalkine and CX3CR1 mRNAs in rat brain: Does fractalkine play a role in signaling from neuron to microglia?', *FEBS Letters*, 429(2), pp. 167–172. doi: 10.1016/S0014-5793(98)00583-3.

Normann, C. et al. (2017) 'Antidepressants Rescue Stress-Induced Disruption of Synaptic Plasticity via Serotonin Transporter-Independent Inhibition of L-Type Calcium Channels', *Biological Psychiatry*. doi: 10.1016/j.biopsych.2017.10.008.

Ohsawa, K. et al. (2000) 'Involvement of Iba1 in membrane ruffling and phagocytosis of macrophages/microglia.', *Journal of cell science*, 113 (Pt 1, pp. 3073–3084.

Ohsawa, K. et al. (2007) 'Involvement of P2X4 and P2Y12 receptors in ATP-induced microglial chemotaxis', *GLIA*, 55(6), pp. 604–616. doi: 10.1002/glia.20489.

Ohsawa, K. et al. (2010) 'P2Y12 receptor-mediated integrin-β1 activation regulates microglial process extension induced by ATP', *GLIA*, 58(7), pp. 790–801. doi: 10.1002/glia.20963.

Ohsawa, K. and Kohsaka, S. (2011) 'Dynamic motility of microglia: Purinergic modulation of microglial movement in the normal and pathological brain', *GLIA*, 59(12), pp. 1793–1799. doi: 10.1002/glia.21238.

Okabe, Y. and Medzhitov, R. (2014) 'Tissue-specific signals control reversible program of localization and functional polarization of macrophages', *Cell*, 157(4), pp. 832–844. doi: 10.1016/j.cell.2014.04.016.

Okere, C. O. and Kaba, H. (2000) 'Heterogenous immunohistochemical expression of microglia-specific ionized calcium binding adaptor protein (Iba1) in the mouse olfactory bulb', *Brain Research*, 877(1), pp. 85–90. doi: 10.1016/S0006-8993(00)02656-1.

Olah, M. et al. (2018) 'A transcriptomic atlas of aged human microglia', *Nature Communications*, 9(1). doi: 10.1038/s41467-018-02926-5.

References

Oliveira, A. L. R. et al. (2004) 'A role for MHC class I molecules in synaptic plasticity and regeneration of neurons after axotomy', *Proceedings of the National Academy of Sciences of the United States of America*, 101(51), pp. 17843–17848. doi: 10.1073/pnas.0408154101.

Olmos-Alonso, A. et al. (2016) 'Pharmacological targeting of CSF1R inhibits microglial proliferation and prevents the progression of Alzheimer's-like pathology', *Brain*, 139(3), pp. 891–907. doi: 10.1093/brain/awv379.

Olson, M. C. et al. (1995) 'PU.1 is not essential for early myeloid gene expression but is required for terminal myeloid differentiation', *Immunity*, 3(6), pp. 703–714. doi: 10.1016/1074-7613(95)90060-8.

Orellana, J. A. et al. (2015) 'Restraint stress increases hemichannel activity in hippocampal glial cells and neurons', *Frontiers in Cellular Neuroscience*, 9. doi: 10.3389/fncel.2015.00102.

Orkin, S. H. and Zon, L. I. (2008) 'Hematopoiesis: An Evolving Paradigm for Stem Cell Biology', *Cell*, pp. 631–644. doi: 10.1016/j.cell.2008.01.025.

Orłowski, D., Sołtys, Z. and Janeczko, K. (2003) 'Morphological development of microglia in the postnatal rat brain. A quantitative study', *Int J Dev Neurosci*, 21(8), pp. 445–450.

Orthgiess, J. et al (2016) 'Neurons exhibit Lyz2 promoter activity in vivo: Implications for using LysM-Cre mice in myeloid cell research.' *Eur J Immunol.* 46(6):1529-32. doi: 10.1002/eji.201546108.

Orr, A. G. et al. (2009) 'Adenosine A2Areceptor mediates microglial process retraction', *Nature Neuroscience*, 12(7), pp. 872–878. doi: 10.1038/nn.2341.

Ota, K. T. et al. (2014) 'REDD1 is essential for stress-induced synaptic loss and depressive behavior', *Nature Medicine*, 20(5), pp. 531–535. doi: 10.1038/nm.3513.

Otte, C. et al. (2016) 'Major depressive disorder', *Nature Reviews Disease Primers*. doi: 10.1038/nrdp.2016.65.

Pabon, M. M. et al. (2011) 'CX3CL1 reduces neurotoxicity and microglial activation in a rat model of Parkinson's disease', *J Neuroinflammation*, 8, p. 9. doi: 10.1186/1742-2094-8-9.

Pagani, F. et al. (2015) 'Defective microglial development in the hippocampus of Cx3cr1 deficient mice.', *Frontiers in cellular neuroscience*, 9(March), p. 111. doi: 10.3389/fncel.2015.00111.

Pan, Y. et al. (1997) 'Neurotactin, a membrane-anchored chemokine upregulated in brain inflammation.', *Nature*, 387(6633), pp. 611–7. doi: 10.1038/42491.

Pandya, H. et al. (2017) 'Differentiation of human and murine induced pluripotent stem cells to microglia-like cells', *Nature Neuroscience*, 20(5), pp. 753–759. doi: 10.1038/nn.4534.

Paolicelli, R. C., Bisht, K. and Tremblay, M.-È. (2014) 'Fractalkine regulation of microglial physiology and consequences on the brain and behavior.', *Frontiers in cellular neuroscience*, 8(May), p. 129. doi: 10.3389/fncel.2014.00129.

Paolicelli, R. C. et al. (2011) 'Synaptic pruning by microglia is necessary for normal brain development.', *Science (New York, N.Y.)*, 333(6048), pp. 1456–8. doi: 10.1126/science.1202529.

Paolicelli, R. C. et al. (2017) 'TDP-43 Depletion in Microglia Promotes Amyloid Clearance but Also Induces Synapse Loss', *Neuron*. doi: 10.1016/j.neuron.2017.05.037.

Paolicelli. RC, and Ferretti, MT (2017) 'Function and Dysfunction of Microglia during Brain Development: Consequences for Synapses and Neural Circuits.' Front Synaptic Neurosci. 10;9:9. doi: 10.3389/fnsyn.2017.00009.

References

Papakostas, G. I. and Ionescu, D. F. (2015) 'Towards new mechanisms: An update on therapeutics for treatment-resistant major depressive disorder', *Molecular Psychiatry*, pp. 1142–1150. doi: 10.1038/mp.2015.92.

Paques, M. et al. (2010) 'In vivo observation of the locomotion of microglial cells in the retina', *GLIA*, 58(14), pp. 1663–1668. doi: 10.1002/glia.21037.

Paris, I. et al. (2018) 'ProMoIJ: A new tool for automatic three-dimensional analysis of microglial process motility', *GLIA*, 66(4), pp. 828–845. doi: 10.1002/glia.23287.

Parkhurst, C. N. et al. (2013) 'Microglia promote learning-dependent synapse formation through brain-derived neurotrophic factor', *Cell*, 155(7), pp. 1596–1609. doi: 10.1016/j.cell.2013.11.030.

