
# MICROGLIAL PURINERGIC SIGNALING IN MOUSE MODELS OF CD39 DEFICIENCY AND SCHIZOPHRENIA

Dissertation zur Erlangung des akademischen Grades des Doktors der Naturwissenschaften (Dr.rer.nat.)

eingereicht im Fachbereich Biologie, Chemie, Pharmazie der Freien Universität Berlin

vorgelegt von

Petya Georgieva
*aus*
*Sofia, Bulgarien*

2014

Diese Arbeit wurde am Max-Delbrück-Zentrum für Molekulare Medizin von März 2011 bis Dezember 2014 unter der Leitung von Prof. Helmut Kettenmann angefertigt.

1. Gutachter: Prof. Helmut Kettenmann

2. Gutachter: Prof. Fritz Rathjen

Disputation am 26.03.2015

# Acknowledgements

I thank **Prof. Dr. Helmut Kettenmann** for the opportunity to work on many exciting projects, for the supervision and funding and to **Prof. Dr. Fritz Rathjen** for being my university supervisor. Thanks to **Prof. Dr. Brian MacVicar** for the supervision and funding during the first part of my PhD as well. I am very grateful to both my supervisors, **Dr. Susanne Wolf** and **Dr. Vitali Matyash**, for the excellent supervision, many discussions and ideas and a lot of support throughout the development of my project, as well as for critical comments on this thesis.
Special thanks to **Birgit Jarchow** for her support and invaluable help in all organisational matters and bureaucratic challenges.
For the technical support **Regina Piske**, **Irene Haupt** and **Hanna Schmidt** were a great help, thank you! I am also very grateful to **Philipp Jordan** and **Ignacio Delgado** for the huge help with the statistical analysis of the calcium waves data, to **Frank Szulzewsky** for the phagocytosis assays, to **Adriana Rocha** for help with the Cd39 project and to **Daniele Mattei** for the breedings of the Poly I:C animals.
One of the greatest things during my PhD was my lab team of creative, inspiring, incredibly smart and funny people! Thank you, **Nadine Richter**, **Katyayni Vinnakota**, **Maria Pannell**, **Felipe Sassi**, **Feng Hu** and **Stefan Wendt** – and everyone else who I forgot to mention here! It was really great to work with all of you!
Last but not least I want to thank my parents **Mariyana and Boyan** for believing in me and always supporting the way I have chosen to go.
This thesis is dedicated to my sister **Eli** and her daughter **Veronika**, two amazing, strong and beautiful girls!

# Contents

# List of abbreviations

| | |
|---|---|
| ACSF | Artificial cerebro-spinal fluid |
| AD | Alzheimer's disease |
| ADP | Adenosine-5'-Diphosphate |
| AM | Acetoxy Methyl (Ester) |
| AMP | Adenosine-5'-Monophosphate |
| ATP | Adenosine-5'-Triphosphate |
| BAI1 | Brain angiogenesis inhibitor 1 |
| BCA | Bicinchoninic Acid |
| BSA | Bovine Serum Albumine |
| CD39 | Cluster of Differentiation 39 |
| cDNA | Complementary Deoxyribonucleic Acid |
| CNS | Central nervous system |
| DAMP | Damage-associated molecular pattern |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | Dimethyl Sulfoxide |
| DNA | Deoxyribonucleic Acid |
| EDTA | Ethylenediaminetetraacetic acid |
| ENTPDase1 | Ectonucleoside Triphosphate Diphosphohydrolase 1 |
| FCS | Fetal calf serum |
| GABA | $\gamma$-aminobutyric acid |
| GDNF | Glial derived neurotrophic factor |
| GFAP | Glial fibrillary acidic protein |
| GJ | Gap junction |
| GM-CSF | Granulocyte-macrophage colony stimulating factor |
| HBSS | Hank's balanced salt solution |
| ICAM1 | Intercellular Adhesion Molecule-1 |
| ICW | Intercellular calcium wave |
| IFN$\gamma$ | Interferon $\gamma$ |
| IL4 | Interleukin 4 |
| IL10 | Interleukin 10 |
| IL13 | Interleukin 13 |
| $IP_3$ | Inositol triphosphate |

| | |
|---|---|
| LPC | Lysophosphatidylcholine |
| LPS | Lipopolysaccharide |
| LTD | Long-Term Depression |
| LTP | Long-Term Potentiation |
| MACS | Magnetic Assisted Cell Sorting |
| M-CSF | Macrophage colony stimulating factor |
| MFG-E8 | Milk fat globule-EGF-factor 8 |
| MIA | Maternal immune activation |
| NECA | 1-(6-Amino-9H-purin-9-yl)-1-deoxy-N- ethyl-$\beta$-D-ribofuranuronamide |
| NMDA | *N*-Methyl-*D*-aspartic acid |
| OD | Optical density |
| PAMP | Pathogen-associated molecular pattern |
| PANX1 | Pannexin 1 |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain reaction |
| PFA | Paraformaldehyd |
| PFC | Pre-frontal cortex |
| Poly I:C | Polyinosinic-polycytidylic acid |
| PPADS | Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium salt |
| PPI | Pre-Pulse Inhibition |
| PRR | Pattern-recognition receptor |
| PS | Phosphatidylserine |
| qPCR | Quantitative Polymerase Chain Reaction |
| rFI | Relative Fluorescence Intensity |
| RNA | Ribonucleic Acid |
| ROI | Region Of Interest |
| RT | Room temperature |
| S1P | Sphingosine 1-phosphate |
| SD | Standard deviation |
| SDS | Sodium Dodecyl Sulfate |
| SEM | Standard error of the mean |
| TCA | Trichloroacetic Acid |
| TGF$\beta$ | Transforming Growth Factor Beta |
| TIM | T cell immunoglobulin and mycin |
| TLR | Toll-Like Receptor |
| UDP | Uridine-5'-Diphosphate |
| UTP | Uridine-5'-Triphosphate |
| VNUT | Vesicular nucleotide transporter |

# List of Figures

# List of Tables

# 1. Introduction

## 1.1 Microglia, the gatekeepers of the brain

Microglia were first described by the Spanish neuroscientist Pio del Rio Hortega [Del Rio-Hortega, 1932], who named them *microgliocytes* and postulated that they invade the brain during early development, have an amoeboid form during migration and acquire a ramified form with many branches upon colonization of the brain. Each cell occupies a distinct territory and upon a pathological event, they undergo transformation into a different phenotype (see fig.1.1). All these postulates still hold true today, 80 years after they were first published. The exact origin of microglia was under debate until recently, when Ginhoux and colleagues showed in an *in vivo* lineage tracing study that microglia arise from myeloid progenitor cells and migrate from the yolk sac to the central nervous system (CNS) between embryonic day 8.5 (E8.5) and E9.5 [Ginhoux et al., 2010]. Upon invading the brain as amoeboid cells, microglia extend their branches and acquire a ramified morphology, characterized by small soma and long, thin processes. This phenotype was previously considered as a "resting" state, however, this view has been challenged by Nimmerjahn and colleagues, who discovered that microglia constantly scan their environment and rapidly respond to injury [Davalos et al., 2005; Nimmerjahn et al., 2005]. Microglial processes are highly motile and protrude and retract rapidly, while the cell bodies remain immobile. The endings of the processes often contact other cells, as well as synapses, and play a crucial role in synaptic pruning during development [Wake et al., 2009; Paolicelli et al., 2011]. Microglia are considered to be the immune cells of the brain and represent the first line of defense against an immune challenge in the CNS. In fact, any disturbance in the normal brain physiology, like trauma, ischemia, neurodegenerative disease or neuronal signal is sensed by microglia and induces a rapid shift of state toward activation [Hanisch and Kettenmann, 2007; Kettenmann et al., 2013]. The cells acquire an amoeboid morphology with less branches and bigger cell soma and can start migrating, become phagocytic, proliferate and release a large number of molecules, such as different cytokines and chemokines.

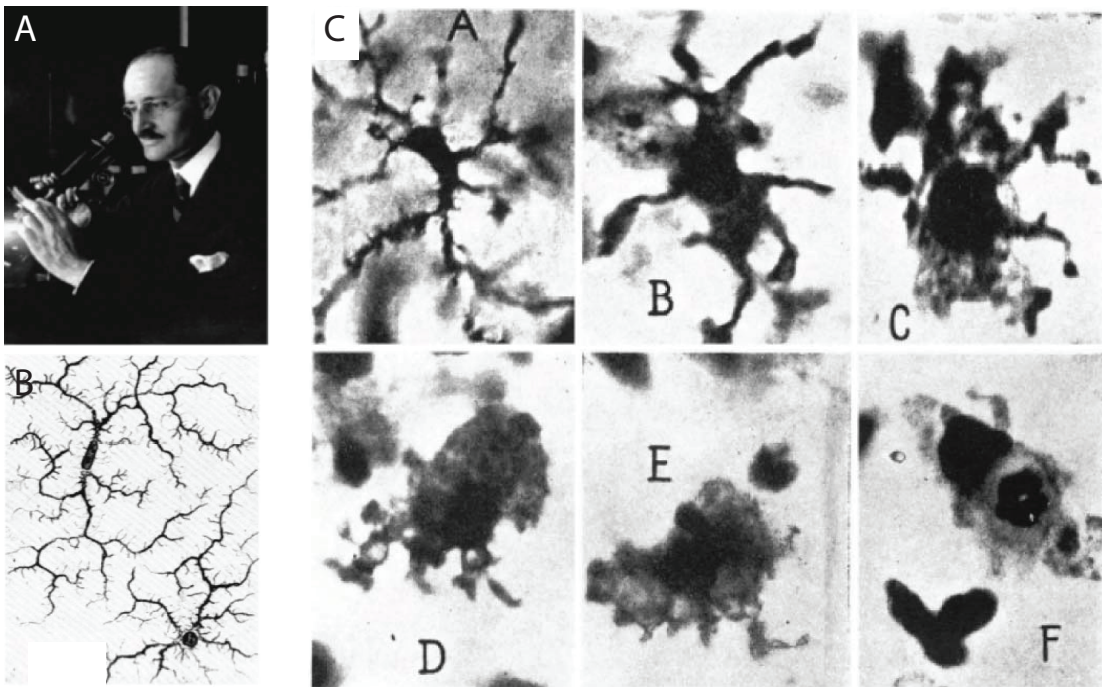


**Figure 1.1: The work of the spanish neuroscientist Pio del Rio Hortega. A: Portrait of Del Rio Hortega (1882-1945) at work. B: Drawing of ramified microglia. C: Silver carbonate impregnation pictures of microglial cells during different stages of activation after entorhinal cortex lesion. Original pictures were published in Del Rio-Hortega [1919] and Del Rio-Hortega [1932].**

### 1.1.1 Factors controlling microglia activation

The concept that microglial transition from "resting" to "activated" state occurs like a switch is no longer valid today. It is rather considered that instead of an all-or-nothing process, activation occurs as a gradual shift of activity states, which is adaptable and context-dependent [Hanisch and Kettenmann, 2007]. What factors can induce this change in microglial function and phenotype? Signals can be generally divided into two main categories: molecules, which are either not present under physiological conditions, or normally do not reach critical concentrations, act as "ON" signals, whereas molecules, which are released from neurons under normal conditions and have a calming effect on microglia, serve as "OFF" signals [Biber et al., 2007]. Both the presence of ON signals, as well as the disturbance or decrease of OFF signals, are sensed by microglial cells and lead to their activation. ON signals are mostly recognized by pattern-recognition receptors (PRRs), which can recognize both pathogen-associated molecular patterns (PAMPs), usually microbial components, and damage-associated molecular patterns (DAMPs), danger indicators released in the context of tissue injury. One of the most prominent PRR families is the toll-like receptor (TLR) family. So far, 13 TLRs have been described, which are expressed on the cell surface or in the endosome. TLRs can be activated by both exogenous factors such as cell wall compo-

nents and surface structures, microbial, bacterial or viral DNA or RNA or endogenous molecules, e.g. HMGB1, fibronectin, versican, fatty acids and heat shock proteins, to name a few. Most insights in TLR-mediated microglia activation come from *in vitro* studies of cultured microglia, which have been treated with lipopolysaccharide (LPS), a cell-wall component of gram-negative bacteria. Other components, which can lead to microglial activation or modulate microglial responses, are cytokines, chemokines, complement factors, neurotrophic factors, neurotransmitters, neurohormones and many more [Kettenmann and Ransom, 2013]. Well-studied OFF signals are members of the immunoglobulin family (IgGS) such as CD200 and CD47, which suppress activation of myeloid cells, as well as fractalkine (CX3CL1) [Biber et al., 2007].

### 1.1.2 Microglial migration

Upon activation, microglia can acquire a migratory phenotype and instead of only extending and retracting their processes, can translocate the whole cell body to a site of injury. It has been shown that microglia can already start migrating 24 hours after tissue damage and can reach a speed of up to 10 $\mu$m/min, depending on the stimulus which triggered migration [Carbonell et al., 2005]. Microglial migration can be triggered by chemokines, such as C5a, neurotrophic factors, like glial cell-derived neurotrophic factor (GDNF) [Ku et al., 2013] and the danger signal adenosine-5'-triphosphate (ATP).

### 1.1.3 Microglial phagocytosis

Microglia serve as the intrinsic macrophages of the brain and can also acquire phagocytic activity. The uptake of extracellular elements is called endocytosis and can be divided into three main types:

- **Receptor-mediated endocytosis:** molecules bind to their specific receptors and the complex is internalized by coated pits-mediated vesicle formation in the cell membrane.
- **Pinocytosis:** describes the uptake of soluble molecules via invaginations of the cell membrane which become vesicles upon closure
- **Phagocytosis:** the internalization of solid particles in endosomes which later fuse with lysosomes for enzymatic digestion [Napoli and Neumann, 2009].

Phagocytic microglia acquire an amoeboid form and express macrophage markers like CD68 on the surface [Streit et al., 1999]. Phagocytosis can be triggered by different factors, like TLR activation by bacterial components, and is important for removal of apoptotic cells, necrotic tissue or microbes. "Find me" signals are diffusible molecules, released from apoptotic cells to attract phagocytic cells to their proximity [Ravichandran, 2011]. Described "find me" signals include lipid lysophosphatidylcholine (LPC), sphingosine 1-phosphate (S1P), the fractalkine CX3CL1 (which binds to the CX3CR1

receptor), and the nucleotides ATP and UTP (uridine-5'-triphosphate), recognized by purinergic receptors [Lauber et al., 2004; Koizumi et al., 2007; Gude et al., 2008; Truman et al., 2008]. Interestingly, "find me" signals are usually released during the early stages of apoptosis, when the apoptotic cells are still intact, so that fast recruitment of phagocytes is ensured. In contrast to necrosis, when vast amounts of nucleotides leak out in the extracellular space, during apoptosis ATP and UTP are subject to regulated release via the plasma membrane channel pannexin 1 (PANX1). Silencing of PANX1 expression in apoptotic Jurkat cells resulted in reduction of both nucleotide release and monocyte recruitment [Chekeni et al., 2010]. When the phagocyte reaches the apoptotic cell vicinity, it needs to specifically recognize the dying cell among all the living ones in the surroundings. Apoptotic cells express surface markers which ensure proper recognition by the phagocytes - the so called "eat me" signals. Typical "eat me" signals are phosphatidylserine (PS), the intercellular adhesion molecule-1 (ICAM-1), and calreticulin [Lauber et al., 2004; Nagata et al., 2010]. PS is one of the most investigated "eat me" signals: it is usually expressed in the inner leaflet of the plasma membrane and upon apoptosis induction, it is exposed to the outer leaflet to provide a specific marker for recognition by phagocytes [Fadok et al., 1992; 2001]. PS can be recognized by phagocytes either directly via members of the T cell immunoglobulin and mucin (TIM) family (TIM1, TIM3 and TIM4) [Kobayashi et al., 2007; Miyanishi et al., 2007; DeKruyff et al., 2010], brain angiogenesis inhibitor 1 (BAI1) [Park et al., 2007] or Stabilin-2 [Park et al., 2008], or indirectly with the help of the soluble protein milk fat globule-EGF-factor 8 (MFG-E8) which acts as a bridging molecule [Hanayama et al., 2002].
Pathogen-associated non-self factors, like components of bacterial cell walls (e.g. LPS) or viral RNA, are recognized by PRRs such as the TLRs, but also by the complement receptors, FcReceptors and scavenger receptors and induce not only phagocytosis, but also the polarization of the phagocytes towards a pro-inflammatory phenotype, which is accompanied by cytokine release [Napoli and Neumann, 2009].

### 1.1.4 Release of cytokines

Activated microglia are able to produce a variety of cytokines and chemokines, which can then act on neighboring cells (*paracrine* signaling) or on microglia themselves (*autocrine* signaling). Although secreted factors usually only affect the surrounding tissue, long-term effects of microglia-derived cytokines have been shown to have an influence on recruitment of peripheral cells to the brain, on astrocytes, on oligodendrocytic growth and myelination, as well as on neuronal activity [Balasingam et al., 1994; Rothwell et al., 1996; Hanisch, 2002].

### 1.1.5 Proliferation

Activated microglia can not only change their functional properties, but also can start proliferating. Microglial proliferation *in vivo* has been mostly studied in models of acute or chronic nerve injury. As early as 12 hours after a lesion, the cells acquire mitotic activity, which can persist for days and even weeks after [Tonchev et al., 2003; Ladeby et al., 2005a]. Apart by injury, proliferation can also be induced by stroke, infection and neurodegenerative diseases (e.g. prion disease) [Ladeby et al., 2005b; Gómez-Nicola et al., 2013]. It is believed that these stimuli induce the production of promitotic factors, such as macrophage colony stimulating factor (M-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF), but also cytokines, chemokines and purines, like ATP [Kettenmann and Ransom, 2013]. All these factors can subsequently activate signaling pathways which ultimately induce gene transcription and cell cycle regulation leading to increased proliferation.

### 1.1.6 Activation states of microglia

Microglial cells possess a functional plasticity and can commit to different reactive phenotypes depending on the extracellular signals. There is accumulating evidence that the transition to an activation state occurs gradually, rather than as a switch [Hanisch and Kettenmann, 2007]. Moreover, clear-cut activation states cannot be found *in vivo*, where a mix of differentially activated microglia is observed [Scheffel et al., 2012; Hanisch, 2013]. Recent studies based on transcriptome analysis suggest an activation spectrum model for macrophages *in vivo*, extending beyond the current M1/M2 model [Xue et al., 2014]. However, *in vitro* studies provide an opportunity to study cells in an isolated system, where distinct activation states are inducible by cytokine treatment [Pannell et al., 2014]. Microglia share many properties with peripheral tissue macrophages, thus we can employ the well-studied activation states in macrophages as a framework for *in vitro* studies in microglia. In this work, the following three activation states were induced in microglia *in vitro*: **Classical activation** (also termed M1 activation), which occurs following a bacterial infection, probably the best studied activation state, can be induced by activation of TLR signaling, for example through LPS stimulation *in vitro*. It is characterized by the release of pro-inflammatory cytokines, especially TNF$\alpha$, IL1-$\beta$ and nitric oxide, proliferation and decreased migration and phagocytosis [Martinez et al., 2008]. **Alternative activation** (M2a activation) can be induced *in vitro* by the Th2 cytokines IL4 and IL13, released in response to large parasites. Alternatively activated macrophages acquire tissue-repair and phagocytic properties and upregulate mannose, arginase and scavenger receptors [Martinez et al., 2009]. The third phenotype is the least well defined. **Acquired deactivation** (M2c) is induced by the anti-inflammatory cytokines, secreted by $T_{regs}$, IL10 and TGF$\beta$,

but also by apoptotic cells and glucocorticoids. Macrophages in the acquired deactivation state support resolution of inflammation by secretion of anti-inflammatory factors [Martinez et al., 2008; Mosser and Edwards, 2008]. Although many differences between microglial and macrophage reactive phenotypes have been found [Regen et al., 2011], the general properties of the reactive phenotypes are similar in both cell types. Figure 1.2 provides an overview of the activation paradigms, used in this work.