Pascual, O. et al. (2012) 'Microglia activation triggers astrocyte-mediated modulation of excitatory neurotransmission', *Proceedings of the National Academy of Sciences*, 109(4), pp. E197–E205. doi: 10.1073/pnas.1111098109.

Pascual, O. et al. (2005) 'Neurobiology: Astrocytic purinergic signaling coordinates synaptic networks', *Science*, 310(5745), pp. 113–116. doi: 10.1126/science.1116916.

Perdiguero, E. G. and Geissmann, F. (2015) 'The development and maintenance of resident macrophages.', *Nature immunology*, 17(1), pp. 2–8. doi: 10.1038/ni.3341.

Perez-Cerda, F., Sanchez-Gomez, M. V. and Matute, C. (2015) 'Pio del Rio Hortega and the discovery of the oligodendrocytes.', *Frontiers in neuroanatomy*, 9, p. 92. doi: 10.3389/fnana.2015.00092.

Petkau, T. L. and Leavitt, B. R. (2014) 'Progranulin in neurodegenerative disease', *Trends in Neurosciences*, pp. 388–398. doi: 10.1016/j.tins.2014.04.003.

Pixley, F. J. and Stanley, E. R. (2004) 'CSF-1 regulation of the wandering macrophage: Complexity in action', *Trends in Cell Biology*, pp. 628–638. doi: 10.1016/j.tcb.2004.09.016.

Plein A., et al. (2018), 'Erythro-myeloid progenitors contribute endothelial cells to blood vessels. Nature 562(7726):223-228. doi: 10.1038/s41586-018-0552-x.

Pocock, J. M. and Kettenmann, H. (2007) 'Neurotransmitter receptors on microglia', *Trends in Neurosciences*, pp. 527–535. doi: 10.1016/j.tins.2007.07.007.

Popoli, M. et al. (2012) 'The stressed synapse: The impact of stress and glucocorticoids on glutamate transmission', *Nature Reviews Neuroscience*, pp. 22–37. doi: 10.1038/nrn3138.

Powell, N. D. et al. (2013) 'Social stress up-regulates inflammatory gene expression in the leukocyte transcriptome via -adrenergic induction of myelopoiesis', *Proceedings of the National Academy of Sciences*, 110(41), pp. 16574–16579. doi: 10.1073/pnas.1310655110.

Pribiag, H. and Stellwagen, D. (2013) 'TNF-α Downregulates Inhibitory Neurotransmission through Protein Phosphatase 1-Dependent Trafficking of GABAA Receptors', *Journal of Neuroscience*, 33(40), pp. 15879–15893. doi: 10.1523/JNEUROSCI.0530-13.2013.

Price, J. L. and Drevets, W. C. (2012) 'Neural circuits underlying the pathophysiology of mood disorders', *Trends in Cognitive Sciences*, pp. 61–71. doi: 10.1016/j.tics.2011.12.011.

Priller, J. et al. (2001) 'Targeting gene-modified hematopoietic cells to the central nervous system: use of green fluorescent protein uncovers microglial engraftment.', *Nature medicine*, 7(12), pp. 1356–61. doi: 10.1038/nm1201-1356.

References

Prinz, M. et al. (1999) 'Microglial activation by components of gram-positive and -negative bacteria: distinct and common routes to the induction of ion channels and cytokines.', *Journal of neuropathology and experimental neurology*, pp. 1078–1089. doi: 10.1097/00005072-199910000-00006.

Prinz, M. and Priller, J. (2014) 'Microglia and brain macrophages in the molecular age: from origin to neuropsychiatric disease.', *Nature reviews. Neuroscience*, 15(5), pp. 300–12. doi: 10.1038/nrn3722.

Prinz, M. et al. (2011) 'Heterogeneity of CNS myeloid cells and their roles in neurodegeneration', *Nat.Neurosci.*, 14(1546–1726 (Electronic)), pp. 1227–1235. doi: 10.1038/nn.2923.

Qin, Y. et al. (2018) 'A Milieu Molecule for TGF-β Required for Microglia Function in the Nervous System', Cell 174(1):156-171.e16. doi: 10.1016/j.cell.2018.05.027.

Raison, C. L. and Miller, A. H. (2003) 'When not enough is too much: The role of insufficient glucocorticoid signaling in the pathophysiology of stress-related disorders', *American Journal of Psychiatry*, pp. 1554–1565. doi: 10.1176/appi.ajp.160.9.1554.

Rajkowska, G. et al. (1999) 'Morphometric evidence for neuronal and glial prefrontal cell pathology in major depression', *Biological Psychiatry*, 45(9), pp. 1085–1098. doi: 10.1016/S0006-3223(99)00041-4.

Ransohoff, R. M. and Cardona, A. E. (2010) 'The myeloid cells of the central nervous system parenchyma.', *Nature*, 468(7321), pp. 253–62. doi: 10.1038/nature09615.

Ransohoff, R. M. and El Khoury, J. (2015) 'KNIHA - Microglia in health and disease', *Cold Spring Harbor Perspectives in Biology*, pp. 1–16. doi: 10.1101/cshperspect.a020560.

Reshef, R. et al. (2014) 'Microglia and their CX3CR1 signaling are involved in hippocampal- but not olfactory bulb-related memory and neurogenesis', *Brain, Behavior, and Immunity*, 41(1), pp. 239–250. doi: 10.1016/j.bbi.2014.04.009.

Reshef, R. et al. (2017) 'The role of microglia and their CX3CR1 signaling in adult neurogenesis in the olfactory bulb', *eLife*, 6. doi: 10.7554/eLife.30809.

Ressler, K. J. and Mayberg, H. S. (2007) 'Targeting abnormal neural circuits in mood and anxiety disorders: From the laboratory to the clinic', *Nature Neuroscience*, pp. 1116–1124. doi: 10.1038/nn1944.

Réu, P. et al. (2017) 'The Lifespan and Turnover of Microglia in the Human Brain', *Cell Reports*, 20(4), pp. 779–784. doi: 10.1016/j.celrep.2017.07.004.

Reznikov, L. R. et al. (2007) 'Acute stress-mediated increases in extracellular glutamate levels in the rat amygdala: differential effects of antidepressant treatment.', *The European journal of neuroscience*, 25(10), pp. 3109–14. doi: 10.1111/j.1460-9568.2007.05560.x.

Rial, D. et al. (2016) 'Depression as a Glial-Based Synaptic Dysfunction', *Frontiers in Cellular Neuroscience*, 9. doi: 10.3389/fncel.2015.00521.

Riazi, K. et al. (2008) 'Microglial activation and TNF production mediate altered CNS excitability following peripheral inflammation', *Proceedings of the National Academy of Sciences*, 105(44), pp. 17151–17156. doi: 10.1073/pnas.0806682105.

Rimmerman, N. et al. (2017) 'The hippocampal transcriptomic signature of stress resilience in mice with microglial fractalkine receptor (CX3CR1) deficiency', *Brain, Behavior, and Immunity*, 61, pp. 184–196. doi: 10.1016/j.bbi.2016.11.023.

References

Rimmerman, N., Schottlender, N. and Yirmiya, R. (2015) 'CX3CR1 (fractalkine receptor)-deficient mice exhibit resilience to chronic unpredictable stress-induced depressive-like behavior and suppressed neurogenesis', *Brain, Behavior, and Immunity*, 49, p. e10. doi: 10.1016/j.bbi.2015.06.054.

Roberson, E. D. et al. (2013) 'Dissociation of frontotemporal dementia-related deficits and neuroinflammation in progranulin haploinsufficient mice', *Annals of Internal Medicine*, 158(6), pp. 5352–5362. doi:10.1523/JNEUROSCI.

Rogers, J. T. et al. (2011) 'CX3CR1 Deficiency Leads to Impairment of Hippocampal Cognitive Function and Synaptic Plasticity', *Journal of Neuroscience*, 31(45), pp. 16241–16250. doi: 10.1523/JNEUROSCI.3667-11.2011.

Rosenbauer, F. and Tenen, D. G. (2007) 'Transcription factors in myeloid development: balancing differentiation with transformation', *Nature Reviews Immunology*, 7(2), pp. 105–117. doi: 10.1038/nri2024.

Rosin, J.M., Vora S.R. and Kurrasch D.M (2018). Depletion of embryonic microglia using the CSF1R inhibitor PLX5622 has adverse sex-specific effects on mice, including accelerated weight gain, hyperactivity and anxiolytic-like behaviour. *Brain Behavior and Immunity*, 73:682-697. doi: 10.1016/j.bbi.2018.07.023.

Rossi, D. L. et al. (1998) 'Cloning and characterization of a new type of mouse chemokine.', *Genomics*, 47(2), pp. 163–70. doi: 10.1006/geno.1997.5058.

Roumier, A. (2004) 'Impaired Synaptic Function in the Microglial KARAP/DAP12-Deficient Mouse', *Journal of Neuroscience*, 24(50), pp. 11421–11428. doi: 10.1523/JNEUROSCI.2251-04.2004.

Rupprecht, R. et al. (2010) 'Translocator protein (18 kDa) (TSPO) as a therapeutic target for neurological and psychiatric disorders', *Nature Reviews Drug Discovery*, pp. 971–988. doi: 10.1038/nrd3295.

Russo, S. J. and Nestler, E. J. (2014) 'The Brain Reward Circuitry in Mood Disorders', *Nature Reviews Neuroscience*, 14(9), pp. 1–34. doi: 10.1038/nrn3381.The.

Ryan, G. R. et al. (2001) 'Rescue of the colony-stimulating factor 1 (CSF-1)-nullizygous mouse (Csf1op/Csf1op) phenotype with a CSF-1 transgene and identification of sites of local CSF-1 synthesis', *Blood*, 98(1), pp. 74–84. doi: 10.1182/blood.V98.1.74.

Safaiyan, S. et al. (2016) 'Age-related myelin degradation burdens the clearance function of microglia during aging', *Nature Neuroscience*, 19(8), pp. 995–998. doi: 10.1038/nn.4325.

Sahay, A. and Hen, R. (2007) 'Adult hippocampal neurogenesis in depression', *Nature Neuroscience*, 10(9), pp. 1110–1115. doi: 10.1038/nn1969.

Salter, M. W. and Beggs, S. (2014) 'Sublime microglia: Expanding roles for the guardians of the CNS', *Cell*, pp. 15–24. doi: 10.1016/j.cell.2014.06.008.

Samokhvalov, I. M., Samokhvalova, N. I. and Nishikawa, S. (2007) 'Cell tracing shows the contribution of the yolk sac to adult haematopoiesis', *Nature*, 446(7139), pp. 1056–1061. doi: 10.1038/nature05725.

Samuels, B. A. et al. (2015), '5-HT1A receptors on mature dentate gyrus granule cells are critical for the antidepressant response', Nat Neurosci. 18(11):1606-16. doi: 10.1038/nn.4116.

Sanacora, G., Treccani, G. and Popoli, M. (2012) 'Towards a glutamate hypothesis of depression: An emerging frontier of neuropsychopharmacology for mood disorders', in *Neuropharmacology*, pp. 63–77. doi: 10.1016/j.neuropharm.2011.07.036.

References

Sasaki, Y. et al. (2003) 'Selective Expression of Gi/o-Coupled ATP Receptor P2Y12 in Microglia in Rat Brain', *GLIA*, 44(3), pp. 242–250. doi: 10.1002/glia.10293.

Sasaki, Y. et al. (2001) 'Iba1 is an actin-cross-linking protein in macrophages/microglia.', *Biochemical and biophysical research communications*, 286(2), pp. 292–297. doi: 10.1006/bbrc.2001.5388.

Sasmono, R. T. et al. (2003) 'A macrophage colony-stimulating factor receptor-green fluorescent protein transgene is expressed throughout the mononuclear phagocyte system of the mouse', *Blood*, 101(3), pp. 1155–1163. doi: 10.1182/blood-2002-02-0569.

Schafer, D. P. et al. (2016) 'Microglia contribute to circuit defects in Mecp2 null mice independent of microglia-specific loss of Mecp2 expression', *eLife*, 5(2016JULY). doi: 10.7554/eLife.15224.

Schafer, D. P. et al. (2012) 'Microglia Sculpt Postnatal Neural Circuits in an Activity and Complement-Dependent Manner', *Neuron*, 74(4), pp. 691–705. doi: 10.1016/j.neuron.2012.03.026.

Schafer, D. P., Lehrman, E. K. and Stevens, B. (2013) 'The "quad-partite" synapse: Microglia-synapse interactions in the developing and mature CNS', *GLIA*, 61(1), pp. 24–36. doi: 10.1002/glia.22389.

Schmid, L. C. et al. (2016) 'Dysfunction of Somatostatin-Positive Interneurons Associated with Memory Deficits in an Alzheimer's Disease Model', *Neuron*, 92(1), pp. 114–125. doi: 10.1016/j.neuron.2016.08.034.

Schulz, C. et al. (2012) 'A lineage of myeloid cells independent of Myb and hematopoietic stem cells.', *Science (New York, N.Y.)*, 336(6077), pp. 86–90. doi: 10.1126/science.1219179.

Schulz, C. et al. (2007) 'Chemokine fractalkine mediates leukocyte recruitment to inflammatory endothelial cells in flowing whole blood: A critical role for P-selectin expressed on activated platelets', *Circulation*, 116(7), pp. 764–773. doi: 10.1161/CIRCULATIONAHA.107.695189.

Scott, C. L. et al. (2016) 'Bone marrow-derived monocytes give rise to self-renewing and fully differentiated Kupffer cells', *Nature Communications*, 7, p. 10321. doi: 10.1038/ncomms10321.

Scott, E. W. et al. (1997) 'PU.1 functions in a cell-autonomous manner to control the differentiation of multipotential lymphoid-myeloid progenitors.', *Immunity*, 6(4), pp. 437–447. doi: 10.1016/S1074-7613(00)80287-3.

Scott, E. W. et al. (1994) 'Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages.', *Science (New York, N.Y.)*, 265(5178), pp. 1573–7. doi: 10.1126/science.8079170.

Sedgwick, J. D. et al. (1991) 'Isolation and direct characterization of resident microglial cells from the normal and inflamed central nervous system.', *Proceedings of the National Academy of Sciences of the United States of America*, 88(16), pp. 7438–7442. doi: 10.1073/pnas.88.16.7438.