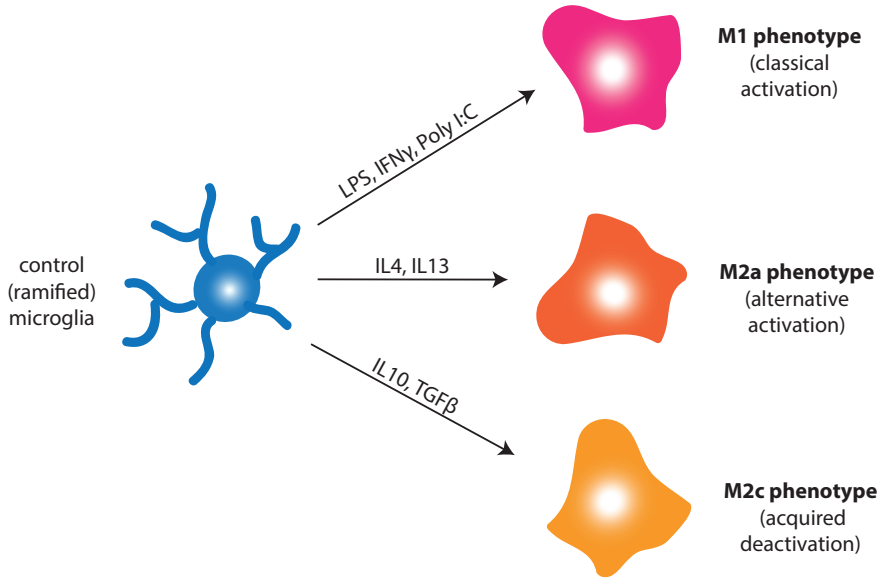


**Figure 1.2: Activation states of microglia.** ***In vitro*** **treatment with LPS, IFN$\gamma$ or Poly I:C induces a transition in the M1 (classical activation) phenotype, while IL4/IL13 induce the M2a (alternative activation) and IL10/TGF$\beta$ induce the M2c (acquired deactivation) phenotype.**

## 1.2 Astrocytes

Astrocytes (from greek: star-(shaped) cell), another type of glial cell, are believed to be the most abundant cells in the CNS. They comprise a rather heterogeneous cell population: fibrous astrocytes are present in the white matter and contact Nodes of Ranvier and blood vessels, while protoplasmic astrocytes are found in the grey matter and enwrap synapses and blood vessels. Radial glia are a type of bipolar astrocytes which are mostly found during development. Most astrocytes express the intermediate filament glial fibrillary acidic protein (GFAP), which is commonly used for their histological identification. Astrocytes have many functions in the healthy CNS, the most important ones so far described being:

- **Metabolic support** of the neurons by producing, storing and supplying them with glycogen and lactate [Bélanger et al., 2011]

- **Release and uptake of neurotransmitters** such as glutamate, GABA and ATP at the tripartite synapse, which ensure a tight regulation of neurotransmitter concentrations in the synaptic cleft and a modulation of synaptic transmission [Ventura and Harris, 1999]
- Participating in the **blood-brain barrier** by contacting blood vessels with their endfeet and regulation of the blood flow by release of vasodilating or constricting molecules [Attwell et al., 2010]
- **Support of myelination** executed by oligodendrocytes [Ishibashi et al., 2006]
- Regulation of the **cation concentration** in the extracellular space by rapidly reducing excess potassium concentrations which might otherwise lead to neuron depolarization [Walz and Hertz, 1983]

Apart from their many functions in the healthy brain, astrocytes also respond to many pathologies through so-called reactive astrogliosis, which may culminate in the formation of a glial scar.

### 1.2.1 Astrocytic calcium waves

Although many important astrocytic functions had been explored for a number of years, the finding that astrocytes express different membrane receptors which enable them to respond to neuronal activity by an increase of calcium levels were paradigm-shifting for the glia field, since they opened the possibility that astrocytes can be involved in CNS information processing [Scemes and Giaume, 2006]. Astrocytes are connected to each other via gap junctions (GJ) and build an electrically coupled syncytium. In 1990, Cornell-Bell and colleagues made the exciting observation that stimulation of a monolayer of hippocampal astrocytic cultures with glutamate leads not only to a local increase of intracellular calcium, but also to activation of adjacent cells in a wave-like fashion [Cornell-Bell et al., 1990]. This phenomenon was termed intercellular calcium wave (ICW). ICWs can be evoked not only *in vitro*, but also in acute brain slices, where fewer cells participate in a wave. *In vivo*, calcium waves occur under physiological conditions in the hippocampus and the cerebellum [Nimmerjahn et al., 2009] and also in pathology, for example in Alzheimer's disease (AD) [Kuchibhotla et al., 2009]. The number of cells participating in a wave depends on the brain region [Blomstrand et al., 1999]. ICWs can occur spontaneously or upon induction via pharmacological (e.g. glutamate application), electrical or mechanical stimulation (pipette touch of a single astrocyte) [Charles, 1998] and they spread to neighboring cells via diffusion of inositol trisphosphate ($IP_3$) through GJs [Charles et al., 1993]. Interestingly, ICWs can spread even if astrocytes are not confluent and cross cell-free areas, thus it was hypothesized that activated astrocytes can release a factor which is sensed by neighboring cells [Hassinger et al., 1996]. Guthrie et al. identified the extracellular component as ATP [Guthrie et al., 1999], which is released by the astrocytes participating in the wave

[Cotrina et al., 1998] and activates other astrocytes in a paracrine fashion by acting on purinergic receptors [Fumagalli et al., 2003]. Nowadays, it is generally accepted that both intra- and extracellular pathways are working in conjunction to enable the spread of an ICW. On the one hand second messengers, which can mobilize $Ca^{2+}$ are transferred via GJs from the cytosol of one cell to its neighbor; on the other hand there is *de novo* generation of second messengers via activation of P2 receptors on the cell surface by extracellular ATP [Scemes and Giaume, 2006].

## 1.3 Purinergic signaling

Purinergic signaling is a form of extracellular signaling which involves the action of nucleotides and nucleosides on purinergic receptors [Burnstock et al., 2010]. It is one of the most widespread intercellular signaling systems in living tissue and has so far been found not only in vertebrates and many invertebrates, but even in plants [Choi et al., 2014]. More than 230 years ago, uric acid, a breakdown product of purine nucleotides, was first isolated by the Swedish pharmacist Scheele [Scheele, 1776], a discovery which is now considered as the birthdate of purinergic signaling. Later on, more purines and pyrimidines were discovered by Ludwig Kossel [Bendich A, 1955]. Of particular importance for the field of purinergic signaling was the work of Emil Fischer, who elucidated the structure of purines and was awarded the Nobel Prize for his discoveries in 1902 [Fischer, 1907]. In 1929, ATP was discovered by Karl Lohmann in Germany [Lohmann, 1929] and in the following decade, its role in the energetics of the cell was established.

### 1.3.1 Extracellular nucleotides and purinergic receptors

Nucleotides are organic molecules – subunits of DNA and RNA. They consist of a sugar (ribose or deoxyribose), nitrogenic base (also called nucleobase) and one to three phosphate groups. A nucleotide without any phosphate groups is called nucleoside. The nucleobase can be either a purine (adenine or guanine) or a pyrimidine (thyamine, uracil or cytosine). Nucleoside triphosphates - ATP, GTP, UTP and CTP - play an important role in the metabolism of the cell. Within the cell, ATP is an energy currency, but once released in the extracellular space, it activates purinergic receptors.
Release of nucleotides can occur via different mechanisms. Large amounts of nucleotides are released upon cell damage, for example in the context of tissue injury or inflammation. Under physiological conditions, small amounts of nucleotides can be released via ATP release channels, nucleotide transporters or via vesicular exocytosis [Yegutkin, 2008] (for an overview, see Fig.1.3).

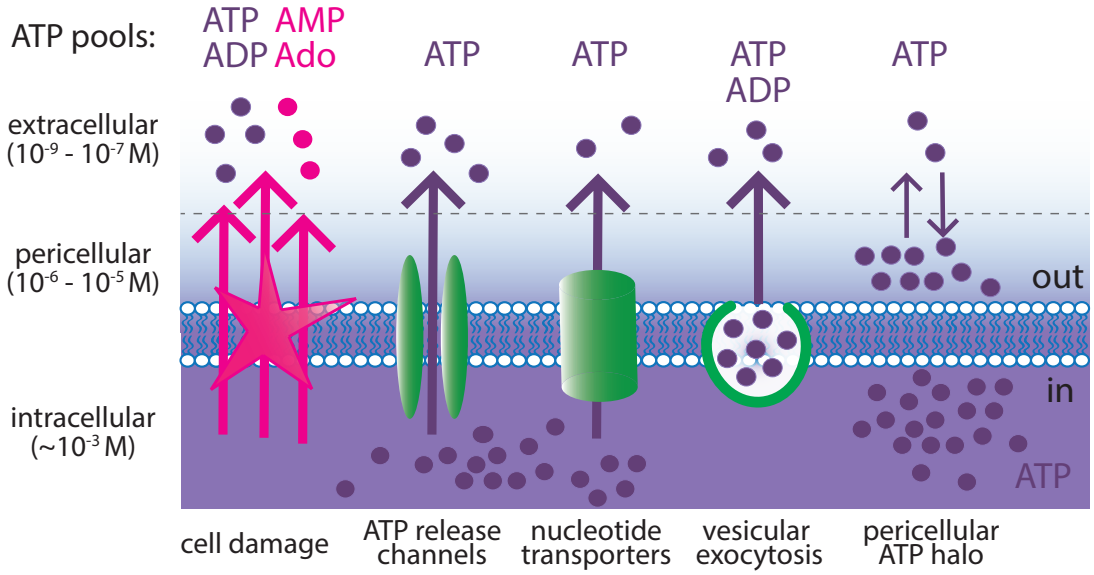


**Figure 1.3: Nucleotide release in the extracellular space can occur via cell damage, ATP release channels, nucleotide transporters or via vesicular endocytosis.** ***Modified from Yegutkin [2008].***

Purinergic receptors are expressed in almost all mammalian tissues and are involved in important physiological processes such as learning and memory, locomotion, feeding and sleep [Burnstock et al., 2010]. They are divided into three classes: P1, P2X and P2Y. P1 receptors are G-protein coupled receptors which respond to adenosine. Four subtypes have been described so far – $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$. P2X receptors are ligand-gated channels which open a pore permeable to cations ($Na^+$, $K^+$ and $Ca^{2+}$) upon binding ATP. The pore is built by three monomers which can be organized as homomers or heteromers. Seven subtypes of P2XR are known so far: $P2X_1$–$P2X_7$ [Abbracchio et al., 2009]. In the context of immunity, $P2X_4$ and $P2X_7$ have received special attention. P2Y receptors are G-protein coupled receptors which consist of seven transmembrane domains and can be activated by different nucleotides including ATP. Currently, eight P2Y receptors have been identified, each having a different affinity to nucleotides. Figure 1.4 provides an insight into the mechanism of action of P2R.

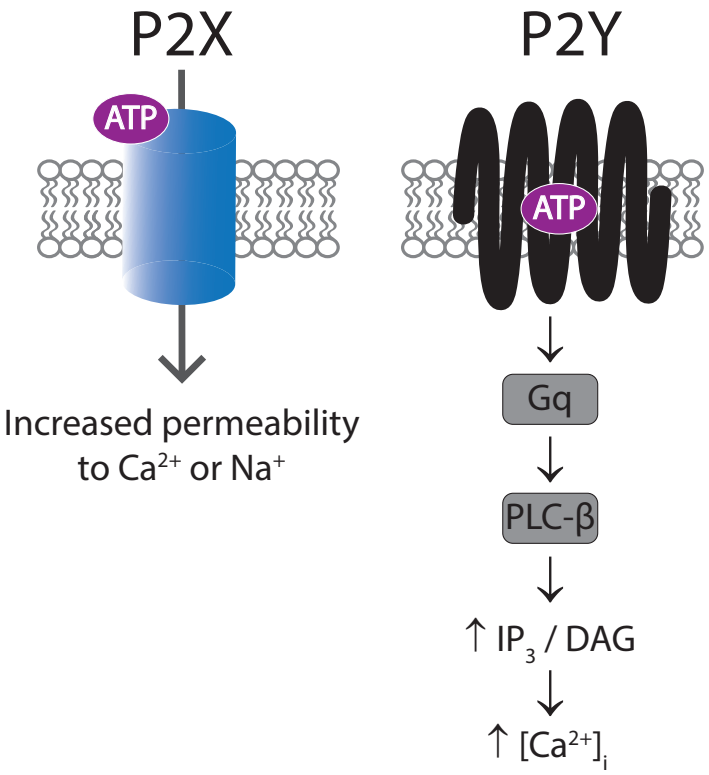


**Figure 1.4: Receptors of the P2 family.** ***Modified from Bucheimer and Linden [2004].***

Functional purinergic receptors are expressed by all glial cells, both in the central (CNS) and the peripheral (PNS) nervous systems. In neuronal-glial and glial-glial communication, purinergic signaling plays a crucial role. ATP has been recognized to act either as a co-transmitter or as a sole transmitter in most neurons of the CNS and PNS [Burnstock, 2007]. ATP released from astrocytes is important for generation and propagation of astrocytic calcium waves. On microglia, ATP and other nucleotides regulate pathological functions like motility, phagocytosis and proliferation. Two main mechanisms ensure the proper coordination of ATP activation of microglia - first, by intrinsic regulation of P2 receptor expression and sensitization and second, by cell surface enzymes, which control the extracellular concentration of nucleotides: ectonucleotidases.

### 1.3.2 Ectonucleotidases

Ecto-enzymes are catalytically active membrane located proteins which have their active sites outside the cell [Goding, 2000]. They can be divided in two main classes: nucleotidases and peptidases. Ecto-enzymes are involved in local control of various factors – cytokines, hormones – which modulate cellular functions like cell growth, differentiation and chemotaxis. Ectonucleotidases are ecto-enzymes, able to hydrolyze nucleotides to nucleosides under the release of inorganic phosphate. Ectonucleoti-

dases can be divided into 4 families: ecto-nucleoside triphosphate dyphosphohydrolases (E-NTPDases), ectonucleotide pyrophosphatase/ phosphodiesterases (E-NPP), alkaline and acid phosphatases (ALP and ACP) and ecto-5-nucleotidase (CD73) [Zimmermann et al., 2007]. The E-NTPDase family comprises of 8 members, termed E-NTPDase 1-8 [Robson et al., 2006]. Four of these proteins are surface-located with their catalytic site facing the extracellular space (NTPDase 1, 2, 3 and 8). NTPDases 4, 5, 6 and 7 have an intracellular localization, and only NTPDases 5 and 6 can be secreted. All NTPDases hydrolyze nucleotide triphosphates, especially ATP and UTP, equally well, but have different substrate specificities to nucleotide diphosphates. The surface-located enzymes are inactive in absence of $Ca^{2+}$ and $Mg^{2+}$ ions [Kukulski et al., 2005]. Differences in catalytic activities between subtypes could be explained as a consequence of their different sequences and structures [Grinthal and Guidotti, 2004]. Functional differences of the enzymes account for different activation of P2 receptors by the respective nucleotides: for instance, NTPDase1 hydrolyzes ATP (almost) directly to adenosine-5'-monophosphate (AMP) while NTPDase2 hydrolysis of ATP leads to an accumulation of adenosine-5'-diphosphate (ADP) which can activate diphosphate-sensitive P2Y receptors. The slower action of NTPDase3 and 8 leads to simultaneously present tri- and diphosphonucleosides [Robson et al., 2006]. Altogether, different substrate specificities of the NTPDases lead to different compositions of tri- and di-phosphonucleotides in the extracellular space, which activate different purinergic receptors, thus a finely-tuned purinergic signaling balance is ensured. Structurally, all NTPDases consist of five "apyrase conserved regions" – ACR1 to 5 – and form homo-oligomeric assemblies, for example dimers, trimers or tetramers [Schulte am Esch et al., 1999]. The surface-located NTPDases (1, 2, 3 and 8) are anchored to the cell membrane by two transmembrane domains (TMD), which may change their position relative to each other upon nucleotide binding and also induce conformational changes, thus influencing enzymatic function [Grinthal and Guidotti, 2006]. NTPDase expression has been found in virtually all tissues and since they play an essential role in the vasculature, the immune system and the CNS, NTPDase research has been mainly focusing on these areas.

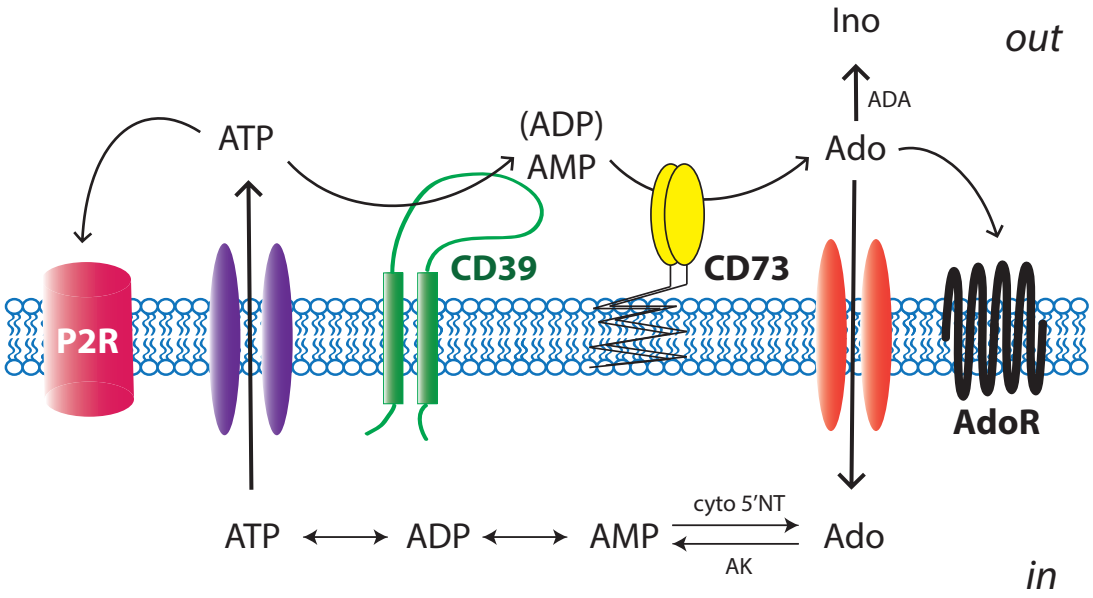


**Figure 1.5: Nucleotide metabolism in the extracellular space. After release, ATP can activate purinergic receptors (P2X or P2Y) and be metabolized to adenosine by the ectonucleotidases CD39 and CD73. Adenosine (Ado) can activate adenosine receptors (AdoR) and get hydrolyzed to inosine (Ino) by adenosinedeaminase (ADA). Within the cell, ATP can be converted to ADP and AMP. AMP can be hydrolyzed to adenosine by cytosolic 5-nucleotidase (cyto 5'NT). Adenosine can be converted to AMP by adenosine kinase (AK). *Modified from Zhou et al. [2010].***

### 1.3.3 The ectonucleotidase CD39

One of the most prominent members of the NTPDase family, CD39 (NTPDase 1), was first identified on the surface of activated natural killer (NK) cells, B cells and subsets of T cells, but not in the resting state of these cells [Kansas et al., 1991]. It was also found on many different types of endothelial cells and it was proposed that it plays a role in lymphocyte adhesion. A few years later, Wang and Guidotti described sequence homology of *Cd39* with that of potato apyrase and were also able to show that COS-7 cells transfected with *Cd39* recombinant cDNA have apyrase activity [Wang and Guidotti, 1996]. Kaczmarek et al. cloned human *CD39* and found that it is functionally identical with the vascular ATPDase [Kaczmarek et al., 1996]. CD39 was then called "ecto-ATP diphosphohydrolase" because it is able to hydrolyze both ATP and ADP [Goding, 2000]. Since the nomenclature so far was quite chaotic, at the Second International Workshop on Ecto-ATPases and Related Ectonucleotidases [Zimmermann et al., 1999] it was decided, that the CD39 family should be termed E-NTPDase family and its members should be numbered in the order of discovery. Since, the official name of CD39 is E-NTPDase 1, although both are commonly used. In this work, I will use the name CD39 for practical reasons.