Sekar, A. et al. (2016) 'Schizophrenia risk from complex variation of complement component 4', *Nature*, 530(7589), pp. 177–183. doi: 10.1038/nature16549.

Sekar, S. et al. (2018), 'Neuro-metabolite profiles of rodent models of psychiatric dysfunctions characterised by MR spectroscopy, Neuropharmacology. 146:109-116. doi: 10.1016/j.neuropharm.2018.11.021.

Sellner, S. et al. (2016) 'Microglial CX3CR1 promotes adult neurogenesis by inhibiting Sirt 1/p65 signaling independent of CX3CL1', *Acta neuropathologica communications*, 4(1), p. 102. doi: 10.1186/s40478-016-0374-8.

References

Serchov, T. et al. (2015) 'Increased Signaling via Adenosine A1Receptors, Sleep Deprivation, Imipramine, and Ketamine Inhibit Depressive-like Behavior via Induction of Homer1a', *Neuron*, 87(3), pp. 549–562. doi: 10.1016/j.neuron.2015.07.010.

Setiawan, E. et al. (2018) 'Association of translocator protein total distribution volume with duration of untreated major depressive disorder: a cross-sectional study', *The Lancet Psychiatry*, 5(4), pp. 339–347. doi: 10.1016/S2215-0366(18)30048-8.

Setiawan, E. et al. (2015) 'Role of translocator protein density, a marker of neuroinflammation, in the brain during major depressive episodes', *JAMA Psychiatry*, 72(3), pp. 268–275. doi: 10.1001/jamapsychiatry.2014.2427.

Shemer, A. et al. (2018) 'Engrafted parenchymal brain macrophages differ from microglia in transcriptome, chromatin landscape and response to challenge', *Nature Communication* 6;9(1):5206. doi: 10.1038/s41467-018-07548-5.

Sheng, J., Ruedl, C. and Karjalainen, K. (2015) 'Most Tissue-Resident Macrophages Except Microglia Are Derived from Fetal Hematopoietic Stem Cells', *Immunity*, 43(2), pp. 382–393. doi: 10.1016/j.immuni.2015.07.016.

Sheridan, G. K. and Murphy, K. J. (2013) 'Neuron-glia crosstalk in health and disease: fractalkine and CX3CR1 take centre stage.', *Open biology*, 3(12), p. 130181. doi: 10.1098/rsob.130181.

Shi, Q. et al. (2017) 'Complement C3 deficiency protects against neurodegeneration in aged plaque-rich APP/PS1 mice', *Science Translational Medicine*, 9(392), p. eaaf6295. doi: 10.1126/scitranslmed.aaf6295.

Shi, Q. et al. (2015) 'Complement C3-Deficient Mice Fail to Display Age-Related Hippocampal Decline', *Journal of Neuroscience*, 35(38), pp. 13029–13042. doi: 10.1523/JNEUROSCI.1698-15.2015.

Shieh, C. H. et al. (2014) 'P2X7-dependent, but differentially regulated release of IL-6, CCL2, and TNF-α in cultured mouse microglia', *GLIA*, 62(4), pp. 592–607. doi: 10.1002/glia.22628.

Shigemoto-Mogami, Y. et al. (2014) 'Microglia Enhance Neurogenesis and Oligodendrogenesis in the Early Postnatal Subventricular Zone', *Journal of Neuroscience*, 34(6), pp. 2231–2243. doi: 10.1523/JNEUROSCI.1619-13.2014.

Sieger, D. et al. (2012) 'Long-Range Ca2+ Waves Transmit Brain-Damage Signals to Microglia', *Developmental Cell*, 22(6), pp. 1138–1148. doi: 10.1016/j.devcel.2012.04.012.

Sierra, A. et al. (2016) 'The "Big-Bang" for modern glial biology: Translation and comments on Pio del Rio-Hortega 1919 series of papers on microglia', *GLIA*, 64(11), pp. 1801–1840. doi: 10.1002/glia.23046.

Sierra, A. et al. (2010) 'Microglia shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis', *Cell Stem Cell*, 7(4), pp. 483–495. doi: 10.1016/j.stem.2010.08.014.

Sierra, A., Tremblay, M.-È. M.-E. and Wake, H. (2014) 'Never-resting microglia: physiological roles in the healthy brain and pathological implications', *Frontiers in Cellular Neuroscience*, 8(August), p. 240. doi: 10.3389/fncel.2014.00240.

Sieweke, M. H. and Allen, J. E. (2013) 'Beyond stem cells: self-renewal of differentiated macrophages.', *Science (New York, N.Y.)*, 342(6161), p. 1242974. doi: 10.1126/science.1242974.

Simard, A. R. et al. (2006) 'Bone marrow-derived microglia play a critical role in restricting senile plaque formation in Alzheimer's disease', *Neuron*, 49(4), pp. 489–502. doi: 10.1016/j.neuron.2006.01.022.

Simen, B. B. et al. (2006) 'TNFα Signaling in Depression and Anxiety: Behavioral Consequences of Individual Receptor Targeting', *Biological Psychiatry*, 59(9), pp. 775–785. doi: 10.1016/j.biopsych.2005.10.013.

References

Sims, R., et al. (2017) 'Rare coding variants in PLCG2, ABI3, and TREM2 implicate microglial-mediated innate immunity in Alzheimer's disease', *Nature Genetics*, 49(9), pp. 1373–1384. doi: 10.1038/ng.3916.

Sipe, G. O., et al. (2016) 'Microglial P2Y12 is necessary for synaptic plasticity in mouse visual cortex', *Nature Communications*, 7, p. 10905. doi: 10.1038/ncomms10905.

Small, J. V. et al. (2002) 'The lamellipodium: Where motility begins', *Trends in Cell Biology*, pp. 112–120. doi: 10.1016/S0962-8924(01)02237-1.

Small, S. A., et al. (2011) 'A pathophysiological framework of hippocampal dysfunction in ageing and disease', *Nature Reviews Neuroscience*, pp. 585–601. doi: 10.1038/nrn3085.

Smith, A. M. and Dragunow, M. (2014) 'The human side of microglia', *Trends in Neurosciences*, pp. 125–135. doi: 10.1016/j.tins.2013.12.001.

Smolders, S. M. T. et al. (2017) 'Age-specific function of α5β1 integrin in microglial migration during early colonization of the developing mouse cortex', *GLIA*, 65(7), pp. 1072–1088. doi: 10.1002/glia.23145.

Soreq, L., et al. (2017) 'Major Shifts in Glial Regional Identity Are a Transcriptional Hallmark of Human Brain Aging', *Cell Reports*, 18(2), pp. 557–570. doi: 10.1016/j.celrep.2016.12.011.

Sosna, J., et al. (2018) 'Early long-term administration of the CSF1R inhibitor PLX3397 ablates microglia and reduces accumulation of intraneuronal amyloid, neuritic plaque deposition and pre-fibrillar oligomers in 5XFAD mouse model of Alzheimer's disease.', *Molecular Neurodegeneration*, 13(1):11. doi: 10.1186/s13024-018-0244-x.

Sousa, C., Biber, K. and Michelucci, A. (2017) 'Cellular and molecular characterization of microglia: A unique immune cell population', *Frontiers in Immunology*. doi: 10.3389/fimmu.2017.00198.

Spangenberg, E. E. et al. (2016) 'Eliminating microglia in Alzheimer's mice prevents neuronal loss without modulating amyloid-β pathology', *Brain*, 139(4), pp. 1265–1281. doi: 10.1093/brain/aww016.

Spiller, K.J. et al. (2018). Microglia-mediated recovery from ALS-relevant motor neuron degeneration in a mouse model of TDP-43 proteinopathy. *Nature Neurosciece*, 21(3):329-340. doi: 10.1038/s41593-018-0083-7.

Squarzoni, P. et al. (2014) 'Microglia Modulate Wiring of the Embryonic Forebrain', *Cell Reports*, 8(5), pp. 1271–1279. doi: 10.1016/j.celrep.2014.07.042.

Stanley, E. R. and Chitu, V. (2014) 'CSF-1 receptor signaling in myeloid cells', *Cold Spring Harbor Perspectives in Biology*. doi: 10.1101/cshperspect.a021857.

Steel, C. D. et al. (2010) 'Distinct macrophage subpopulations regulate viral encephalitis but not viral clearance in the CNS', *Journal of Neuroimmunology*, 226(1–2), pp. 81–92. doi: 10.1016/j.jneuroim.2010.05.034.

Steiner, J. et al. (2008) 'Immunological aspects in the neurobiology of suicide: Elevated microglial density in schizophrenia and depression is associated with suicide', *Journal of Psychiatric Research*, 42(2), pp. 151–157. doi: 10.1016/j.jpsychires.2006.10.013.

Stellwagen, D. et al. (2005) 'Differential Regulation of AMPA Receptor and GABA Receptor Trafficking by Tumor Necrosis Factor-α', *The Journal of Neuroscience*, 25(12), pp. 3219–3228. doi: 10.1523/JNEUROSCI.4486-04.2005.

Stellwagen, D. and Malenka, R. C. (2006) 'Synaptic scaling mediated by glial TNF-α', *Nature*, 440(7087), pp. 1054–1059. doi: 10.1038/nature04671.

References

Stence, N., Waite, M. and Dailey, M. E. (2001) 'Dynamics of microglial activation: A confocal time-lapse analysis in hippocampal slices', *GLIA*, 33(3), pp. 256–266. doi: 10.1002/1098-1136(200103)33:3<256::AID-GLIA1024>3.0.CO;2J.

Stephan, A. H., Barres, B. A. and Stevens, B. (2012) 'The Complement System: An Unexpected Role in Synaptic Pruning During Development and Disease', *Annual Review of Neuroscience*, 35(1), pp. 369–389. doi: 10.1146/annurev-neuro-061010-113810.

Stephan, A. H. et al. (2013) 'A Dramatic Increase of C1q Protein in the CNS during Normal Aging', *Journal of Neuroscience*, 33(33), pp. 13460–13474. doi: 10.1523/JNEUROSCI.1333-13.2013.

Stevens, B. et al. (2007) 'The Classical Complement Cascade Mediates CNS Synapse Elimination', *Cell*, 131(6), pp. 1164–1178. doi: 10.1016/j.cell.2007.10.036.

Stremmel, C. et al. (2018) 'Yolk sac macrophage progenitors traffic to the embryo during defined stages of development', *Nature Communications*, 9(1). doi: 10.1038/s41467-017-02492-2.

Sugama, S. et al. (2007) 'Stress induced morphological microglial activation in the rodent brain: Involvement of interleukin-18', *Neuroscience*, 146(3), pp. 1388–1399. doi: 10.1016/j.neuroscience.2007.02.043.

Sun, P. et al. (2011) 'Increase in cortical pyramidal cell excitability accompanies depression-like behavior in mice: a transcranial magnetic stimulation study.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 31(45), pp. 16464–72. doi: 10.1523/JNEUROSCI.1542-11.2011.

Svensson, M. and Aldskogius, H. (1993) 'Synaptic Density of Axotomized Hypoglossal Motorneurons Following Pharmacological Blockade of the Microglial Cell Proliferation', *Experimental Neurology*, 120, pp. 123–131. doi: 10.1006/exnr.1993.1046.

Swinnen, N. et al. (2013) 'Complex invasion pattern of the cerebral cortex bymicroglial cells during development of the mouse embryo', *GLIA*, 61(2), pp. 150–163. doi: 10.1002/glia.22421.

Takata, K. et al. (2017) 'Induced-Pluripotent-Stem-Cell-Derived Primitive Macrophages Provide a Platform for Modeling Tissue-Resident Macrophage Differentiation and Function', *Immunity*, 47(1), p. 183–198.e6. doi: 10.1016/j.immuni.2017.06.017.

Tarozzo, G. et al. (2003) 'Fractalkine protein localization and gene expression in mouse brain.', *Journal of neuroscience research*, 73(1), pp. 81–88. doi: 10.1002/jnr.10645.

Tay, T. L. et al. (2018) 'Microglia Gone Rogue: Impacts on Psychiatric Disorders across the Lifespan', *Frontiers in Molecular Neuroscience*, 10. doi: 10.3389/fnmol.2017.00421.

Tay, T. L. et al. (2017) 'A new fate mapping system reveals context-dependent random or clonal expansion of microglia', *Nature Neuroscience*. doi: 10.1038/nn.4547.

Thams, S., Oliveira, A. and Cullheim, S. (2008) 'MHC class I expression and synaptic plasticity after nerve lesion', *Brain Research Reviews*, pp. 265–269. doi: 10.1016/j.brainresrev.2007.06.016.

Thompson, S. M. et al. (2015) 'An excitatory synapse hypothesis of depression', *Trends in Neurosciences*, pp. 279–294. doi: 10.1016/j.tins.2015.03.003.

Toescu, E. C. et al. (1998) 'Long-term activation of capacitative Ca2+ entry in mouse microglial cells', *Neuroscience*, 86(3), pp. 925–935. doi: 10.1016/S0306-4522(98)00123-7.

References

Torres-Platas, S. G. et al. (2014) 'Evidence for increased microglial priming and macrophage recruitment in the dorsal anterior cingulate white matter of depressed suicides', *Brain, Behavior, and Immunity*, 42, pp. 50–59. doi: 10.1016/j.bbi.2014.05.007.

Torres, L. et al. (2016) 'Dynamic microglial modulation of spatial learning and social behavior', *Brain, Behavior, and Immunity*, 55, pp. 6–16. doi: 10.1016/j.bbi.2015.09.001.

Toth, A. B. et al. (2013) 'Synapse Maturation by Activity-Dependent Ectodomain Shedding of SIRPα', *Nature Neuroscience*, 16(10): 1417–1425. doi: 10.1038/nn.3516

Trapp, B. D. et al. (2007) 'Evidence for synaptic stripping by cortical microglia', *GLIA*, 55(4), pp. 360–368. doi: 10.1002/glia.20462.

Treccani, G. et al. (2014) 'Stress and corticosterone increase the readily releasable pool of glutamate vesicles in synaptic terminals of prefrontal and frontal cortex', *Molecular Psychiatry*, 19(4), pp. 433–443. doi: 10.1038/mp.2014.5.

Tremblay, M.-E. et al. (2011) 'The Role of Microglia in the Healthy Brain', *Journal of Neuroscience*, 31(45), pp. 16064–16069. doi: 10.1523/JNEUROSCI.4158-11.2011.