#### 1.3.3.1 Distribution and functional properties

CD39 expression was found to be present not only on immune cells, but also in human placenta, lung, skeletal muscle, kidney and heart, but not in the brain [Kaczmarek et al., 1996]. Later studies suggested CD39 expression in neurons and astrocytes, however, Braun et al. and colleagues suspected cross-reactivity of antibodies, binding to other N-TPDases, and assigned CD39 expression in the CNS exclusively to microglial cells and the vascular endothelium [Braun et al., 2000]. Interestingly, the N-terminal domain of the protein, which is located in the plasma membrane, is palmitoylated, which raises the possibility that CD39 can be recycled and does not always require *de novo* formation [Robson et al., 2006]. Similarly to the other members of the NTPDase family, CD39 requires calcium ($Ca^{2+}$) or magnesium ($Mg^{2+}$) ions (ca. 1.8 mM) and a pH range between 7 and 9.5 for maximal activity [Kukulski et al., 2005]. A very important property of CD39 is its Michaelis constant ($K_m$). In enzyme kinetic reactions, $K_m$ describes the concentration of the substrate at which half of the $V_{max}$ (the maximum rate, achieved by the system) is reached. CD39 has a $K_m$ (for ATP) of 17 $\mu$M (human) and 12 $\mu$M (murine), it is thus much more effective than other NTPDases in removing very low concentrations of nucleotides from the extracellular space and in terminating purinergic signaling, because the $EC_{50}$ values of the P2 receptors lie in the range of activity of the enzyme. The importance of CD39 for control of purinergic signaling is further underlined by a FRET study, showing that G-protein coupled purinergic receptors form heterooligomers not only with each other, but also with CD39 [Schicker et al., 2009].

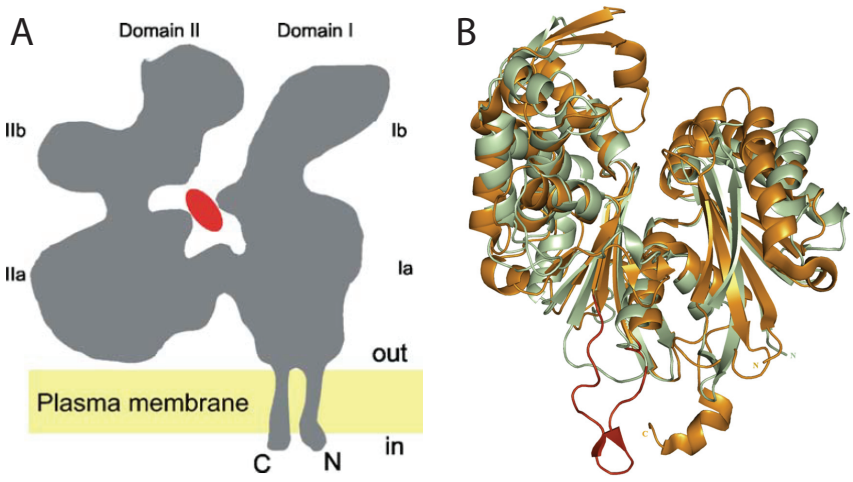


**Figure 1.6: Structure of the ectonucleotidase CD39. A: Membrane topology of the surface-located CD39 with two transmembrane domains. *From Robson et al. [2006].* B: Proposed crystal structure of the prokaryotic NTPDase from *Legionella pneumophila*, which is a functional and structural homolog to the eukaryotic one. *From Vivian et al. [2010].***

#### 1.3.3.2 CD39 in the immune system

ATP can be released after cell lysis (in very high concentrations) upon injury, trauma or inflammation – or via several non-lytic mechanisms at lower concentrations [Yegutkin, 2008]:

- Exocytosis
- Nucleotide-permeable channels (hemichannels, $P2X_7R$ etc.)
- Transport vesicles
- Lysosomes

Upon release, ATP is rapidly metabolized on the cell surface to AMP by CD39 and further to adenosine by CD73. The presence of ATP is characteristic of a pro-inflammatory environment, while adenosine has an anti-inflammatory effect. In this perspective, CD39 and CD73 are regarded as an "immunological switch", crucial for maintaining the balance between ATP and adenosine in the extracellular space and thus for fine-tuning cellular functions [Antonioli et al., 2013]. There is increasing evidence that the CD39/CD73 pathway can change dynamically in the pathophysiological context of many diseases, such as diseases of the immune system, cancer and even schizophrenia [Schetinger et al., 2007; Aliagas et al., 2013; Antonioli et al., 2013; Bastid et al., 2013]. CD39 was first discovered and described as an activation marker on lymphocytes [Kansas et al., 1991]. $Foxp3^+$ regulatory T cells ($T_{reg}$), which are responsible for limiting the size of inflammation and for preventing autoimmunity, show very high surface expression of CD39 and its enzymatic activity correlates with the activation status of the cell [Schuler et al., 2011]. The ATP metabolizing activity is crucial for the immunosuppressive function of $T_{reg}$ cells and also supports their invasion into the inflamed tissue [Borsellino et al., 2007]. Adenosine generation is part of an autocrine loop, through which $T_{reg}$ cells expand and reinforce their immunosuppressive activity [Ohta et al., 2012]. T helper cells ($T_h17$) also express both CD39 and CD73 and this expression is regulated by factors, which induce $T_h17$ differentiation - IL6 and TGF$\beta$ [Chalmin et al., 2012]. Neutrophils, the first cells to respond in acute inflammation and to migrate toward a source of chemokines, express CD39, which is involved in creating an ATP gradient, supporting their chemotaxis [Chen et al., 2006].

### 1.3.4 Purinergic signaling and microglia

Purinergic signaling is involved in a variety of microglial properties, including phagocytosis, motility and chemotaxis. The first observations on purinergic modulation of microglial motility were published by Honda et al. [2001], who saw that application of ATP or ADP, but not adenosine, induces membrane ruffling in primary rat cultured microglia. Furthermore, they observed that ATP and ADP induce chemokinesis in the Boyden chamber assay as well as chemotaxis in the Dunn chamber. By using purinergic

signaling blockers, these effects were assigned to G-coupled P2Y receptors. The next crucial study on microglial motility was published by Nimmerjahn et al. [2005]. In an *in vivo* setting of the mouse brain where in the microglia one copy of the fractalkine gene locus (*Cx3cr1*) was exchanged for enhanced green fluorescent protein (*EGFP*), they observed that the microglia are in fact highly motile in their previously assumed "resting" state. While the cell somata remain immotile, the processes are constantly extended and retracted, an observation which was termed as a "scanning" behaviour. Upon a laser-induced micro-stroke, the microglia nearby extended their processes towards the site of the damage, while retracting the processes on the opposite side. These results were confirmed by Davalos et al. in a more extensive study. Since a local ATP application via a pipette induces a similar response, which is abolished by apyrase (an enzyme, which rapidly degrades ATP), ATP was recognized as the molecule triggering the microglial response to the laser lesion. In the laser lesion setting, damaged cells release high amounts of ATP which is sensed by microglia in the vicinity. Since in mice lacking *P2ry12* the microglial response to laser lesion is abolished [Haynes et al., 2006], this response was attributed to the $P2Y_{12}$ receptor. The process extension is very rapid and within minutes, the lesion site is "engulfed" by the microglial growth cones and the damage site is reduced. If the response of the microglia is blocked, for example by application of $Cl^-$ channel blocker tamoxifen or actin polymerization inhibitor Latrunculin B, the damage spreads further in the brain parenchyma [Hines et al., 2009]. Later it was also shown that $P2Y_{12}$ receptor activation by ATP/ADP requires phosphatidylinositol-3'-kinase (PI3K) and phospholipase C (PLC) pathway activation and that the process extension depends on integrin-$\beta$ activation which then accumulates in the tips of the microglial processes to facilitate their extension [Ohsawa et al., 2010]. While microglia extend their processes toward an ATP source, they simultaneously retract the processes on the opposite side, which is mediated by activation of the adenosine $A_{2A}$ receptor by the breakdown product of ATP – adenosine [Orr et al., 2009]. Microglia, lacking the $A_1$ receptor or the ectonucleotidase CD39 do not migrate upon stimulation of $P2Y_{12}$ receptors, even though adenosine alone has no effect [Farber et al., 2008]. Migration is restored by adding either adenosine or soluble CD39. Thus, microglial migration depends on a finely balanced interplay between ATP activating $P2Y_{12}$ receptor and its metabolite adenosine acting on the $A_1$ receptor [Koizumi et al., 2013]. Upon migrating to the site of injury, microglia can become phagocytic, a process mediated by UDP that leaks from dying or injured cells and activates microglial $P2Y_6$ receptors [Koizumi et al., 2007]. UDP is considered as a "find me" phagocytic signal, as it is transiently available upon release and quickly metabolized by cell surface enzymes and does not activate other P2 receptors besides $P2Y_6$ [Koizumi et al., 2013]. Interestingly, UDP differs from ATP in one crucial aspect: it is a spatiotemporally restricted signal. This and the fact that UDP does not activate $P2Y_{12}$ receptors means that microglial migra-

tion and phagocytosis are regulated by two distinct mechanisms. Koizumi et al. [2013] proposed a model for a modal shift of microglial function during activation: the migrational phenotype triggered by $P2Y_{12}$ receptor activation transitions to a phagocytic phenotype by downregulating *P2ry12* expression and upregulating *P2ry6* expression, as shown in figure 1.7.

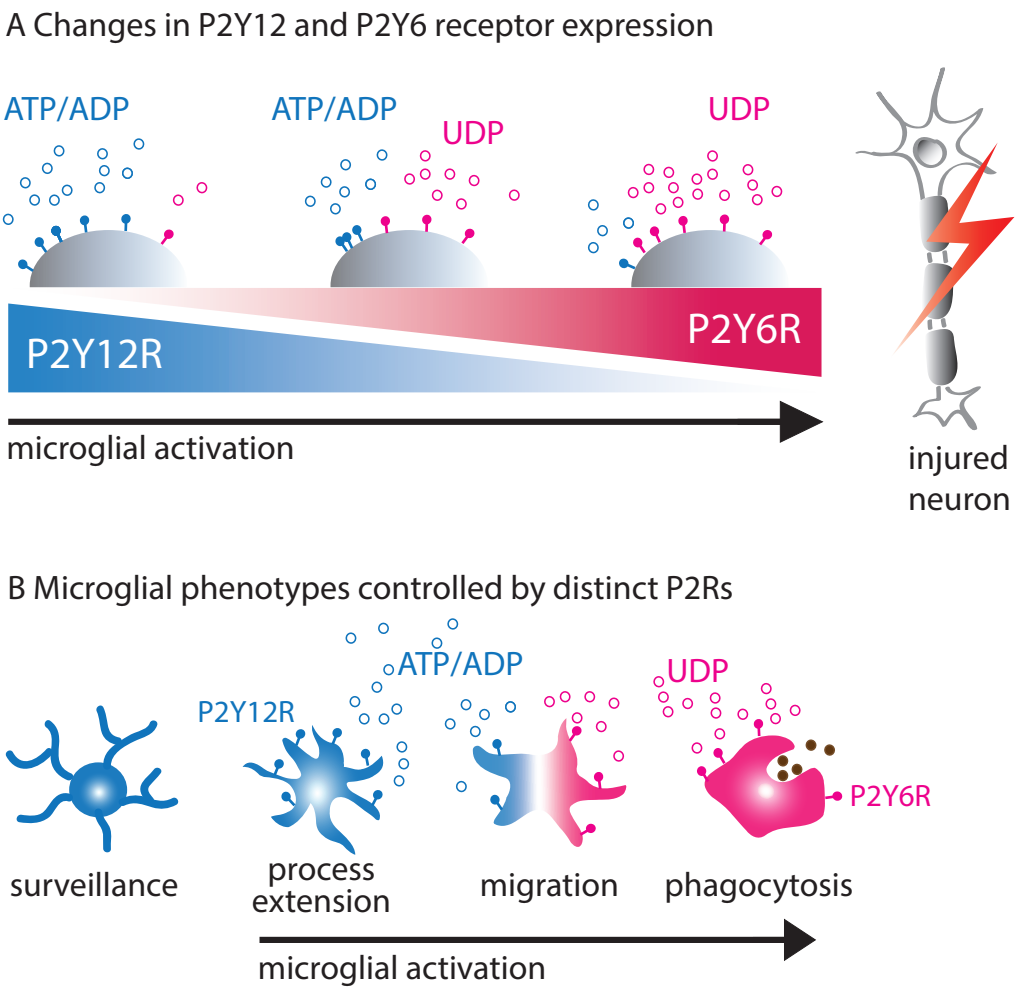


**Figure 1.7: A proposed model for a modal shift of microglial receptor expression, phenotype and function during activation. A: Upon nucleotide binding, *P2ry12* expression is downregulated and *P2ry6* expression is upregulated. B: Changes in *P2ry* expression are key for the transition of activated microglial cells from the resting via the migratory to the phagocytic phenotype. *Modified from Koizumi et al. [2013].***

.

#### 1.3.4.1 CD39 and microglia

One major player in purinergic signaling apart from the nucleotides and their respective receptors, are the ectonucleotidases which regulate the concentration of nucleotides

in the extracellular space. CD39 is the only ectonucleotidase expressed by microglia and its importance for proper microglial function has been extensively studied in our lab. In 2006, Farber et al. observed that in *Cd39*$^{-/-}$ cells, ATP does not induce migration, in contrast to wildtype cells [Farber et al., 2008]. ATP-induced migration could be restored either by the addition of soluble CD39 (apyrase), or by NECA, a non-hydrolysable analogue of adenosine. Moreover, microglial migration was also disrupted in microglia derived from mice lacking the $A_1$ adenosine receptor. The authors conclude that migration depends on both ATP and its metabolite adenosine. CD39 seems to be instrumental for achieving the nucleotide/nucleoside balance necessary for microglial migration. Microglial CD39 is also important for the phagocytic activity of the cells [Bulavina et al., 2013]. *Cd39*$^{-/-}$ microglia have higher phagocytic activity, which cannot be further enhanced by the addition of ATP in contrast to wildtype cells. Either blocking of purinergic receptors with PPADS or activation of adenosine receptors with NECA decreases phagocytosis in both wildtype and knockout microglia. Since *Cd39*$^{-/-}$ microglia are unable to metabolize ATP, which reflects on the overall ATP degrading activity of brain tissue, the authors proposed that the lack of CD39 leads to chronically elevated ATP levels which induce constant phagocytic activity.

#### 1.3.4.2 The *Cd39*$^{-/-}$ mouse model

Mice deficient in CD39 in all tissues were generated by homologous recombination and characterized by Keiichi Enjyoji in 1999 [Enjyoji et al., 1999]. Developmental or reproductive abnormalities were detected neither in the homozygous *Cd39*$^{-/-}$ nor in the heterozygous *Cd39*$^{+/-}$ animals. Plasma levels of both ATP and ADP were normal. However, these mice showed abnormal counts and function of peripheral blood platelets, resulting in significantly prolonged bleeding times from 1 minute in wildtype animals to up to 7 minutes or even 20 minutes in the knockouts, probably due to $P2Y_1$ receptor desensitization on the platelets.

Purinergic signaling is a fundamental system, found in all cell types. There is emerging evidence for purinergic disbalance in many CNS pathologies, which are tightly linked to microglial function. For instance, in schizophrenia brains, a shift in the ATP/adenosine ratio has been proposed [Boison et al., 2012].

## 1.4 Schizophrenia

Schizophrenia (from Greek, "splitting of the mind") was first termed by the Swiss psychiatrist Paul Eugen Bleuler in 1908, when he realized that the previously accepted name of the disease, *dementia praecox*, was rather inaccurate. Schizophrenia is a debilitating and deteriorating disorder, mainly characterized by five groups of symptoms

[van Os and Kapur, 2009]:

1) **Positive** symptoms (psychosis and hallucinations)
2) **Negative** symptoms (such as social withdrawal and motivation loss)
3) **Cognitive deficits** (memory and attention are usually affected)
4) Affective dysregulation consisting of both
   a. **Depression** and/ or
   b. **Mania**.

It is also termed as a non-affective psychosis, with a higher prevalence of the negative symptoms and less depression and mania compared to bipolar disorder. Schizophrenia is usually diagnosed in early adulthood in 0,3-0,7 % of the general population, and men are more likely to be affected than women [McGrath et al., 2008]. Life expectancy in patients is decreased by 12-15 years, not only due to higher suicide rates but also due to socioeconomic and lifestyle factors, influenced by the disease [Saha et al., 2007] or the treatment. Schizophrenia is currently treated with antipsychotic drugs, which block the dopamine $D_2$ receptor, however they only ameliorate the positive symptoms of the disease and often induce metabolic or motor side-effects [Leucht et al., 2009].