Tremblay, M. Ě., Lowery, R. L. and Majewska, A. K. (2010) 'Microglial interactions with synapses are modulated by visual experience', *PLoS Biology*, 8(11). doi: 10.1371/journal.pbio.1000527.

Tsou, C. L., Haskell, C. A. and Charo, I. F. (2001) 'Tumor Necrosis Factor-α-converting Enzyme Mediates the Inducible Cleavage of Fractalkine', *Journal of Biological Chemistry*, 276(48), pp. 44622–44626. doi: 10.1074/jbc.M107327200.

Tye, KM. et al. (2013) 'Dopamine neurons modulate neural encoding and expression of depression-related behaviour. Nature 24;493(7433):537-541. doi: 10.1038/nature11740.

Tynan, R. J. et al. (2010) 'Chronic stress alters the density and morphology of microglia in a subset of stress-responsive brain regions', *Brain, Behavior, and Immunity*, 24(7), pp. 1058–1068. doi: 10.1016/j.bbi.2010.02.001.

Ueno, M. et al. (2013) 'Layer V cortical neurons require microglial support for survival during postnatal development.', *Nature neuroscience*, 16(5), pp. 543–51. doi: 10.1038/nn.3358.

Ulrich, J. D. et al. (2017) 'Elucidating the Role of TREM2 in Alzheimer's Disease', *Neuron*, pp. 237–248. doi: 10.1016/j.neuron.2017.02.042.

Vallieres, L. and Sawchenko, P. E. (2003) 'Bone marrow-derived cells that populate the adult mouse brain preserve their hematopoietic identity', *J Neurosci*, 23(12), pp. 5197–5207. doi: 23/12/5197 [pii].

Varol, D. et al. (2017) 'Dicer Deficiency Differentially Impacts Microglia of the Developing and Adult Brain', *Immunity*, 46(6), p. 1030–1044.e8. doi: 10.1016/j.immuni.2017.05.003.

Vasek, M. J. et al. (2016) 'A complement–microglial axis drives synapse loss during virus-induced memory impairment', *Nature*, 534(7608), pp. 538–543. doi: 10.1038/nature18283.

Videbech, P. and Ravnkilde, B. (2004) 'Hippocampal volume and depression: a meta-analysis of MRI studies.', *The American journal of psychiatry*, 161(11), pp. 1957–66. doi: 10.1176/appi.ajp.161.11.1957.

Vinet, J. et al. (2012) 'Neuroprotective function for ramified microglia in hippocampal excitotoxicity', *Journal of Neuroinflammation*, 9(1), p. 515. doi: 10.1186/1742-2094-9-27.

References

Waisman, A. et al. (2015) 'Homeostasis of Microglia in the Adult Brain: Review of Novel Microglia Depletion Systems', *Trends in Immunology*, pp. 625–636. doi: 10.1016/j.it.2015.08.005.

Wake, H. et al. (2009) 'Resting Microglia Directly Monitor the Functional State of Synapses In Vivo and Determine the Fate of Ischemic Terminals', *Journal of Neuroscience*, 29(13), pp. 3974–3980. doi: 10.1523/JNEUROSCI.4363-08.2009.

Wake, H. et al. (2013) 'Microglia: Actively surveying and shaping neuronal circuit structure and function', *Trends in Neurosciences*, pp. 209–217. doi: 10.1016/j.tins.2012.11.007.

Wakselman, S. et al. (2008) 'Developmental neuronal death in hippocampus requires the microglial CD11b integrin and DAP12 immunoreceptor', *J Neurosci*, 28(32), pp. 8138–8143. doi: 10.1523/JNEUROSCI.1006-08.2008.

Walker, F. R. et al. (2014) 'Dynamic structural remodelling of microglia in health and disease: A review of the models, the signals and the mechanisms', *Brain, Behavior, and Immunity*, pp. 1–14. doi: 10.1016/j.bbi.2013.12.010.

Walz, W. et al. (1993) 'Extracellular ATP activates a cation conductance and a K+ conductance in cultured microglial cells from mouse brain.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 13(10), pp. 4403–4411.

Wang J. et al. (2015) 'Wild-type microglia do not reverse pathology in mousemodels of Rett syndrome', *Nature*, 521. doi: 10.1038/nature10907.

Wang, X., Zhang, D. and Lu, X. Y. (2015) 'Dentate gyrus-CA3 glutamate release/NMDA transmission mediates behavioral despair and antidepressant-like responses to leptin', *Molecular Psychiatry*, 20(4), pp. 509–519. doi: 10.1038/mp.2014.75.

Wang, Y. et al. (2012) 'IL-34 is a tissue-restricted ligand of CSF1R required for the development of Langerhans cells and microglia', *Nature Immunology*, 13(8), pp. 753–760. doi: 10.1038/ni.2360.

Wang, Y. et al. (2016) 'TREM2-mediated early microglial response limits diffusion and toxicity of amyloid plaques.', *The Journal of experimental medicine*, 213(5), pp. 667–75. doi: 10.1084/jem.20151948.

Weber, M. D. et al. (2018), 'The Influence of Microglial Elimination and Repopulation on Stress Sensitization Induced by Repeated Social Defeat', Biol Psychiatry. doi: 10.1016/j.biopsych.2018.10.009.

Węgiel, J. et al. (1998) 'Reduced number and altered morphology of microglial cells in colony stimulating factor-1-deficient osteopetrotic op / op mice', *Brain Research*, 804(1), pp. 135–139. doi: 10.1016/S0006-8993(98)00618-0.

Wei, S. et al. (2010) 'Functional overlap but differential expression of CSF-1 and IL-34 in their CSF-1 receptor-mediated regulation of myeloid cells', *Journal of Leukocyte Biology*, 88(3), pp. 495–505. doi: 10.1189/jlb.1209822.

Weinhard, L. et al. (2017) 'Microglia remodel synapses by presynaptic trogocytosis and spine head filopodia induction', *doi.org*, p. 190975. doi: 10.1101/190975.

Wes, P. D. et al. (2016) 'Next generation transcriptomics and genomics elucidate biological complexity of microglia in health and disease', *GLIA*, 64(2), pp. 197–213. doi: 10.1002/glia.22866.

Wieghofer, P., Knobeloch, K. P. and Prinz, M. (2015) 'Genetic targeting of microglia', *GLIA*, pp. 1–22. doi: 10.1002/glia.22727.

Wieghofer, P. and Prinz, M. (2015) 'Genetic manipulation of microglia during brain development and disease', *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, 1862(3), pp. 299–309. doi: 10.1016/j.bbadis.2015.09.019.

References

Winkler, Z. et al. (2017) 'Impaired microglia fractalkine signaling affects stress reaction and coping style in mice', *Behavioural Brain Research*, 334, pp. 119–128. doi: 10.1016/j.bbr.2017.07.023.

Wlodarczyk, A. et al. (2017) 'A novel microglial subset plays a key role in myelinogenesis in developing brain', *The EMBO Journal*, p. e201696056. doi: 10.15252/embj.201696056.

Wohleb, E. S. et al. (2016) 'Integrating neuroimmune systems in the neurobiology of depression', *Nature Reviews Neuroscience*, 17(8), pp. 497–511. doi: 10.1038/nrn.2016.69.

Wohleb, E. S. et al. (2014) 'Re-establishment of anxiety in stress-sensitized mice is caused by monocyte trafficking from the spleen to the brain', *Biological Psychiatry*, 75(12), pp. 970–981. doi: 10.1016/j.biopsych.2013.11.029.

Wohleb, E. S. et al. (2015) 'Monocyte trafficking to the brain with stress and inflammation: A novel axis of immune-to-brain communication that influences mood and behavior', *Frontiers in Neuroscience*. doi: 10.3389/fnins.2014.00447.

Wohleb, E. S. et al. (2013) 'Stress-Induced Recruitment of Bone Marrow-Derived Monocytes to the Brain Promotes Anxiety-Like Behavior', *Journal of Neuroscience*, 33(34), pp. 13820–13833. doi: 10.1523/JNEUROSCI.1671-13.2013.

Wolf, S. A., Boddeke, H. W. G. M. and Kettenmann, H. (2017) 'Microglia in Physiology and Disease', *Annual Review of Physiology*, 79(1), pp. 619–643. doi: 10.1146/annurev-physiol-022516-034406.

Wolf, Y. et al. (2017) 'Autonomous TNF is critical for in vivo monocyte survival in steady state and inflammation', *The Journal of Experimental Medicine*, 214(4), pp. 905–917. doi: 10.1084/jem.20160499.

Wolf, Y. et al. (2013) 'Microglia, seen from the CX3CR1 angle.', *Frontiers in cellular neuroscience*, 7(March), p. 26.

Wong, K. et al. (2017) 'Mice deficient in NRROS show abnormal microglial development and neurological disorders', *Nature Immunology*. doi: 10.1038/ni.3743.

Wu, C. H. et al. (1993) 'A quantitative study of the differentiation of microglial cells in the developing cerebral cortex in rats', *J Anat*, pp. 403–413.

Wu, L. J., Vadakkan, K. I. and Zhuo, M. (2007) 'ATP-induced chemotaxis of microglial processes requires P2Y receptor-activated initiation of outward potassium currents', *GLIA*, 55(8), pp. 810–821. doi: 10.1002/glia.20500.

Wu, Y. et al. (2015) 'Microglia: Dynamic Mediators of Synapse Development and Plasticity', *Trends in Immunology*, pp. 605–613. doi: 10.1016/j.it.2015.08.008.

Wynne, A. M. et al. (2010) 'Protracted downregulation of CX3CR1 on microglia of aged mice after lipopolysaccharide challenge', *Brain, Behavior, and Immunity*, 24(7), pp. 1190–1201. doi: 10.1016/j.bbi.2010.05.011.

Xu, J. et al. (20015). 'Temporal-Spatial Resolution Fate Mapping Reveals Distinct Origins for Embryonic and Adult Microglia in Zebrafish'. *Dev Cell.* 28;34(6):632-41. doi: 10.1016/j.devcel.2015.08.018.

Yamada, M. et al. (2006) 'X-ray Structures of the Microglia/Macrophage-specific Protein Iba1 from Human and Mouse Demonstrate Novel Molecular Conformation Change Induced by Calcium binding', *Journal of Molecular Biology*, 364(3), pp. 449–457. doi: 10.1016/j.jmb.2006.09.027.

Yeh, F. L., Hansen, D. V. and Sheng, M. (2017) 'TREM2, Microglia, and Neurodegenerative Diseases', *Trends in Molecular Medicine*, pp. 512–533. doi: 10.1016/j.molmed.2017.03.008.

Yin, F. et al. (2010) 'Behavioral deficits and progressive neuropathology in progranulin-deficient mice: a mouse model of frontotemporal dementia', *The FASEB Journal*, 24(12), pp. 4639–4647. doi: 10.1096/fj.10-161471.

References

Yin, Z. et al. (2016) 'Immune hyperreactivity of Aβ plaque-associated microglia in Alzheimer's disease', *Neurobiology of Aging*. doi: 10.1016/j.neurobiolaging.2017.03.021.

Yirmiya, R., Rimmerman, N. and Reshef, R. (2015) 'Depression as a Microglial Disease', *Trends in Neurosciences*, pp. 637–658. doi: 10.1016/j.tins.2015.08.001.

Yona, S. et al. (2013) 'Fate Mapping Reveals Origins and Dynamics of Monocytes and Tissue Macrophages under Homeostasis', *Immunity*, 38(1), pp. 79–91. doi: 10.1016/j.immuni.2012.12.001.

Yuen, E. Y. et al. (2009) 'Acute stress enhances glutamatergic transmission in prefrontal cortex and facilitates working memory', *Proceedings of the National Academy of Sciences*, 106(33), pp. 14075–14079. doi: 10.1073/pnas.0906791106.

Zanos, P. et al. (2016) 'NMDAR inhibition-independent antidepressant actions of ketamine metabolites', *Nature*, 533(7604), pp. 481–486. doi: 10.1038/nature17998.

Zelante, T. and Ricciardi-Castagnoli, P. (2012) 'The yin-yang nature of CSF1R-binding cytokines', *Nature Immunology*, 13(8), pp. 717–719. doi: 10.1038/ni.2375.

Zhan, Y. et al. (2014) 'Deficient neuron-microglia signaling results in impaired functional brain connectivity and social behavior', *Nature Neuroscience*, 17(3), pp. 400–406. doi: 10.1038/nn.3641.

Zhang, J. et al. (2014) 'Microglial CR3 activation triggers long-term synaptic depression in the hippocampus via NADPH oxidase', *Neuron*, 82(1), pp. 195–207. doi: 10.1016/j.neuron.2014.01.043.

Zhang, Y. et al. (2014) 'An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. *Journal of Neuroscience.* 3;34(36):11929-47. doi: 10.1523/JNEUROSCI.1860-14.2014.

Zhao, C., Deng, W. and Gage, F. H. (2008) 'Mechanisms and functional implications of adult neurogenesis', *Cell*, 132(4), pp. 645–660. doi: 10.1016/j.cell.2008.01.033.

Zöller, T. *et al.* (2018) 'Silencing of TGFβ signalling in microglia results in impaired homeostasis', *Nature Communication*, 1;9(1):4011. doi: 10.1038/s41467-018-06224-y.

Zujovic, V. et al. (2000) 'Fractalkine modulates TNF-α secretion and neurotoxicity induced by microglial activation', *GLIA*, 29(4), pp. 305–315. doi: 10.1002/(SICI)1098-1136(20000215)29:4<305::AID-GLIA2>3.0.CO;2-V.