### 1.4.1 The dopaminergic / glutamatergic hypothesis

Our current understanding of the pathophysiology of schizophrenia is based largely on the theory of neurotransmitter disbalance. In particular, dopamine hyperfunction and glutamate hypofunction seem to play a major role. Dopamine is a neurotransmitter, which is involved in motor control, motivation and reward. The dopaminergic hypothesis bases on the observation that many antipsychotic drugs are in fact blockers of the $D_2$ receptors, whereas amphetamine, which induces dopamine release, exacerbates psychotic symptoms [Seeman et al., 2006]. Positron emission tomography (PET) imaging studies have found increased basal occupation of $D_{2A}R$ [Abi-Dargham et al., 2000], higher dopamine turnover [Lindström et al., 1999] and increased amphetamine-induced dopamine release in the brains of schizophrenic patients [Laruelle and Abi-Dargham, 1999]. Glutamate, the main excitatory neurotransmitter in the vertebrate CNS, acts on ionotropic (AMPA, NMDA and kainate) and metabotropic (mGlu) receptors. The glutamatergic system dysfunction hypothesis in schizophrenia has been proposed, because NMDA receptor antagonists induce the positive, the negative and the cognitive effects of schizophrenia [Olney and Farber, 1995]. In addition, treatment with glycine or glycine reuptake inhibitor leads to a reduction in the negative symptoms [Tsai et al., 2004].

### 1.4.2 The adenosine hypothesis

Current pharmacotherapy of schizophrenia relies on dopaminergic treatment, which only affects the positive symptoms of the disease and is accompanied by unwanted side effects. On the other hand, inhibition of the glycine receptor Glyt1 has shown to be promising for ameliorating the negative and cognitive symptoms of schizophrenia [Yee et al., 2006]. Thus, an approach which would modulate both the dopaminergic and glutamatergic system dysfunctions is required. Adenosine, a homeostatic bioenergetic network regulator, would make an excellent candidate for restoring the neurotransmitter disbalance in the schizophrenia brain [Boison et al., 2012]. The adenosine hypothesis was first proposed by Lara and colleagues [Lara and Souza, 2000; Lara et al., 2006]. Adenosine is a purine ribonucleoside and a break-down product of ATP. Upon release of ATP, either by passive leakage out of injured cells, or via vesicles or hemichannels, mainly from astrocytes, it is rapidly dephosphorylated to AMP and further to adenosine. Another source of adenosine is its direct release by nucleoside transporters. Adenosine acts on four different types of receptors: $A_1R$ and $A_3R$ are inhibitory, while $A_{2A}R$ and $A_{2B}R$ are stimulatory receptors. $A_1R$ is the most abundantly expressed throughout the CNS, especially in the neocortex, cerebellum and hippocampus [Bauer et al., 2003], while $A_{2A}R$ is mainly found in the striatum [Ferré, 1997]. Interestingly, $A_1R$, which inhibits release of dopamine and glutamate [Dunwiddie and Masino, 2001; Fredholm et al., 2005], builds heterodimers with $A_{2A}R$, which promotes neurotransmitter release [Fredholm et al., 2005]. Furthermore, $A_{2A}R$ has been shown to build heteromers with the dopamine $D_{2A}R$ receptor [Ferré, 1997]. Thus adenosine is claimed to be "strategically positioned to affect several molecular pathways synergistically" [Boison et al., 2012]. Glutamatergic activity is regulated by adenosine as well. For instance, in the hippocampus, adenosine has an inhibitory action on glutamate release [Dunwiddie and Masino, 2001] and NMDA receptor function thus inhibiting long-term potentiation (LTP) and depression (LTD) [Rebola et al., 2008]. Adenosine-mediated inhibition of NMDAR function underlies the neuroprotective effect of adenosine against excitotoxicity during hypoxia [Dunwiddie and Masino, 2001]. Reciprocally, NMDAR activation can have inhibitory effects on $A_1R$ agonist action [Bartrup and Stone, 1990]. Several clinical studies provide further support for the adenosine hypothesis of schizophrenia. Kurumaji and colleagues have found an increased density of $A_{2A}R$ in post-mortem tissue of schizophrenia patients [Kurumaji and Toru, 1998], which might be explained as a compensatory effect for reduced extracellular levels of adenosine [Lara et al., 2006]. A connection has been established between $A_1R$ polymorphism and schizophrenia pathophysiology [Gotoh et al., 2009]. A clinical study with adjunctive allopurinol (a purine degradation inhibitor) therapy has shown some moderate effects, probably due to elevated adenosine levels [Brunstein et al., 2005]. Beneficial effects have been reported after treatment with an adenosine transport in-

hibitor, dipyramidole [Akhondzadeh et al., 2000]. Preclinical data show that $A_1R$ and $A_{2A}R$ agonists can inhibit the effect of psychostimulant drugs and rescue the pre-pulse inhibition (PPI) deficits, induced by activation of $D_2R$ or blocking of NMDAR [Boison et al., 2012]. Several knockout mouse models for adenosine signaling provide further insights into the link between schizophrenia-related cognitive and behavioral deficits and adenosine system dysfunction (for a review, see [Boison et al., 2012]).

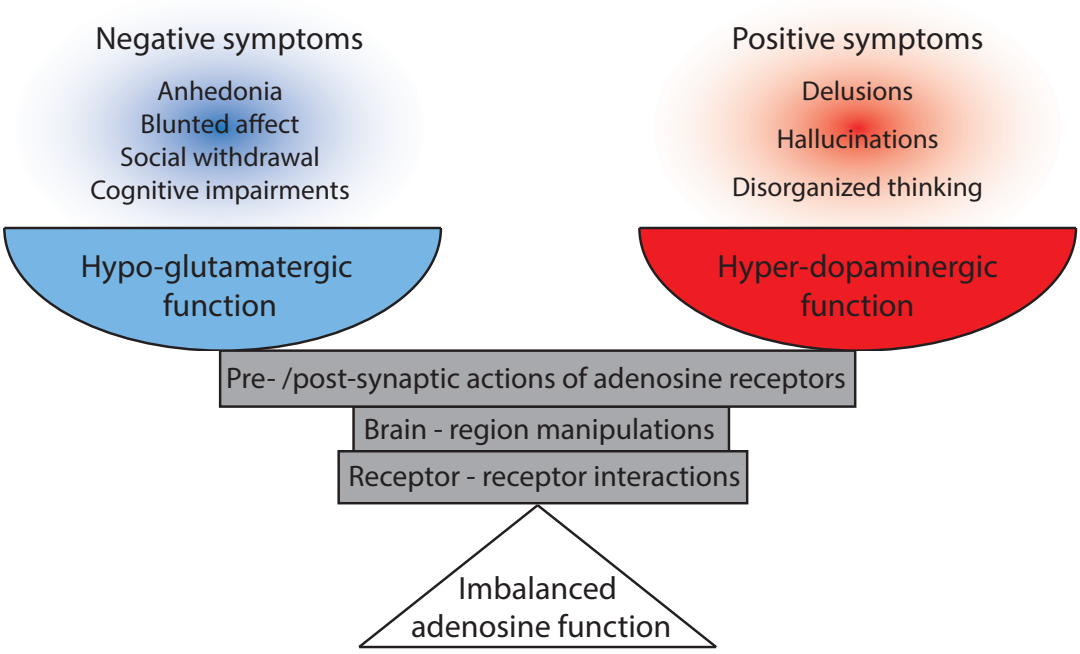


**Figure 1.8: The adenosine hypothesis of schizophrenia: adenosine is a neurotransmitter, which is strategically positioned to restore the balance in both glutamatergic and dopaminergic dysfunction in schizophrenia.** ***Modified from Boison et al. [2012].***

### 1.4.3 Microglia in schizophrenia

Although the number of studies focusing on the involvement of microglia in the pathophysiology of schizophrenia has been rapidly increasing in the recent years, there is still a lot of controversy on certain issues. Microglial density has been regarded as a hallmark of brain pathology. Studies of post-mortem brains of schizophrenic patients have found increased microglial density in the frontal and temporal association cortex [Garey, 2010], in the hippocampus [Busse et al., 2012] and in the prefrontal cortex (PFC) [Fillman et al., 2013]. Another study showed activation of microglial cells in the prefrontal cortex and the visual cortex [Uranova et al., 2010]. On the other hand, Steiner at al. did not find any differences between microglial density in schizophrenic and healthy human brains [Steiner et al., 2006]. Similarly, in animal models of schizophrenia, contrasting observations have been made: Juckel et al. found

increased microglial density in the hippocampus and the striatum, but not in the PFC of schizophrenic mice [Juckel et al., 2011], whereas Van den Eynde reported increased microglial density and activation in the corpus callosum, hippocampus and thalamus, but not in the striatum and the cortex [Van den Eynde et al., 2014]. A recent study conducted in our lab described increased microglial density in the nucleus accumbens, but not in the ventral striatum, cingulate gyrus, PFC, dentate gyrus or cerebellum [Mattei et al., 2014]. These contradicting reports could be attributed to different analysis methods or the age and gender of the subjects or animals used in the different studies. Evidence for activated microglia in the grey matter was also found in an *in vivo* PET study with a microglial tracer [van Berckel et al., 2008]. It has also been proposed, that microglial density and/or activation may be associated with the acuity and the severity of the disease symptoms, rather than with the disease *per se* [Steiner et al., 2006; Doorduin et al., 2009; Busse et al., 2012]. A recent functional gene data set analysis of genome-wide association data revealed that microglia gene sets are not associated with increased risk of schizophrenia [Goudriaan et al., 2014]. All these data point toward a strong influence of environmental factors on the regulation of microglial activity in the context of schizophrenia [Bernstein et al., 2014] and that microglial activation could be a secondary event in the pathophysiology of the disease.

### 1.4.4 The Poly I:C schizophrenia model

There is emerging evidence from epidemiological, clinical and pre-clinical studies, that maternal infection during gestation can be a risk factor of schizophrenia [Brown and Susser, 2002]. Brown et al. conducted a nested case-control study of cytokine levels in pregnant mothers, in which they found that elevated Il-8 levels in the blood serum of the mothers is associated with diagnosed schizophrenia-spectrum disorder in the offspring [Brown et al., 2004]. Further studies have confirmed that maternal infections of different types can increase up to sevenfold the risk of schizophrenia in the progeny [Brown and Derkitis, 2010]. Inspired by these findings, a rodent model for schizophrenia was established, based on the maternal immune activation (MIA) principle. Infection in the maternal host during pregnancy can disturb normal brain development and exert profound and long-lasting effects on the brain function of the progeny [Meyer, 2014]. In animal models, MIA can be induced by viral or bacterial components or by pro-inflammatory cytokines. Poly I:C (polyriboinosinic-polyribocytidilic acid) is a synthetic analogue of double-stranded RNA, a viral byproduct, which is recognized by the innate immune system via the TLR3 receptor [Alexopoulou et al., 2001]. Upon binding it induces production and release of classical pro-inflammatory cytokines like TNF$\alpha$ and IL6, but also of type I interferons – IFN$\alpha$ and IFN$\beta$ [Kimura et al., 1994]. Poly I:C administration during gestation mimics a viral infection. One particular advantage of this model is that the schizophrenic symptoms only emerge when the offspring have reached

early adulthood, similar to the characteristic maturational delay of schizophrenia in humans [Zuckerman et al., 2003]. The MIA offspring have impaired sensorimotor gating and display deficits in selective attention, social behavior, exploration behavior, working memory and cognitive flexibility and an enhanced sensitivity to psychotomimetic drugs [Meyer, 2014]. The model also shows histopathological and neuropathological changes comparable to the ones evident in schizophrenic patients [Kirkpatrick, 2013], as well as abnormalities in the levels of dopamine, serotonin, and the amino acid taurin as determined by high-performance liquid chromatography [Winter et al., 2008; 2009]. In our experiments, pregnant dams were injected intraperitoneally with either Poly I:C or saline at gestational day 15. In all experiments, I only used male offspring, because they display a higher prevalence of schizophrenic symptoms than female offspring.

## 1.5 Aims of the work

Microglial functions are modulated by molecules in the extracellular space, in particular, ATP is an important signal which influences microglial motility, phagocytosis and response to laser lesion. Microglia are able to degrade ATP and its metabolite ADP via the very potent enzyme CD39, expressed on their surface. Microglia lacking this enzyme have increased phagocytosis and disturbed migration. In this work, I investigate the following questions:

- How is microglial CD39 regulated in the course of microglial activation? Do the *Cd39* gene expression and protein activity change upon an inflammatory challenge? (Project 1)
- How does microglial CD39 control ATP levels under stress conditions - is the ATP release of CD39 deficient microglia different? Is there accumulation of ATP in absence of CD39 and how does this influence astrocytic calcium waves? (Project 2)
- Is microglial purinergic signaling and consequently microglial function altered in microglia, derived from the Poly I:C mouse model of schizophrenia? (Project 3)

# 2. Materials and methods

## 2.1 Materials

### 2.1.1 Chemicals and reagents

| Chemical | Company |
|---|---|
| Adenosine-5'-Diphosphate (ADP) | Sigma Aldrich, St. Louis, USA |
| Adenosine-5'-Triphosphate (ATP) | Sigma Aldrich, St. Louis, USA |
| $\beta$ -mercaptoethanol | Sigma Aldrich, St. Louis, USA |
| AquaPolymount | Polysciences GmbH, Germany |
| Bovine serum albumine (BSA) | Carl Roth, Karlsruhe, Germany |
| Calcium chloride dihydrate ($CaCl_2$) | Carl Roth, Karlsruhe, Germany |
| CD11b beads | Miltenyi Biotec, Germany |
| DNase | Life technologies, USA |
| dNTPs | Life technologies, USA |
| Dimethyl Sulfoxide (DMSO) | Sigma Aldrich, St. Louis, USA |
| DirectPCR® Lysis Reagent Ear | Peqlab Biotechnologie, Germany |
| D-luciferin | Life technologies, USA |
| DTT | Life technologies, USA |
| Dulbeccos Modified Eagles Medium | Life technologies, USA |
| Ethylenediaminetetraacetic acid (EDTA) | Carl Roth, Karlsruhe, Germany |
| Fetal calf serum (FCS) | Life technologies, USA |
| Firefly luciferase | Life technologies, USA |
| Fluo 4 AM | Life technologies, USA |
| Fluorescent latex beads | Polysciences GmbH, Germany |
| Glucose monohydrate | |
| ($C_6H_{12}O_6.H_2O$) | Carl Roth, Karlsruhe, Germany |
| Hank's balanced salt solution (HBSS) | Life technologies, USA |
| HEPES | Carl Roth, Karlsruhe, Germany |
| IFN$\gamma$, recombinant murine | Peprotech, Rocky hill, USA |
| Interleukin 4 (IL4), recombinant murine | PeproTech, Rocky hill, USA |
| Interleukin 10 (IL10), recombinant murine | PeproTech, Rocky hill, USA |
| Interleukin 13 (IL13), recombinant murine | PeproTech, Rocky hill, USA |

| Chemical | Company |
|---|---|
| Lipopolysaccharide (LPS) from E.coli | Enzo Life sciences, Farmingdale, USA |
| Lucifer yellow | Sigma Aldrich, St. Louis, USA |
| Magnesium chloride ($MgCl_2$) | Carl Roth, Karlsruhe, Germany |
| Oligo DT | Life technologies, USA |
| Penicillin-Streptomycin-Glutamine | Life technologies, USA |
| Percoll | Th. Geyer, Renningen, Germany |
| Phosphate- buffered saline (PBS) | Life technologies, USA |
| Pluronic F127 | Sigma Aldrich, St. Louis, USA |
| Poly I:C | Invivogen, San Diego, USA |
| Poly-L-lysine | Sigma Aldrich, St. Louis, USA |
| Potassium chloride (KCl) | Carl Roth, Karlsruhe, Germany |
| Proteinase K solution | Peqlab Biotechnologie, Germany |
| RNase Out | Life technologies, USA |
| Sodium chloride (NaCl) | Carl Roth, Karlsruhe, Germany |
| Sodium Dodecyl Sulfate (SDS) | Sigma Aldrich, St. Louis, Germany |
| di- Sodium hydrogen phosphate dihydrate | |
| ($Na_2HPO_4.2H_20$) | Carl Roth, Karlsruhe, Germany |
| Sodium hydrogen phosphate monohydrate | |
| ($NaHPO_4.H_20$) | Carl Roth, Karlsruhe, Germany |
| Superscript II Reverse Transcriptase | Life technologies, USA |
| SYBR green Select Mastermix | Life technologies, USA |
| Trichloroacetic Acid (TCA) | Merck, Darmstadt, Germany |
| Transforming Growth Factor Beta (TGF$\beta$) | PeproTech, Rocky hill, USA |
| Trypsin | Biochrom, Berlin, Germany |

**Table 2.1: List of chemicals.**

### 2.1.2 Antibodies

| Antibody | Company |
|---|---|
| Cy3-labeled donkey-anti rabbit | Invitrogen, Karlsruhe, Germany |
| Iba1 primary antibody | Wako Pure Chemicals, Japan |

**Table 2.2: List of antibodies.**

### 2.1.3 Buffers

| Buffer | Composition |
|---|---|
| ACSF buffer | NaCl 134 mM, KCl 2.5 mM, $MgCl_2$ 1.3 mM |
| | $CaCl_2$ 2 mM, $K_2HPO_4$ 1.26 mM |
| | D-glucose 10 mM, $NaHCO_3$ 26 mM; pH 7.4 |
| Dissection buffer | HBSS, Glucose 30 mM, HEPES 13 mM |
| DMEM complete | DMEM, 10 % FCS, 2 mM L- glutamin |
| | 100 U/ml penicillin, 100 $\mu$g/ml streptomycin |
| HEPES buffer (Imaging) | HEPES 4.95 mM, NaCl 150 mM, KCl 5.36 mM |
| | $MgCl_2$ 1 mM, $CaCl_2$ 2 mM, Glucose 10 mM |
| HEPES buffer (Malachite green) | HEPES 20 mM, NaCl 135 mM, KCl 5 mM |
| | $CaCl_2$ 1.8 mM, $MgCl_2$ 1 mM, Glucose 5.6 mM |
| L929 fibroblast cond. medium | 1/3 DMEM from confluent L929 fibroblast grown |
| | for 2 days mixed with 2/3 DMEM |
| MACS buffer | PBS, 0.5% BSA, 2mM EDTA |
| Myelin gradient buffer | $NaHPO_4.H_20$ 5.66 mM |
| | $Na_2HPO_4.2H_20$ 11.8 mM |
| | NaCl 137 mM, KCl 5.36 mM, Glucose 10 mM |
| PB buffer | $Na_2HPO_4$ 100 mM, $NaH_2PO_4$ 100 mM |
| Percoll solution | 75% (v/v) myelin gradient buffer |
| | 22% (v/v) Percoll |
| | 2.4% (v/v) 1.5M NaCl |
| Permeabilization buffer | 2 % Triton-X, 2 % bovine serum albumin (w/v) |
| | 10% normal donkey serum in 0.1 M PB |

**Table 2.3: List of buffers.**

### 2.1.4 Commercial kits

| Kit | Company |
|---|---|
| ATP determination kit | Life technologies, USA |
| BCA protein assay kit | Thermo Fisher scientific, Rockford, USA |
| Malachite green phosphate assay | Bioassay Systems, Hayward, USA |
| InviTrap Spin universal RNA mini kit | Invitek, Berlin, Germany |

**Table 2.4: List of commercial kits.**

### 2.1.5 Tools and plasticware

| Tool | Company |
|---|---|
| 4-well plate | Thermo scientific, Rockford, USA |
| 6-well plate | BD Biosciences, Heidelberg, Germany |
| 12-well plate | BD Biosciences, Heidelberg, Germany |
| 24-well plate | BD Biosciences, Heidelberg, Germany |
| 96-well plate (transparent) | BD Biosciences, Heidelberg, Germany |
| 96-well plate (black) | Brand, Wertheim, Germany |
| Cell strainer, 70 $\mu$M | NeoLab, Heidelberg, Germany |
| Glass coverslip | Thermo Fischer Scientific, Walldorf, Germany |
| LS MACS columns | Miltenyi Biotec, Bergisch Gladbach, Germany |
| Magnetic stand for MACS sorting | Miltenyi Biotec, Bergisch Gladbach, Germany |
| Pasteur pipette | Carl Roth, Karlsruhe, Germany |
| T75 cell culture flask | Greiner Bio-one, Frickenhausen, Germany |

**Table 2.5: List of tools and plasticware.**

### 2.1.6 Devices

| Device | Company |
|---|---|
| Camera *PCO sensicam* | PCO AG, Kehlheim, Germany |
| Centrifuges *5417R* and *5810R* | Eppendorf, Hamburg, Germany |
| Confocal microscope *Leica TCS SPE* | Leica Microsystems, Wetzlar, Germany |
| Incubator *Steri-Cult Forma* | Thermo-Scientific, Rockford, USA |
| Microplate reader *Infinite M200* | Tecan, Männedorf, Switzerland |
| Monochromator *Polychrome II* | Till photonics, Gräfelfing, Germany |
| qPCR machine | |
| *7500 Fast Real Time PCR System* | AB Applied Biosciences, Foster City, USA |
| Shaking platform *SSM4* | Stuart scientific, Stone, United Kingdom |
| Spectrophotometer *Nanodrop 1000* | PeqLab Biotechnologie, Erlangen, Germany |
| Thermocycler *T3000* | Biometra, Göttingen, Germany |
| Thermomixer Compact | Eppendorf, Hamburg, Germany |
| Vibratome *Microm HM 650V* | Thermo Scientific, Rockford, USA |

**Table 2.6: List of devices.**

### 2.1.7 Software

| Program | Company |
|---|---|
| Adobe Design Standard CS6 | Adobe Inc, San Jose, USA |
| GraphPad Prism | GraphPad Software Inc., La Jolla, USA |
| ImageJ | National Institute of Health, USA |
| Imaging Cells Easily | own development |
| Microsoft Office (Word, Excel) | Microsoft, Redmond, USA |
| R statistics package | R Foundation for Statistical Computing, Austria. |
| TIDA | HEKA, Lambrecht/Pfalz, Germany |

**Table 2.7: List of software.**

## 2.2 Methods

### 2.2.1 Animals

#### 2.2.1.1 Breeding

Animals were bred at the animal facilities of the Max-Delbrück-Center für molekulare Medizin according to the LaGesO (Landesamt für Gesundheit und Soziales) regulations. All animal experiments were conducted according to the German guidelines for animal care and were approved in advance by the LaGeSo. C57/Bl6 mice were purchased by Charles River Laboratories and *Cd39*$^{-/-}$ mice [Braun et al., 2000] were a kind donation by Prof. Dr. Simon Robson (Gastroenterology Department, Harvard Medical School, Boston, USA).

#### 2.2.1.2 Poly I:C injections

Maternal immune stimulation is an established animal model for schizophrenia [Mattei et al., 2014]. We subjected C57/Bl6 mice to timed mating. Females were checked daily for plug development, considered as gestation day 1. On gestation day 15, pregnant dams received a single injection intraperitoneally of either 150 $\mu$l Poly I:C (5 mg/kg, Sigma-Aldrich, Germany) dissolved in 0.9 % NaCl, or vehicle (Fig.2.1). At the age of 21 days, animals were weaned and females and males were separated. Only male offspring were taken for experiments.

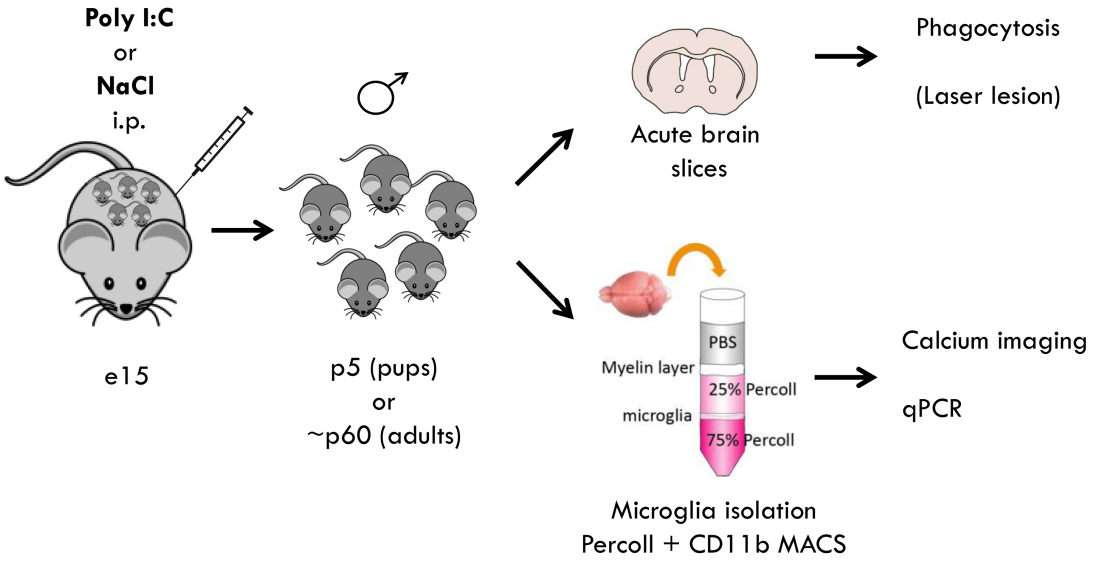


**Figure 2.1: Experimental design of the Poly I:C schizophrenia mouse model: pregnant C57/Bl6 mice received an i.p. injection of either Poly I:C or NaCl solution at gestational day 15. Male pups were then taken at either postnatal day 5 or as adults for acute brain slices or isolation of microglial cells.**

#### 2.2.1.3 Genotyping

*Cd39*$^{-/-}$ mice on the C57Bl/6x129svj background were backcrossed with C57Bl/6 mice. For genotyping, immediately after birth a tailcut was lysed with the DirectPCR® Lysis Reagent Ear. Briefly, the tailcut was incubated for 3 h at 55°C in 100 $\mu$l DirectPCR®-Ear, supplemented with 10 $\mu$l 0.2 mg/ml proteinase K. The reaction mix was then incubated for 50 min at 85°C for proteinase inactivation. 1 $\mu$l of the resulting lysate was employed directly in the PCR reaction, which was set up as follows:

| Reagent | Volume |
|---|---|
| $H_2O$ | 19.85 $\mu$l |
| Buffer | 2.5 $\mu$l |
| dNTPs | 0.5 $\mu$l |
| D3M | 0.5 $\mu$l |
| NEOP2(KO) | 0.5 $\mu$l |
| MEC8P (WT) | 0.5 $\mu$l |
| Taq polymerase | 0.15 $\mu$l |
| DNA | 1 $\mu$l |

**Table 2.8: PCR reaction mix for genotyping of *Cd39*$^{-/-}$ mice.**

The primers used were as follows: for the wildtype Com2 5' CAGAGACCCA-CAAGTCTAGCATCCAGTGG 3' and MEC8P 5'GAC AGA CGA GGG AAG AGG AAG G 3 ', for the knockout Com2 5' CAGAGACCCACAAGTCTAGCATCCAGTGG 3' and NEOP2 5' TAC CCG TGA TAT TGC TGA AGA GCT TGG CGG 3'. The product size is 700 bp (knockout) and 1000 bp (wildtype). The genotyping program used was:

| Cycles | Time | Temperature |
|---|---|---|
| 1 | 3 min | 94°C |
| | 15 s | 94°C |
| 31 | 1 min 30 s | 66°C |
| | 1 min | 72°C |
| 1 | 10 min | 72°C |
| | $\infty$ | 4°C |

**Table 2.9: PCR program for genotyping of *Cd39*$^{-/-}$ mice.**

The PCR products were run on a 1.5 % agarose gel and analyzed under ultraviolet light at a wavelength of 254 nm. All animals used for experiments were genotyped a second time for validation of the genotype.

### 2.2.2 Cell culture

#### 2.2.2.1 Primary microglia and astrocyte preparation

Neonatal C57/Bl6 or *Cd39*$^{-/-}$ mice (P0-P3) were decapitated, the brains were removed and transferred into ice-cold PBS. The meningues were removed carefully and the brains were incubated with a trypsin/ DNase mix for 2 min at 37°C for tissue dissociation. The reaction was stopped with DMEM supplemented with 10 % FCS, 2 mM L-glutamin, 100 U/ml penicillin and 100 $\mu$g/ml streptomycin (DMEM complete). The suspension was then incubated with DNase and dissociated mechanically with a fire-polished Pasteur pipette. After one wash and centrifugation step, the resulting pellet was resuspended in complete DMEM, plated out in poly-L-lysine coated T75 flasks and cultured for 9-12 days. The mixed glial cultures were washed with PBS every 3 days and the medium was subsequently replaced. When reaching confluency, the medium was exchanged with medium containing 30 % L929 fibroblast conditioned medium to induce microglial proliferation and migration to the top of the astrocytic layer. After 3, 5, or 7 days, microglial cells were separated from the astrocytic layer by shaking at 37°C for 1 h. Then the cells were washed, centrifuged, resuspended in DMEM complete and plated onto 6-well, 96-well plates or on glass coverslips in 24-well plates. Cells were used for experiments 24 or 48 h after seeding, unless stated otherwise. The remaining

astrocytic layer, which still contained many microglia cells, was then trypsinized, centrifuged, resuspended and plated onto PLL-coated glass coverslips. Astrocyte-microglia co-cultures were grown until confluency prior to calcium wave experiments.

#### 2.2.2.2 Cell stimulation

Microglial cells, seeded onto 6-well plates (for RNA isolation) or 96-well plates (for malachite green phosphate assay, were stimulated for 6, 24 or 48 h with LPS (10 and 100 ng/ ml), Poly I:C (10 or 100 $\mu$g/ml), IFN$\gamma$ (10 or 100 ng/ml), TGF$\beta$ (1 or 10 ng/ml), IL10 (10 or 100 ng/ml), IL4 (10 or 100 ng/ml) or IL13 (10 or 100 ng/ml), dissolved in DMEM complete.

#### 2.2.2.3 Isolation of microglia via Percoll gradient and MACS separation

Adult mice were decapitated and the brains were collected in ice-cold PBS. Tissue was mechanically dissociated first with a scalpel and then homogenized in a glass potter with 2 ml of dissection buffer. The resulting suspension was filtered through a 70 $\mu$m cell strainer and pelleted for 20 minutes at 400 g, 4°C. The pellet was then resuspended in 25 ml Percoll solution and a layer of 5 ml PBS was carefully added on top, avoiding mixing of the two solutions. After 20 min of centrifugation at 950 rcf, acceleration 4 and brake 0, the myelin layer was visible as a cloud in the middle of the Percoll solution and could be removed by suction. The remaining cell pellet was resuspended in MACS buffer and centrifuged 5 minutes at 500 g. The pellet was then resuspended in 180 $\mu$l MACS buffer and 20 $\mu$l CD11b beads were added to the suspension, mixed well and incubated for 15 minutes at 4°C. Cells were washed by adding 2 ml MACS buffer and centrifuged 5 minutes at 500 g. LS columns were fixed to a magnetic stand and washed with 3 ml MACS buffer. The cell pellet was resuspended in 3 ml MACS buffer and applied to the columns, followed by 3 x 3 ml washing steps. The columns were then removed from the magnetic stand, inserted into new tubes, filled with 5 ml MACS buffer and the CD11b positive cells attached to the beads were flushed out and pelleted at 500 g for 5 minutes. The resulting microglia pellet was then either resuspended in DMEM complete and plated onto glass coverslips for calcium imaging experiments or lysed in TLR buffer for RNA isolation.

### 2.2.3 Biochemical methods

#### 2.2.3.1 RNA isolation

After stimulation, cells were washed 1x with PBS, lysed with Lysis Solution TR supplemented with 1 % $\beta$-mercaptoethanol and frozen at -80°C. RNA isolation was performed with the Invitrap Universal RNA mini kit (Invitek, Berlin, Germany) according to the

manufacturer's instructions. Total yield and quality of RNA was quantified by the NanoDrop 1000 spectrophotometer (PeqLab Biotechnologie GmBH, Germany).

#### 2.2.3.2 cDNA preparation

After RNA isolation, complementary DNA was synthesized with 200 U/$\mu$l Superscript II reverse transcriptase and 0.5 $\mu$g/ml oligodeoxythymidine $_{12-18}$ primers (Life technologies).

#### 2.2.3.3 Quantitative PCR

Relative gene expression changes were determined via quantitative real-time PCR. The primers used were as follows:

| **Gene** | | **Primer sequence** |
|---|---|---|
| *Cd39* | sense | TACCACCCCATCTGGTCATT |
| | antisense | GGACGTTTTGTTTGGTTGGT |
| *P2ry1* | sense | AGCAGAATGGAGACACGAGTTTG |
| | antisense | GGGATGTCTTGTGACCATGTTACA |
| *P2ry2* | sense | GAAGAACTGGAGCAGGCGCT |
| | antisense | CCATTGCCCTGGACCTGATC |
| *P2ry4* | sense | CTGCAAGTTCGTCCGCTTTC |
| | antisense | GTATTGCCCGCAGTGGATG |
| *P2ry6* | sense | TGAAAACAACGAGGAACACCAA |
| | antisense | CAGCCTTTCCTATGCTCGGA |
| *P2ry12* | sense | CACAGAGGGCTTTGGGAACTTA |
| | antisense | TGGTCCTGCTTCTGCTGAATC |
| *P2ry13* | sense | CAGCTGAGTCTCTTCCAAAACAAA |
| | antisense | TGCATCCCAGTGGTGTTGAT |
| *P2ry14* | sense | CCACCACAGACCCTCCAAAC |
| | antisense | CAACACGGGAATGATCTGCTTT |
| *P2rx1* | sense | CAGAAAGGAAAGCCCAAGGTATT |
| | antisense | CACGTCTTCACAGTGCCATTG |
| *P2rx3* | sense | AAGGCTTCGGACGCTATGC |
| | antisense | GATGACAAAGACAGAAGTGCCCT |
| *P2rx4* | sense | AGACGGACCAGTGATGCCTAAC |
| | antisense | TGGAGTGGAGACCGAGTGAGA |
| *P2rx5* | sense | GATGTGGCAGACTTTGTCATTCC |
| | antisense | CCTTCACGCTCAGCACAGATG |
| *P2rx6* | sense | ACGTGTTCTTCCTGGTAACCAACT |
| | antisense | TGGACATCTGCCCTGGACTT |
| *P2rx7* | sense | ACAATGTGGAAAAGCGGACG |
| | antisense | TCAATGCACACAGTGGCCA |
| *$\beta$-actin* | sense | CCCTGAAGTACCCCATTGAA |
| | antisense | GTGGACAGTGAGGCCAAGAT |

**Table 2.10: Primer sequences used for gene expression quantification.**

Gene amplification was performed in triplicates using the SYBR green master mix with the ABI Biosystems qPCR machine. The program used was:

| Process | Cycles | Time | Temperature |
|---|---|---|---|
| Holding stage | 1 | 15 min | 95°C |
| Cycling stage | 45 | 15 s | 95°C |
| | | 1 min | 60°C |
| Melt curve stage | 45 | 15 s | 95°C |
| | | 1 min | 60°C |

**Table 2.11: qPCR program for *Cd39*$^{-/-}$ and *P2r* expression quantification.**

Gene expression was always normalised to gene expression levels of the housekeeping gene *β-actin*. Data analysis was performed using the ΔΔct-method.

### 2.2.4 Functional and enzymatic assays

#### 2.2.4.1 Malachite green phosphate assay

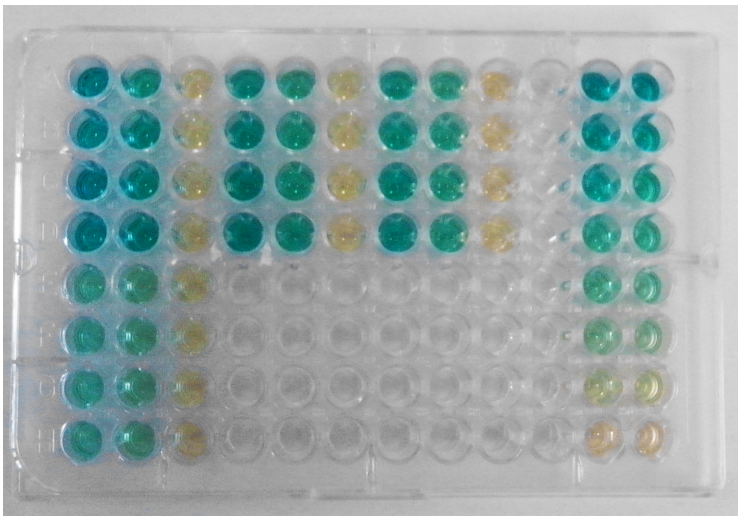

**Figure 2.2: Malachite green assay in 96-well format. Free orthophosphate in the solution builds a green complex with malachite green and molybdate. Phosphate concentration in cell supernatants, a product of ATP degradation, can be estimated with colorimetric measurements and plotting the unknown values against a standard curve.**

Enzymatic activity of CD39 was measured with the malachite green phosphate assay (Bioassay Systems). Primary neonatal microglial cells seeded in 96-well plates and stimulated as described above were washed 3 times with HEPES buffer at RT and incubated with 1 mM ATP or ADP for 10 minutes at 37°C. In parallel, cells without nucleotides and nucleotides without cells were incubated as well to control for spontaneous degradation of nucleotides without the contribution of cells. The reaction was

stopped by adding 10 % TCA and putting the plate on ice. The supernatants were then transferred to a new plate and diluted 1:5. A phosphate standard was pipetted on to the plate for absolute quantification of phosphate concentrations. Reagent A and reagent B were mixed at a ratio of 100:1 according to the manufacturers' instructions and added to the diluted samples. The plate was incubated 30 minutes at room temperature on a shaking platform allowing the colorimetric reaction to take place. The optical density was then measured at 620 nm on a spectrophotometer (Tecan, Germany). The resulting OD values of the samples were then plotted against the standard phosphate concentrations after subtraction of the negative control sample values. The determined phosphate concentrations were then normalized for protein amount in the cells measured by the BCA assay and presented as $\mu$M phosphate/mg protein/min.

#### 2.2.4.2 ATP determination assay

ATP concentrations in the cells supernatant were determined with the ATP determination kit (Life technologies). Primary microglia cultures were seeded onto 96-well plates and allowed to adhere overnight. Cells were washed once with HEPES buffer and after addition of 50 $\mu$l buffer per well, the plate was incubated for 1 h at 37°C. The supernatants were collected at 1, 5, 10, 30, and 60 minutes after medium change. In the meantime, ATP standard was prepared. Standard reaction solution was prepared consisting of 1x reaction buffer, 1 $\mu$M DTT, 500 $\mu$M D-luciferin and 12,5 $\mu$g firefly luciferase in dd$H_2O$. The solution was mixed well and kept in the dark. 10 $\mu$l of the cell supernatants or the ATP standard were added to the wells, followed by 100 $\mu$l of the reaction solution and luminescence of each well was measured, integrating over 10 seconds. For quantification of ATP concentration a standard curve was created and the luminescence values for the unknowns were plotted against the standard curve after subtraction of the background luminescence. All experiments were performed in triplicates from three independent cultures.