Curriculum Vitae

Personal details

Name/Surname:	Simone Brioschi	
Date and place of birth:	June 23, 1986, Milano (Italy)	
Current Address:	4467 Maryland Ave. 63108 St. Louis (USA)	
Professional background:	Biotechnologist	
Contact information:	Email:	s.brioschi@wustl.edu
	Cell phone:	+1-314-600-3573

Education

June 2014 – Present — **PhD candidate in Biology**

Albert-Ludwigs University of Freiburg, Faculty of Biology Schänzlestraße 1, 79104 Freiburg (Germany)

October 2010 – February 2013 — **Master's Degree in Medical Biotechnology**

University of Milano-Bicocca, Faculty of Medicine and Surgery Via Cadore 48, 20900 Monza (Italy). Grade: 110/110 Summa Cum Laude

October 2006 – June 2010 — **Bachelor's Degree in Biotechnology**

University of Milano-Bicocca, Faculty of Life-Science, Piazza della Scienza, 20126 Milano (Italy). Grade: 96/110

September 2002 – June 2006 — **High School**

Scientific Gymnasium Enrico Fermi, Via Giovanni XXIII, 22063 Cantù (Italy). Grade: 83/100

Curriculum Vitae

Research Experiences

February 2018 – Present	**Institute for Multiple Sclerosis Research (IMSF), Lab of Neuroimmunology**
Position covered:	Guest scientist
Address:	Von-Siebold-Straße 3A, 37075 Göttingen
Area of research:	Analysis of microglia motility in two-photon *in vivo* imaging, thesis preparation

January 2014 – December 2017	**University of Freiburg Medical Centre, Department of Psychiatry and Psychotherapy, Lab of Molecular Psychiatry**
Position covered:	PhD student
Address:	Hauptstraße 5, 79104 Freiburg (Germany)
Area of research:	Mechanisms of microglia-neurons communication in a mouse model of depressive-like behavior
Supervisor:	Prof. Knut Biber

April 2016 – May 2016	**Washington University School of Medicine St. Louis, Department of Pathology and Immunology**
Position covered:	Guest scientist
Address:	South Euclid Ave 660, MO 63110 St. Louis (USA)
Area of research:	Behavioral tests on IL34 knock-out mice
Supervisor:	Prof. Marco Colonna

Curriculum Vitae

March 2013 – December 2013	**University of Milano-Bicocca, Department of Health Sciences, Lab of Applied Biophysics**
Position covered:	Research fellow
Address:	Via Cadore 48, 20900 Monza (Italy)
Area of research:	Aggregation kinetics of amyloid-β_{42} peptides, biophysical characterization of multi-functionalized liposomes, investigation of DNA's nanomechanical properties in single-molecule manipulation techniques
Supervisor:	Prof. Francesco Mantegazza

January 2012 – February 2013	**University of Milano-Bicocca, Department of Health Sciences, Lab Neurobiology**
Position covered:	Master student
Address:	Via Cadore 48, 20900 Monza (Italy)
Area of research:	Mechanisms of amyloid-β induced cytotoxicity, development of amyloid-β binding liposomes
Supervisor:	Prof. Carlo Ferrarese

September 2011 – November 2011	**University of Pittsburgh School of Medicine, Department of Pharmacology**
Position covered:	Guest student
Address:	Lothrop Street 200, PA 15261 Pittsburgh (USA)
Area of research:	Role of Caveolin-1 protein in cellular senescence and bioenergetics.
Supervisor:	Prof. Flavio Galbiati

Curriculum Vitae

List of publications Current H-index: 5

Manuscripts in preparation/submission

- **Brioschi S**, Wieghofer P, Amann LS, Janova H, d'Errico P, Wojcik SM, Meyer-Luehmann M, Rajendran L, Paolicelli RC and Biber K. *A FACS-based assessment of synaptic material in microglia during brain development and disease.* **Simone Brioschi performed the experiments, analyzed the data and prepared the manuscript**

- **Brioschi S**, Kracht L, Nebeling F, Wolf Y, Fuhrmann M, Colonna M, Biber K. *Microglia promote synaptic loss and depressive-like behavior under chronic-stress exposure.* **Simone Brioschi performed the experiments, analyzed the data and prepared the manuscript**
  .
- Garcia P, Wemheuer W, **Brioschi S**, Masuch A, Masliah E, Koziel V, Pillot T, Koncina E, Jaeger C, Weihofen A, Balling R, Biber K, Buttini M. *Neurodegeneration and microgliosis are independent of α-synuclein aggregation in a mouse model of "prion-like" α-synuclein spreading.* **Simone Brioschi performed the imaging experiments**

- **Brioschi S**, Walker R and Biber K. *Microglia and mood disorders: misleading interpretation, caveats and drawbacks.* **Simone Brioschi prepared the manuscript**

2018

- **Brioschi S** and Colonna M. *The CNS Immune-Privilege Goes Down the Drain(age).* Trends Pharmacol Sci. 2019 Jan;40(1):1-3. **Simone Brioschi prepared the manuscript**

- Yousif NM, Oliveira A, **Brioschi S**, Huell M, Biber K and Fiebich B. *Activation of EP2 receptor suppresses Poly(I:C) and LPS-mediated inflammation in primary microglia and organotypic hippocampal slice cultures: Contributing role for MAPKs.* Glia 66 (4), 708-724. **Simone Brioschi performed the imaging experiments**

2017

- Ardura-Fabregat A, Boddeke EWGM, Boza-Serrano A, **Brioschi S**, Ceyzériat K, Dansoko C, Dierkes T, Gelders G, Heneka MT, Hoeijmakers L, Hoffmann A, Iaccarino L, Jahnert S, Kuhbandner K, Landreth G, Lonnemann N, Löschmann PA, McManus R, Paulus A, Reemst K, Sanchez-Caro JM, Van der Perren A, Vautheny A, Venegas C, Webers A, Weydt P, Wijasa T, Xianyuan X, Yiyi Y. *Targeting Neuroinflammation to Treat Alzheimer's Disease.* CNS drugs, 1-26. **Simone Brioschi prepared one chapter of the review**

Curriculum Vitae

2016

- Safaiyan S, Kannaiyan N, Snaidero N, **Brioschi S**, Biber K, Yona S, Edinger AL, Jung S, Rossner MJ, Simons M. *Age-related myelin degradation burdens microglia clearance function during aging.* Nat. Neurosci. 19 (8), 995. **Simone Brioschi performed the FACS experiments**

- Hellwig S, **Brioschi S**, Dieni S, Frings L, Masuch A, Blank T, Biber K. *Altered microglia morphology and higher resilience to stress-induced depression-like behavior in CX3CR1-deficient mice.* Brain Behav Immun. 55, 126-137. **Simone Brioschi performed the experiments and analyzed the data**

- Salerno D, Beretta GL, Zanchetta G, **Brioschi S**, Cristofalo M, Missana N, Nardo L, Cassina V, Tempestini A, Giovannoni R, Cerrito MG, Zaffaroni N, Bellini T, Mantegazza F. *Platinum-Based Drugs and DNA Interactions Studied by Single-Molecule and Bulk Measurements.* Biophys J. 110 (10), 2151-2161. **Simone Brioschi performed the magnetic tweezer experiments**

- Nardo L, Re F, **Brioschi S**, Cazzaniga E, Orlando A, Minniti S, Lamperti M, Gregori M, Cassina V, Brogioli D, Salerno D, Mantegazza F. *Fluorimetric detection of the earliest events in amyloid β oligomerization and its inhibition by pharmacologically active liposomes.* Biochim Biophys Acta. 1860 (4), 746-756. **Simone Brioschi performed the Aβ aggregation experiments**

- Cassina V, Manghi M, Salerno D, Tempestini A, Iadarola V, Nardo L, **Brioschi S**, Mantegazza F. *Effects of cytosine methylation on DNA morphology: An atomic force microscopy study.* Biochim Biophys Acta. 1860 (1), 1-7. **Simone Brioschi prepared the introduction of the manuscript**