#### 2.2.4.3 BCA assay for protein determination

For quantification of protein amount in each well of the 96-well plate after the malachite green assay, cells were lysed in 10 % SDS for 60 minutes at a shaking platform. Working reagent consisting of Reagent A and Reagent B in a 50:1 ratio (Thermo fischer scientific, USA) was prepared and a protein standard of BSA samples was pipetted in a separate 96-well plate. The lysate was transferred to a new plate and working reagent was added on the lysate and the standard. The plate was incubated at 37°C in a wet chamber for 30 minutes. After cooling down, the absorbance was measured at 562 nm on a Tecan spectrophotometer. OD values of the unknowns were plotted against the BSA standard curve to obtain the amount of protein per well.

#### 2.2.4.4 Calcium imaging

For calcium imaging experiments, microglia cells were seeded on glass coverslips at a density of 50,000 cells per coverslip and allowed to adhere to the glass for 24 hours. Cells were loaded with the calcium indicator Fluo 4 AM (5 $\mu$M in DMSO with 1 % pluronic acid, dissolved in HEPES buffer) for 30 minutes at RT and washed once in buffer. The coverslip was then transferred to a chamber with constant perfusion of buffer with a speed of 4 ml/min. After 1 minute, the test substance was applied for 1 minute, followed by 5 minutes washout and 30 seconds application of 300 $\mu$M ATP as a positive control. The whole protocol lasted 11 minutes including the final washout. Fluo 4 AM was excited at 488 nm wavelength by a Polychrome II monochromator (Till photonics, Martinsried, Germany) and pictures were taken at a rate of 0,5 Hz by an inverted CCD camera. For analysis, cell somata were selected as regions of interest (ROI) and the mean relative fluorescent intensity (rFI) for each ROI and frame was determined with the program Imaging cells easily (own development). The monochromator, the camera, the perfusion system and data acquisition were controlled by the TIDA software (HEKA, Lambrecht/Pfalz, Germany). Further analysis was performed with Excel. Only cells which responded to the positive control were analyzed. A cell was categorized as responding to the test substance if the response maximum during application of the test substance was higher than the baseline calculated over a range of 10 frames before stimulation plus 3 times the standard deviation of the baseline. Each cell trace of relative fluorescent intensity was checked manually for exclusion of false positives. All experiments were performed in quadruplicates from three independent cultures.

#### 2.2.4.5 Astrocytic calcium waves

Microglia-astrocytic co-cultures derived from either C57/Bl6 or *Cd39*$^{-/-}$ neonatal mice were seeded on glass coverslips and grown until confluent. For selective microglia depletion, some of the co-cultures were treated with 50 $\mu$l/ml clodronate for 24 hours before seeding on coverslips. The cells were incubated with Fluo 4 AM dye for 30 minutes at RT and washed in HEPES buffer. The coverslip was transferred to a bath chamber filled with HEPES buffer. A patch clamp pipette with a resistance of 7-8 MOhm, filled with Lucifer Yellow dye for visualization, was lowered over the cell layer and a single astrocyte was activated by mechanical touch, which induced a concentrical propagation of an astrocytic calcium wave. Movies were recorded at a rate of 2 frames/s with a 10x objective and 200 ms exposure. Per coverslip, 4 calcium waves were induced in different areas.

#### 2.2.4.6 Data analysis of the astrocytic calcium waves

For measurement of the maximal spread of the calcium wave, a z-stack of the maximal intensity for all frames was generated for each wave, thresholded and the area of the wave was determined with ImageJ software. For single cell analysis, the coordinates of most cells participating in the calcium wave (usually between 50 and 300 cells per wave) were manually selected in ImageJ and their distance from the stimulation site and time-point of fluorescence increase was determined with a custom-written R script (see fig.2.3, B). Statistical analysis was further performed with the R software. For each wave and each frame, the intensity for each cell was determined to acquire an intensity-per-time curve. The baseline intensity before the initiation of the wave was calculated as average intensity ± SD over twenty frames. The threshold was calculated as average intensity ± 2,5-fold SD. The cell response was defined as the timepoint when the intensity becomes higher than the threshold for at least five consecutive frames, to exclude spontaneous peaks of fluorescence (see fig.2.3, C and D). Cells which did not respond at all or responded with spontaneous activity were excluded from the analysis. A wave was regarded as having stopped propagating when the frequency of events dropped below one event per ten frames. The intensity-per-time curve for each wave was then fitted to an exponential function described by the following formula:

$$distance = span \times \left(1 - e^{\frac{-1 \times frame}{\tau}}\right) \tag{2.1}$$

where
**distance** = the distance of the cell from the stimulation point
**span** = the maximal distance reached by the wave
$\tau$ = the change of velocity over time
**frame** = the number of the frame after start of stimulation, i.e. the time[sec]*2

Statistical analysis was then performed to compare the parameters, describing the properties of the wave propagation.

#### 2.2.4.7 Phagocytosis assay in acute brain slices and immunohistochemistry

*In situ* microglial phagocytosis assay was performed as described previously [Bulavina et al., 2013; Krabbe et al., 2013]. 130 $\mu$m thick coronal brain slices were prepared in ice-cold ACSF buffer with the Microm HM 650V vibratome (Thermo Scientific) and carbogenated for 2h at RT before experiments. Yellow-green fluorescent latex beads (3 $\mu$m size, Polysciences Europe GmbH, Eppelheim, Germany) were incubated for 30 minutes with fetal calf serum (FCS) at RT and 1000 rpm shaking, centrifuged (2 min at 3000 rpm), washed in PBS, centrifuged (2 min at 3000 rpm) and resuspended in Hanks Balanced Salt Solution (HBSS, Gibco) with or without 100 $\mu$M ATP. Slices

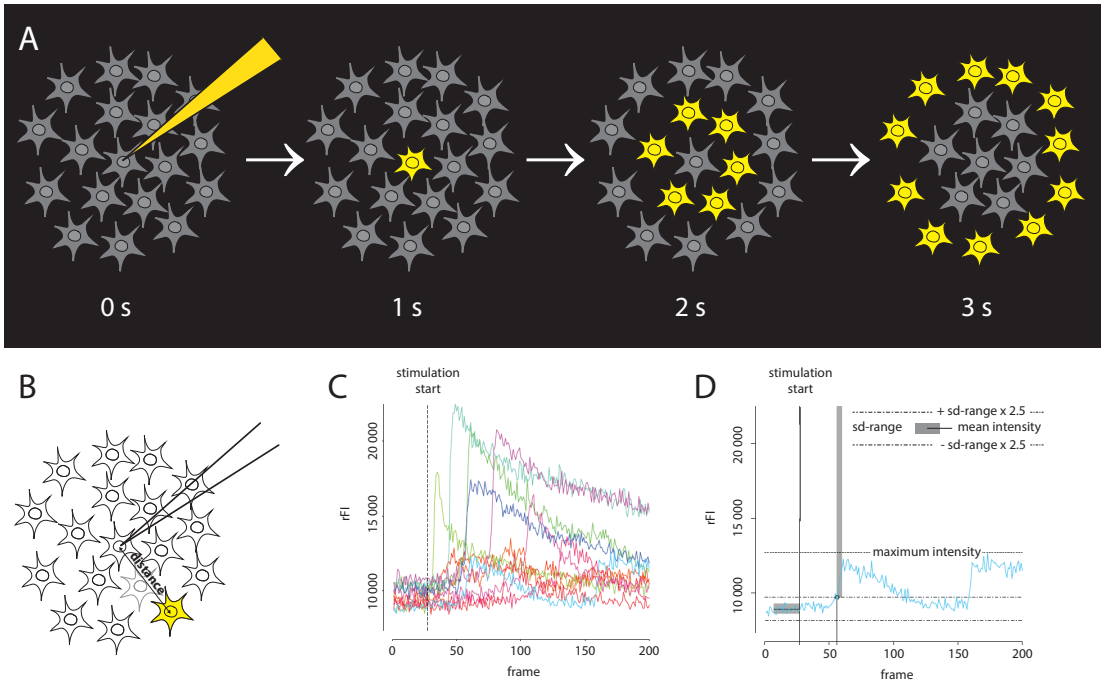


**Figure 2.3: Data analysis of the astrocytic calcium waves. A: A single astrocyte in an astrocytic monolayer is touched with a lucifer yellow-loaded pipette. The cell becomes activated and a calcium wave begins to spread, involving more and more cells. B: The distance from the stimulation point is calculated for each cell. C: Example fluorescence intensities for different cells participating in one calcium wave. D: Analysis parameters, used for automatic determination of the time-point of response for each cell.**

were transferred to 24-well plates and incubated with 500 $\mu$l HBSS containing 8.4 x $10^6$ beads for 60 min at 37°C. Slices were washed twice with 0.1 M PB and fixed with 4 % paraformaldehyde (PFA). For staining, slices were permeabilized with blocking/permeabilization buffer for 4 h, stained with Iba1 primary antibody (0.75 $\mu$g/ml, from Wako Pure Chemicals, Japan) for 24 h at RT. Cy3-labeled donkey-anti rabbit (4 $\mu$g/ml, from Invitrogen, Karlsruhe, Germany) was applied as a secondary antibody for 2 h at RT. Slices were mounted in AquaPolymount (Polysciences Europe GmbH, Eppelheim, Germany) and kept at 4°C until confocal microscopy.

#### 2.2.4.8 Confocal microscopy

For evaluation of microglial phagocytosis, confocal z-stacks were prepared from the stained slices using a Leica TCS SPE confocal microscope (Leica Microsystems, Wetzlar, Germany). Per experiment and condition 15 z-stacks (21 $\mu$m depth, 1 $\mu$m steps) were imaged in the cortex area of slices prepared from at least 4 different animals. Phagocytic microglia were counted manually using Fiji ImageJ software. The phago-

cytic index was calculated as:

$$\text{phagocytic index} = \left(\frac{a}{b}\right) \times c \times 100 \tag{2.2}$$

where
$a$= number of phagocytic Iba1$^+$ cells
$b$= total number of Iba1$^+$ cells
$c$= average number of beads phagocytosed.
For statistical analysis a non-parametric one-way ANOVA test was performed using GraphPad Prism 5.

### 2.2.5 Statistical analysis

Statistical analysis was performed with Microsoft Excel and GraphPad Prism software, unless stated otherwise. qPCR data for *Cd39* and *P2r* expression is presented as average $2^{-\Delta\Delta ct}$ values and treated groups were compared to controls using the Students' t-test for unequal variances. For statistical analysis of the malachite green phosphate assay and ATP release assays the One way ANOVA Kruskal-Wallis test was used followed by the Dunn's multiple comparison post-hoc test. For the calcium imaging experiments, the percentage of cells responding in the different groups were compared using the 2-sample test for equality of proportions with continuity correction, performed with the R statistics package software. Students' t-test was also used for determination of statistical differences between the response sizes in the calcium imaging experiments. For the calcium wave experiments and the phagocytosis experiments, different groups were compared with the One way ANOVA followed by the Bonferroni's multiple comparison test. Statistical significance is shown as *$\leq$0,05, **$\leq$0,01, ***$\leq$0,001.

# 3. Results

## 3.1 Project 1: *Cd39* mRNA expression and protein activity in different activation states of microglia

### 3.1.1 Pro- and anti-inflammatory stimuli modulate *Cd39* mRNA expression in primary neonatal microglia

The ecto-ATPase CD39 is important for microglial functions such as phagocytosis and migration, however it is not yet known which factors regulate *Cd39* expression in microglia. To study whether *Cd39* expression is influenced by the activation state of the cell, primary neonatal microglial cultures were stimulated for 6, 24, or 48 hours with LPS, Poly I:C, IFN$\gamma$ (classical activation), TGF$\beta$, IL10 (acquired deactivation), IL4 or IL13 (alternative activation). Subsequently, the cells were lysed and total RNA was isolated. Complementary DNA was synthesized and *Cd39* expression was assessed by means of qPCR. All experiments were performed in triplicates from at least 3 independent cultures. *Cd39* expression was always normalized to the housekeeping gene *$\beta$-actin* and analysis was performed via the $\Delta\Delta$ct-method. Upon LPS stimulation, *Cd39* expression was strikingly reduced to 5,3 % (10 ng/ml) and 9,5 % (100 ng/ml) of control after 6 hour stimulation. *Cd39* levels remained significantly reduced after 24 h (9,5 % and 3,3 %, respectively) and after 48 h (29 % and 52 % of controls) (Fig. 3.1 A). Similar results were observed after stimulation with the viral component Poly I:C. *Cd39* expression was significantly reduced after 6h incubation with 10 $\mu$g /ml Poly I:C (to 9,3 % of control) and 100 $\mu$g/ml (to 6,2 % of control), as well as after 24 h (16 % and 3,6 % of control respectively). After 48 h of Poly I:C, *Cd39* levels returned to control levels (Fig. 3.1 B). Interestingly, treatment with INF$\gamma$, which is also released in pro-inflammatory conditions, induced an opposite effect - treatment with 10 or 100 ng/ml IFN$\gamma$ led to an upregulation of *Cd39* expression, which was significant after 6h stimulation with 100 ng/ml (Fig. 3.1 C). Treatment with the acquired deactivation stimuli TGF$\beta$ and IL10 both led to a small but significant reduction in *Cd39* expression after 6 h, but not after 24 h and 48 h stimulation (Fig. 3.1 D and E). Induction of alternative activation in microglia with IL4 significantly reduced *Cd39* RNA after 24 h to 48 % (10 ng/ml) and 52% (100 ng/ml), but after 6h or 48 h no change was observed. Another alternative activation stimulus, IL13, did not induce changes in *Cd39* expres-

sion (Fig. 3.1 F and G). Taken together, these results show that treatment with pro- and anti-inflammatory stimuli modulate *Cd39* mRNA expression in primary microglial cells in a time- and dose-dependent manner.

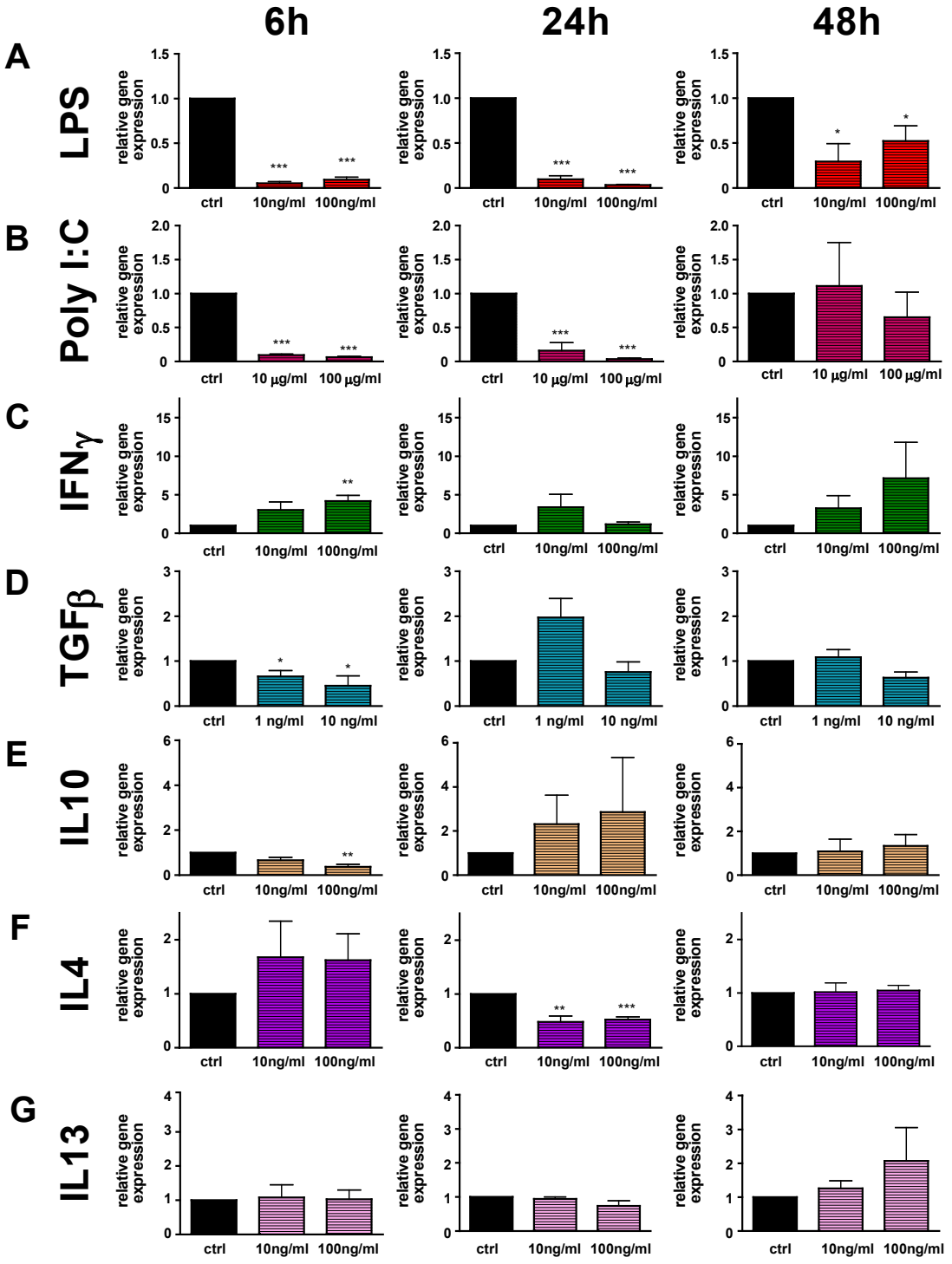


**Figure 3.1:** ***Cd39*** **expression in primary cultured microglia** ***in vitro*** **upon stimulation for 6h, 24h or 48h with A: LPS, B: Poly I:C, C: IFN$\gamma$, D: TGF$\beta$, E: IL10, F: IL4 or G: IL13. Gene expression was normalized to levels of the housekeeping gene** $\beta$***-actin*** **and presented as average** $2^{-\Delta\Delta ct}$ **values from at least three different microglial cultures. Data is shown as mean±SEM. Statistical significance is shown as *≤0,05,**≤0,01,***≤0,001.**

### 3.1.2 CD39 enzymatic activity is modulated by pro- and anti-inflammatory stimulation of primary microglia *in vitro*

To investigate whether changes in *Cd39* RNA expression affect the ability of the enzyme to metabolize ATP, primary microglia were treated for 6, 24 or 48 h with LPS, Poly I:C, IFN$\gamma$ (classical activation), TGF$\beta$, IL10 (acquired deactivation), IL4 or IL13 (alternative activation). The cells were then incubated for 10 minutes with 1 mM ATP and the released free phosphate was determined via a colorimetric reaction with the malachite green phosphate assay. Phosphate concentration was normalized to total protein content, assessed by the Pierce BCA protein assay kit. Experiments were performed in triplicates from three or four independent cultures. Similarly to the *Cd39* expression, CD39 activity was also modulated by pretreatment of the cells. Treatment with LPS significantly reduced CD39 activity after 6 h (from 6,07 to 4,15 $\mu$M phosphate/min/mg protein), 24 h (from 10,46 to 4,88 $\mu$M phosphate/min/mg protein) and 48 h (from 10,45 to 1,4 $\mu$M phosphate/min/mg protein) (Fig. 3.2 A). Poly I:C stimulation reduced strongly CD39 activity, the most prominent effect being after 48 h stimulation with 100 $\mu$g/ml (from 8,73 to 2,87 $\mu$M phosphate/min/mg protein) (Fig. 3.2 B). Treatment with 10 ng/ml IFN$\gamma$ for 24 h significantly increased CD39 activity (Fig. 3.2 C). 10 ng/ml TGF$\beta$ significantly decreased CD39 activity after 24 h, while IL10 led to an increase after 6 h only (Fig. 3.2 D and E). 100 ng/ml IL4 decreased enzymatic activity both after 24 h and 48 h, whereas IL13 stimulation increased phosphate degradation after 6 h (Fig. 3.2 F and G). To summarize, CD39 enzymatic activity changes in different activation states in microglia and the pattern of activity modulation is similar to the *Cd39* expression changes.

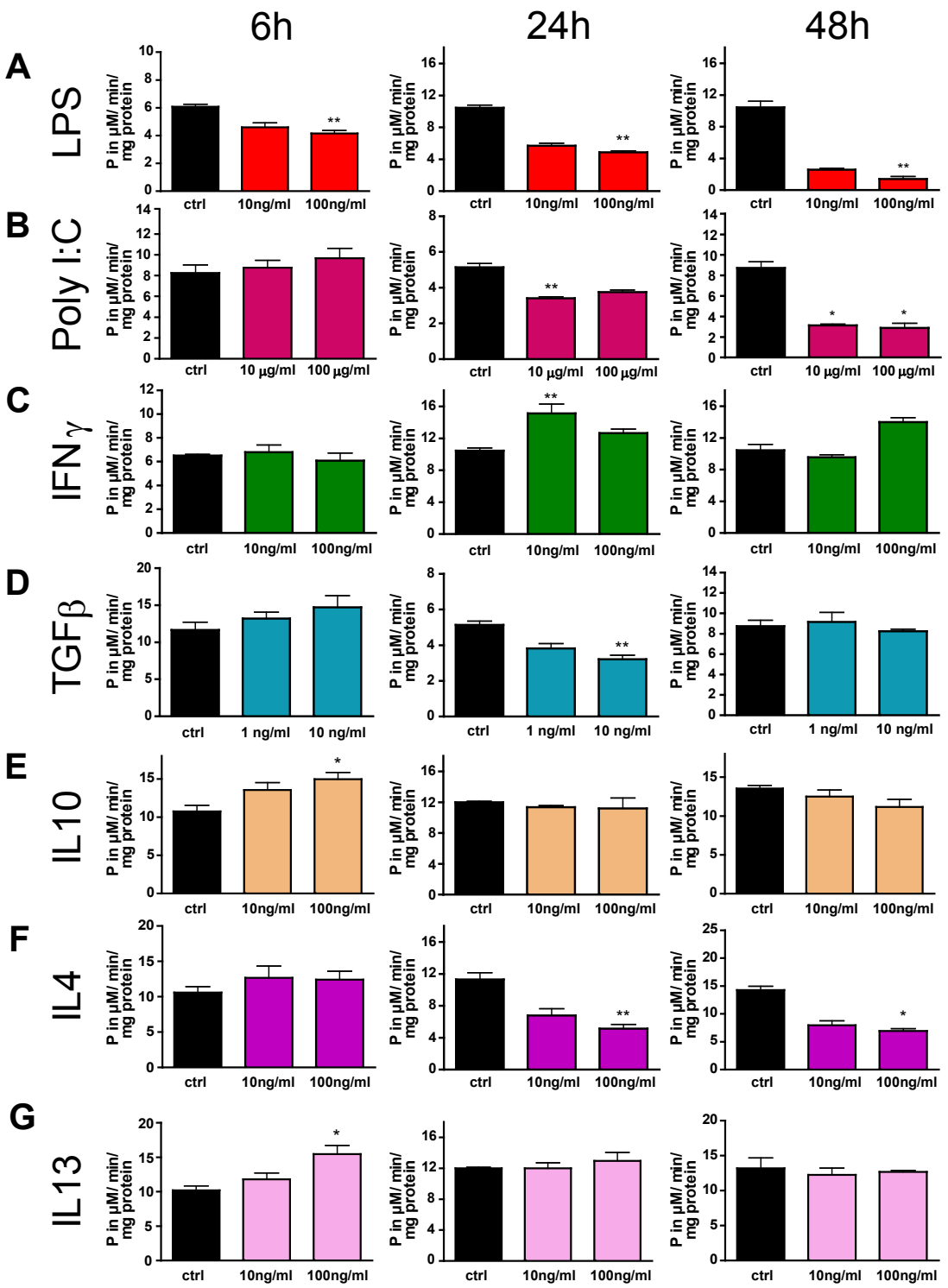


**Figure 3.2: Enzymatic activity of microglial CD39 changes upon activation *in vitro*.** CD39 activity was assessed by malachite green phosphate assay in primary cultured microglia upon stimulation for 6 h, 24 h, or 48 h with A: LPS, B: Poly I:C, C: IFN$\gamma$, D: TGF$\beta$, E: IL10, F: IL4 or G: IL13. Data is presented as mean phosphate in $\mu$M/min/mg total protein from at least 3 independent experiments. Statistical significance is shown as: *$\leq$0,05,**$\leq$0,01,***$\leq$0,001.

## 3.2 Project 2: Microglial purinergic signaling in the absence of the enzyme CD39 and its influence on astrocytic calcium waves

### 3.2.1 *Cd39*$^{-/-}$ microglia release more ATP upon medium change and degrade added ATP slower than wildtype microglia

To study the role of microglial CD39 in keeping the balance of extracellular ATP, I measured ATP concentrations in the supernatant of cultured primary microglial cells upon medium change with medium containing either no or very low concentrations (100 nM, 1 $\mu$M or 10 $\mu$M) of ATP. After medium replacement, aliquots of the supernatants were collected after incubation for 1, 5, 10, 30, and 60 minutes, and ATP concentrations were determined by means of the ATP assay, as described in the Materials and Methods section. Medium change induced a small release of ATP leading to extracellular concentration of 2,5±0,4 nM (n=12) after 1 min in wildtype cells, which declined to 1,5±0,17 nM after 30 min and remained at that level after 60 min. In *Cd39*$^{-/-}$ cells, medium change induced a much higher release of ATP (108,9± 4,84 nM after 1 min), which was reduced to 4,7 (±0,6) nM after 60 min.

To test the degradation activity of defined ATP levels by microglial CD39, I added 100 nM, 1 $\mu$M or 10 $\mu$M ATP and measured ATP levels after 1, 5, 10, 30 and 60 min. 100 nM of ATP were degraded to 2,7±0,24 nM after 1 min, 1 $\mu$M ATP to 10,1±2,6 nM and 10 $\mu$M ATP to 2,5±0,4 $\mu$M. 10 min after addition of 1 $\mu$M ATP, it was degraded to baseline level (3,5±0,7 nM). 10 $\mu$M ATP was reduced to 0,99±0,14 $\mu$M after 10 min and to 0,21±0,02 $\mu$M after 30 min (Fig.3.3 A, left row).

When 100 nM ATP were added to the knockout cultures, a similar effect as medium change (69,7±8,6 nM after 1 min, 32,9±2,9 nM after 10 min and 10,6±0,6 nM after 30 min) was induced. 1 $\mu$M and 10 $\mu$M were gradually removed in *Cd39*$^{-/-}$ cells, but the decay of the ATP concentration was much slower compared to wildtype cells and even after 60 min incubation, ATP levels did not return to baseline (132±7,5 nM for 1 $\mu$M and 5,99±0,08 $\mu$M for 10 $\mu$M, respectively) (Fig.3.3 A, right row).

Direct comparison of ATP levels in the medium of wildtype and *Cd39*$^{-/-}$ cells after medium change reveals that in the knockout cultures the ATP level was significantly higher after 1 min (108,9±4,84 nM vs 4,18±0,46 nM). Although ATP levels decreased gradually over 1 hour, ATP concentrations in the *Cd39*$^{-/-}$ medium were significantly higher than in the wildtype medium for every time point studied (62,0±4,45 nM vs 1,94±0,36 nM after 5 min, 37,21±5,08 nM vs 1,09±0,08 nM after 10 min, 13,68±0,75 nM vs 1,13±0,2 nM after 30 min and 4,7±0,63 nM vs 1,04±0,17 nM after 60 min) (Fig.3.3 B).

Next, I added 50 $\mu$M ARL67156, a pharmacological blocker of CD39, to the cultured

cells, in order to investigate whether this would mimic the genetic removal of the enzyme. Indeed, addition of ARL67156 to wildtype cells induced a similar effect as *Cd39*$^{-/-}$. ATP concentration in the medium of wildtype cells in the presence of the blocker was significantly higher than in wildtype cells only after 1 min (12,25±0,95 nM vs 4,96±1,27 nM), 5 min (8,37±1,27 nM vs 1,89±0,26 nM), and 10 min (4,05±0,38 nM vs 2,16±0,07 nM), but not after 30 and 60 min (Fig.3.3C). Pharmacological blocking of CD39 induced a much smaller effect than the knockout cultures, because the blocker does not block CD39 function completely (data not shown).

To determine the background levels of ATP in cultured microglia, I also measured ATP levels in the supernatant 24 h after medium change. Culture medium was replaced by phenol-red free DMEM and after 24 h of incubation, supernatant aliquots were collected and ATP concentrations were determined. ATP levels in wildtype supernatants did not differ significantly from those in *Cd39*$^{-/-}$ supernatants (3,84±3,05 nM versus 5,11±3,58 nM, fig. 3.3 D). Thus, although the *Cd39*$^{-/-}$ cultures are not able to efficiently remove ATP from the extracellular space within 1 h, they still are able to reach baseline levels of ATP, similar to those of wildtype cells, within 24 h.

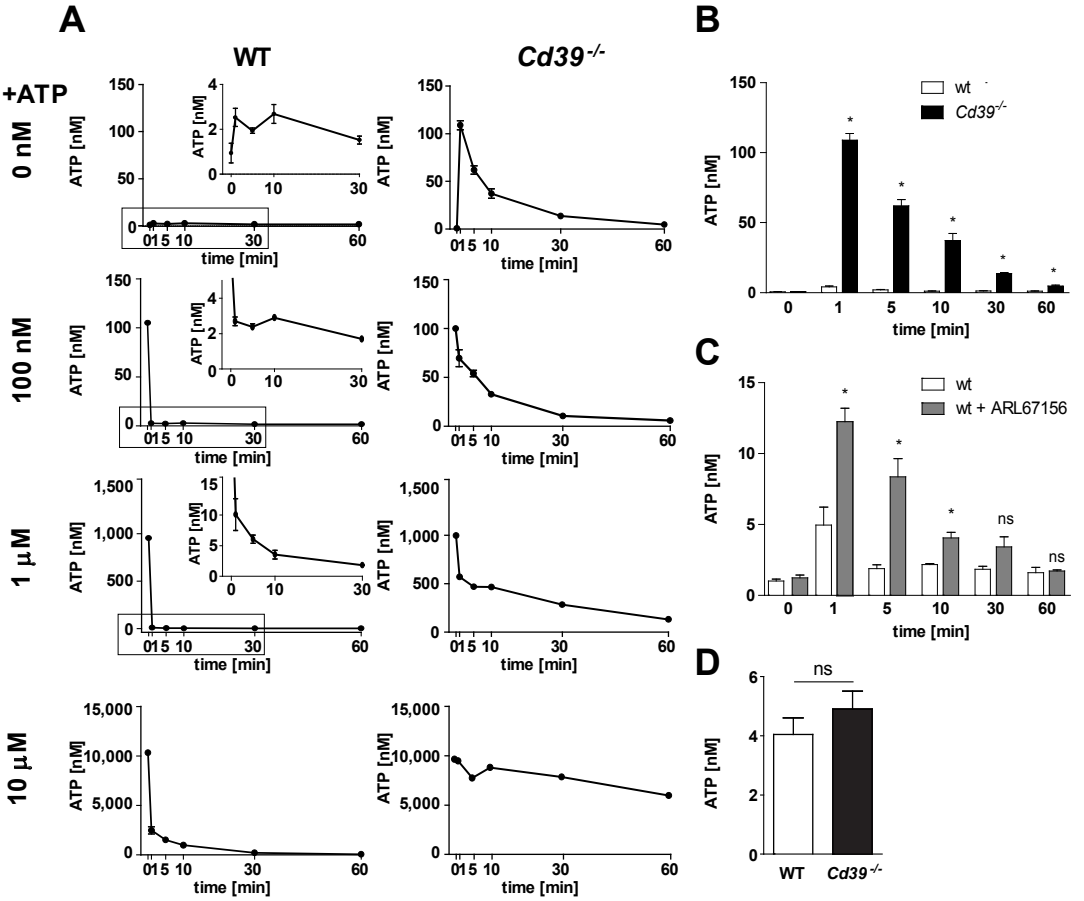


**Figure 3.3: Time-course of ATP concentration in the supernatant of microglial primary cultures after medium change. A: The culture medium of wildtype (left panel) or *Cd39*$^{-/-}$ (right panel) microglia was replaced by HEPES buffer containing either 0, 100 nM, 1 $\mu$M or 10 $\mu$M ATP. Aliquots of the supernatants were taken out after 1, 5, 10, 30 and 60 minutes and ATP concentrations were measured by means of the ATP determination kit. Time point 0 represents the ATP concentration of the buffer before addition to the cells. The inlays show an enlargement of the part of the graph marked with a rectangle. B: Direct comparison of the time-course of extracellular ATP concentration after medium change in microglia cultures derived from *Cd39*$^{-/-}$ and wildtype animals. C: Pharmacological blocking of CD39 function with 50 $\mu$M ARL67156 has a similar effect to CD39 deletion on medium-change-induced ATP release from microglia cells. D: Background ATP levels in wildtype and *Cd39*$^{-/-}$ microglial supernatants measured 24 hours after medium change. Average ATP concentration in nM from 4 culture wells from three independent cell cultures is presented as average ± SEM. Statistical significance is shown as: *$\leq$0,05.**

### 3.2.2 Astrocytes release more ATP than microglia after medium change in a microglia-astrocyte co-culture

Next, I asked which cell type is the major source of ATP – microglia or astrocytes. To investigate this, I compared ATP release in astrocytic cultures, in astrocytic cultures

enriched with microglial cells on top, or in microglial cultures only after medium change. Astrocytes released 56,7±4,7 nM ATP after 1 minute, which was significantly higher than the ATP released by microglia alone – 3,2±0,58 nM. The presence of only a few microglia on top of the astrocytic layer already substantially reduced the ATP concentrations in comparison to astrocytes to 10,4±0,7 nM. Over the course of one hour, the ATP concentrations in the astrocytic supernatants were gradually reduced, but for every time point studied, ATP concentrations remained significantly higher than those in the microglial supernatant (Fig.3.4, A). Since astrocytes have a much bigger cell size than microglia, the ATP values measured were also normalized for total protein amount per well, measured with the BCA assay kit, in order to exclude the possibility that astrocytes release more ATP due to their higher cell volume. The released ATP per $\mu$g protein in astrocytes was significantly higher than in microglia for all time points measured as well, confirming that the higher ATP release in astrocytes is not dependent on cell volume (Fig.3.4, B). Thus, in an astrocyte-microglia co-culture, astrocytes are the main source of ATP, whereas microglia can efficiently remove it to restore the nucleotide balance in the supernatant.

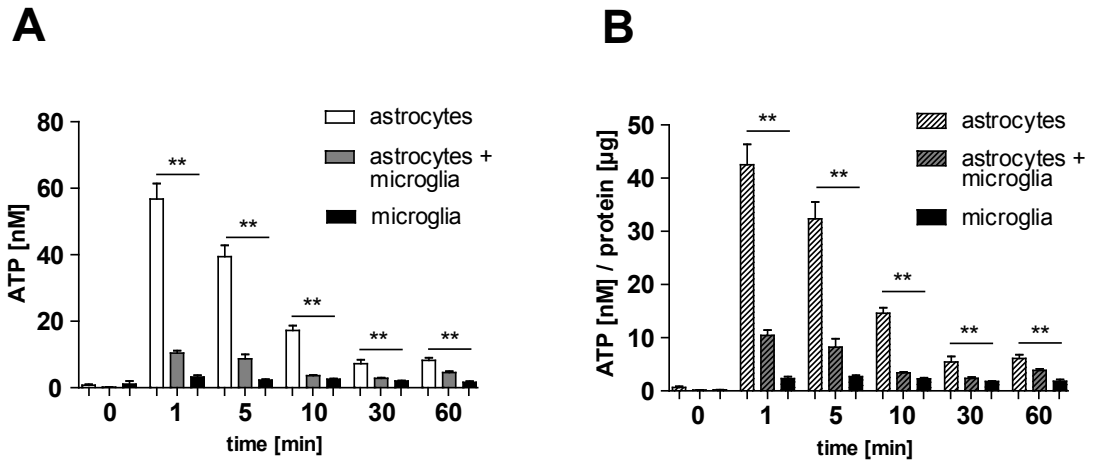


**Figure 3.4: Comparison of the medium change-induced ATP release from pure astrocytic cultures, astrocyte-microglia co-cultures and pure microglia cultures. A: Aliquots of the supernatants from the three culture types were collected after 1, 5, 10, 30, and 60 minutes after medium change and ATP concentrations were determined by means of the ATP determination kit. Time point 0 represents the ATP concentration of the buffer before addition to the cells. B: The ATP concentrations shown in (A) were normalized to protein amounts per well, determined with the BCA protein kit, to control for differences in cell volume and/ or cell number in the different cultures tested. Average ATP concentration in nM or nM/ $\mu$g protein from 4 culture wells from three independent cell cultures is presented as average ± SEM. Statistical significance is shown as: **≤0,01.**

### 3.2.3 *Cd39*$^{-/-}$ microglia are more sensitive to low concentrations of ATP

CD39 terminates purinergic signaling by removing nucleotides from the extracellular space. To study the influence of CD39 in microglial ATP responses, I activated P2 receptors of cultured neonatal microglial cells by application of ATP and measured calcium levels by Fluo-4 AM fluorescence imaging. Primary microglia were loaded with the calcium indicator and the relative changes in fluorescence intensity upon application of different concentrations of ATP for 1 minute were recorded. After washout for 5 minutes, 300 $\mu$M ATP was applied as a positive control for 30 seconds. Only cells responding to the positive control were taken for further analysis. I tested 7 different concentrations of ATP, ranging from 0,1 $\mu$M to 100 $\mu$M. Microglia showed a significantly higher sensitivity to 0,3 $\mu$M and 1 $\mu$M ATP in comparison to wildtype cells: stimulation with 0,3 $\mu$M ATP induced a calcium response in 38,10 % of the *Cd39*$^{-/-}$ cells versus 21,27 % of the wildtype cells. Application of 1 $\mu$M ATP activated 73,74 % of the *Cd39*$^{-/-}$ cells but only 35,65 % of the wildtype cells (Fig.3.5, A). In conclusion, CD39 deletion in microglia increases the sensitivity to low concentrations of ATP. Furthermore, I analyzed the $Ca^{2+}$ signal amplitude of the responding cells. The size of the response was determined by subtracting the average fluorescence intensity before ATP application (the baseline) from the maximal fluorescence intensity of the ATP response. The data are presented as per cent of the maximal response to application of 300 $\mu$M of ATP, performed in independent experiments. Upon application of 1, 3, 10 and 30 $\mu$M ATP, the response amplitudes of the *Cd39*$^{-/-}$ cells were higher than those of the wildtype cells, however the difference in amplitudes was only significant for the 3 $\mu$M application (43,13± 5,46 % for *Cd39*$^{-/-}$ versus 24,21± 3,6 % for *wt* microglia, $p<0,01$). Interestingly, for very high and very low concentrations, the wildtype microglia elicited higher response amplitudes than the *Cd39*$^{-/-}$ microglia: 9,43± 1,94 % in *Cd39*$^{-/-}$ versus 18,95± 3,09 % in *wt* at 0,3 $\mu$M ATP and 61,34± 6,67 % in *Cd39*$^{-/-}$ versus 86,20± 5,05 % in *wt* at 100 $\mu$M ATP (Fig.3.5, B).

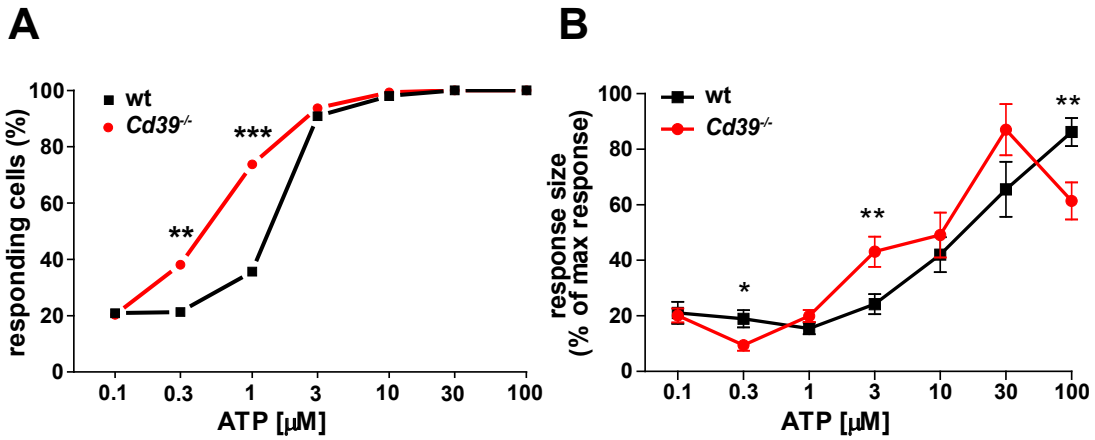


**Figure 3.5: Calcium responses of wildtype and *Cd39*$^{-/-}$ microglia cells upon application of ATP concentrations, ranging from 100 nM to 100 $\mu$M. A: Percent of cells responding to each application of all experiments performed (at least 12 experiments from 3 independent microglial preparations). B: Average response size, presented as % of the maximal response, elicited by application of 300 $\mu$M ATP. Statistical significance is shown as: *≤0,05,**≤0,01,***≤0,001.**

### 3.2.4 Increased sensitivity of *Cd39*$^{-/-}$ microglia to low ATP concentrations is not associated with changes in the expression of purinergic receptors

Microglial cells express a variety of purinergic receptors [Farber et al., 2008]. However, each purinergic receptor has a different sensitivity to ATP and other nucleotides. Since the calcium imaging experiments revealed that microglia lacking CD39 have increased sensitivity to low concentrations of ATP, this might be due to altered expression of purinergic receptors. Total RNA was isolated from three independent microglial cultures derived from either *Cd39*$^{-/-}$ or wildtype mice, and purinergic receptor expression was tested via quantitative PCR. I found *P2ry6*, *P2rx4*, *P2rx7*, *P2ry13* and *P2ry12* to be highly expressed (between 0.1 and 1 % of *β-actin* expression), whereas *P2ry2*, *P2ry14*, *P2rx1* and *P2rx6* showed low expression (less than 0.01 % of *β-actin* expression) and *P2ry1*, *P2rx3*, *P2rx5* and *P2ry4* expression was beyond detection (fig.3.6). These results confirm previous findings [Shieh et al., 2014] with some small differences, concerning *P2ry13*, *P2rx6* and *P2ry1* expression.

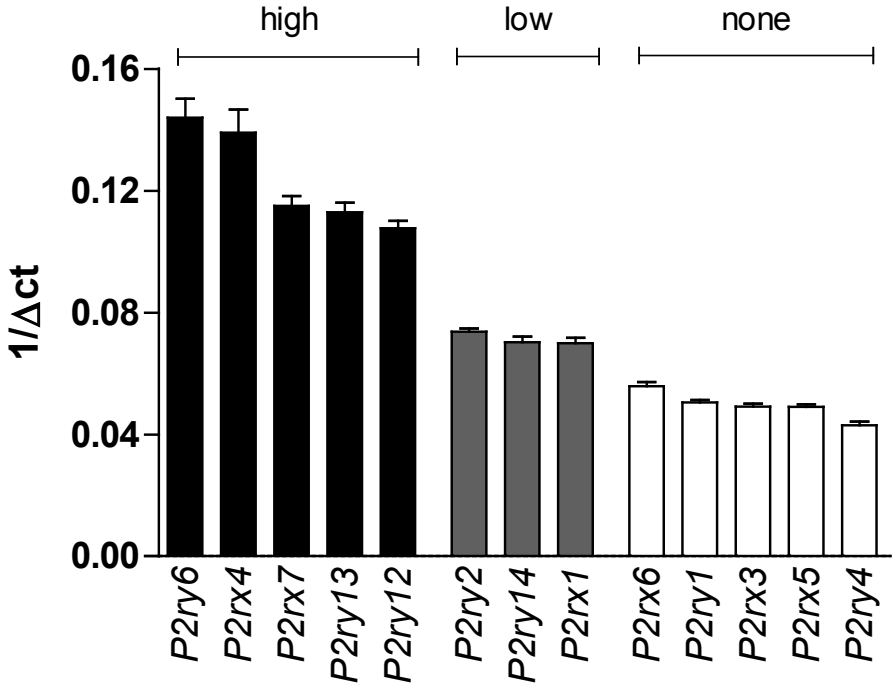


**Figure 3.6: Expression of purinergic receptors in cultured microglia. Gene expression was normalized to the housekeeping gene *β-actin* and presented as 1/Δct values. Mean±SEM of the expression in microglia from at least three independent cultures is shown.**

When I compared P2X and P2Y expression in wildtype and $Cd39^{-/-}$ microglial cultures, I found that all 10 purinergic receptors, expressed by *wt* microglia, were expressed by $Cd39^{-/-}$ microglia to a similar extent (fig.3.7). Thus, increased microglial sensitivity to ATP cannot be attributed to altered purinergic receptor expression.

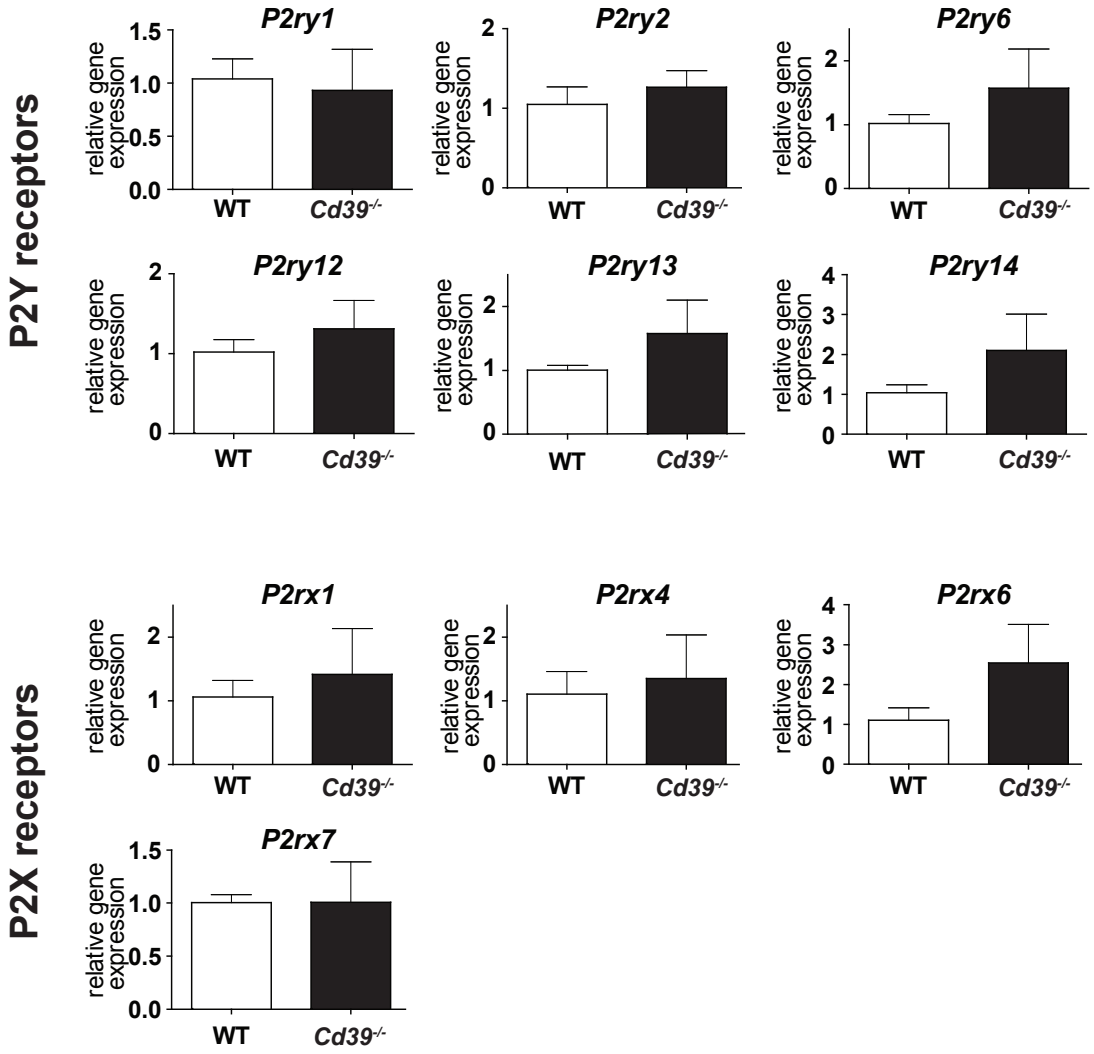


**Figure 3.7: Comparison of purinergic receptors expression in cultured neonatal wildtype and *Cd39*$^{-/-}$ microglia. Gene expression was normalized to the housekeeping gene $\beta$-*actin* and presented as $2^{-\Delta\Delta ct}$ values. Data is shown as mean±SEM from at least three different microglial cultures. Statistical significance is shown as *≤0,05,**≤0,01,***≤0,001.**

### 3.2.5 Microglial CD39 controls the spread of astrocytic calcium waves *in vitro*

Astrocytic calcium waves are concentrically spreading elevations of intracellular calcium in astrocytic cultures or brain slices and can be elicited by mechanical, electrical or chemical stimulation. The spread of the calcium wave is partly dependent on the release of ATP from activated cells, which induce a calcium response in neighbouring cells via activation of purinergic receptors. To study the importance of microglial CD39 for the spread of astrocytic calcium waves, I induced calcium wave propagation by pipette touch in wildtype or *Cd39*$^{-/-}$ astrocyte-microglia co-cultures as well as in microglia-

depleted pure astrocytic cultures, loaded with the fluorescence indicator Fluo-4 AM, and subsequently recorded the spread of the wave. Touching a single astrocyte induced an increase of intracellular calcium with subsequent activation of neighbouring cells and spread concentrically from the initiation point. The relative change of fluorescence intensity for each cell reached a maximum at a time point depending on the distance of the cell from the stimulation pipette.

The maximal area of each calcium wave was determined by generating a Z-stack of the maximal fluorescent intensity of 200 recorded images on average. Representative waves are shown in Fig. 3.8A. In the wildtype co-cultures, the wave spread with a median of 1 244 $\mu$m$^2$ ($25^{th}$ percentile: 987,7 $\mu$m$^2$, $75^{th}$ percentile: 1 665 $\mu$m$^2$, n=75), whereas the spread of the wave in *Cd39*$^{-/-}$ co-cultures was significantly higher median area 1 506 $\mu$m$^2$ ($25^{th}$ percentile: 1 115 $\mu$m$^2$, $75^{th}$ percentile: 2 111 $\mu$m$^2$, n=84, p $\leq$0,05). Selective depletion of microglia by treatment with 50 $\mu$l/ml clodronate for 24 hours led to a similar effect as in the *Cd39*$^{-/-}$ co-cultures - the wave spread with a median of 1 635 $\mu$m$^2$ ($25^{th}$ percentile: 1 241 $\mu$m$^2$, $75^{th}$ percentile: 2 298 $\mu$m$^2$, n=81) which was significantly higher than wildtype as well (p $\leq$ 0,01, Fig.3.8 B). In summary, depletion of either microglial CD39 or the microglial cells themselves both led to a larger spread of the astrocytic calcium waves.

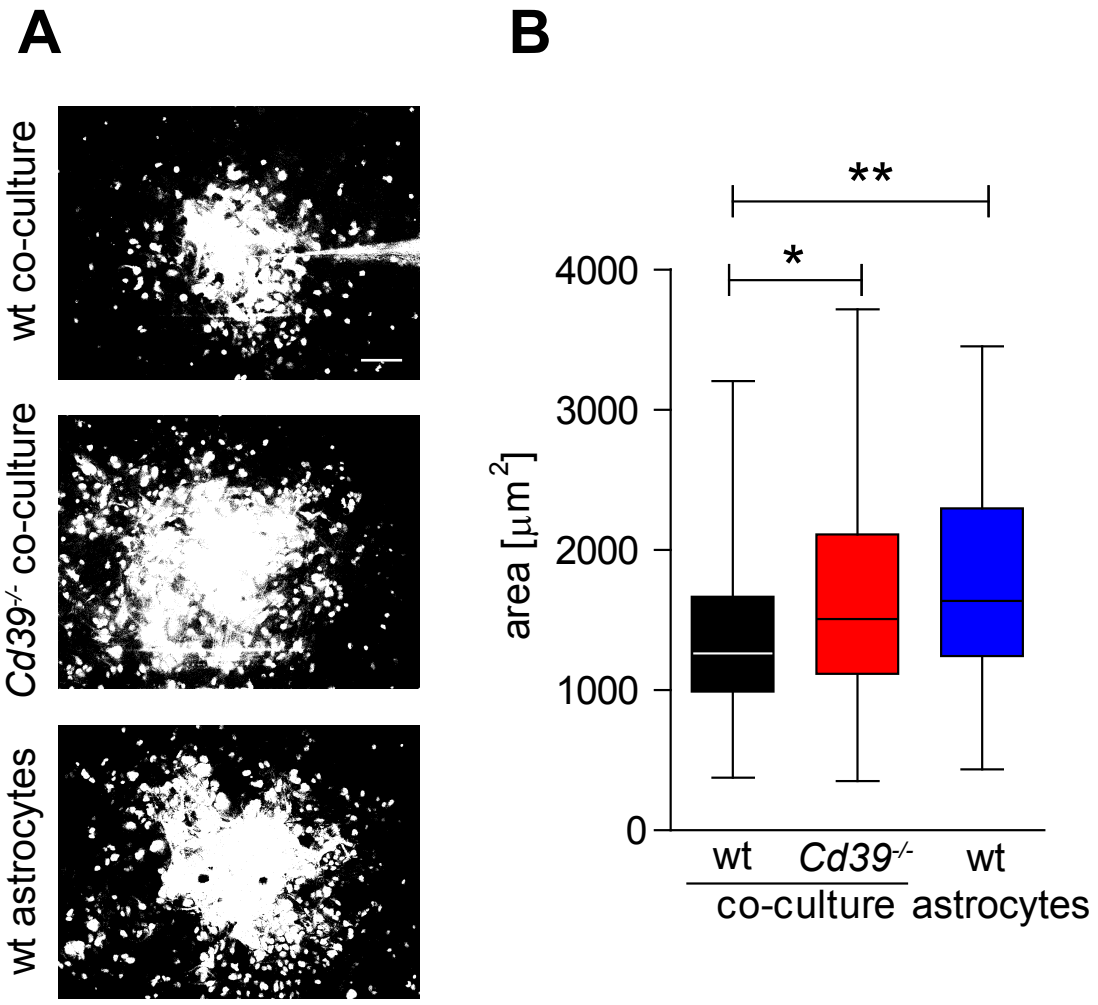


**Figure 3.8: Mechanically induced calcium waves in astrocyte-microglia cultures. Cells were loaded with the calcium indicator Fluo-4 AM and a calcium wave was induced by touching a single astrocyte with a pipette. A: Representative examples of maximal spread of the calcium wave in a wildtype co-culture, *Cd39*$^{-/-}$ co-culture and in astrocytes only. Scale bar: 10 $\mu$M. B: The boxplots represent the median areas of the waves in wildtype, *Cd39*$^{-/-}$ microglia-astrocyte co-cultures and clodronate-treated pure astrocytic cultures. Statistical significance is shown as: *$\leq$0,05; **$\leq$0,01.**

To study whether other parameters of the calcium wave propagation are also affected by deletion of *Cd39*, a single-cell analysis with the R statistics package was performed. For each wave, participating cells were selected manually and the time point of the beginning of the increase in fluorescence as well as the distance from the stimulation point were determined for each single cell. Fig.3.9A shows the calcium wave propagation for the three cultures tested plotted as a function of the distance over the first 70 seconds of the recording. Most waves did not propagate longer than this time. Next, a fitting to the formula 2.1 on page 35 was performed for each wave to determine two parameters,

which describe the calcium wave properties: the span, which is the distance at which the wave reaches a plateau phase, and $\tau$, which describes the acceleration of the wave. The span of the calcium waves in *Cd39*$^{-/-}$ co-cultures was significantly higher than in widltype cultures (median of 664,8 $\mu$m in *Cd39*$^{-/-}$ cells vs 558,7 $\mu$m in wildtype cells, p$\leq$0,01). The median span in wildtype astrocytes, free of microglia, was slightly, but not significantly, higher than the span of wildtype co-cultures (595,4 $\mu$m, Fig.3.9, B). For the $\tau$ parameter, there was no significant difference between the wildtype and the *Cd39*$^{-/-}$ co-cultures (median of 49,55 a.u. for wildtype vs 61,5 a.u. for *Cd39*$^{-/-}$ cells), and in wildtype astrocytes only, $\tau$ was significantly smaller (41,9 a.u., p$\leq$ 0,001, Fig.3.9, C).

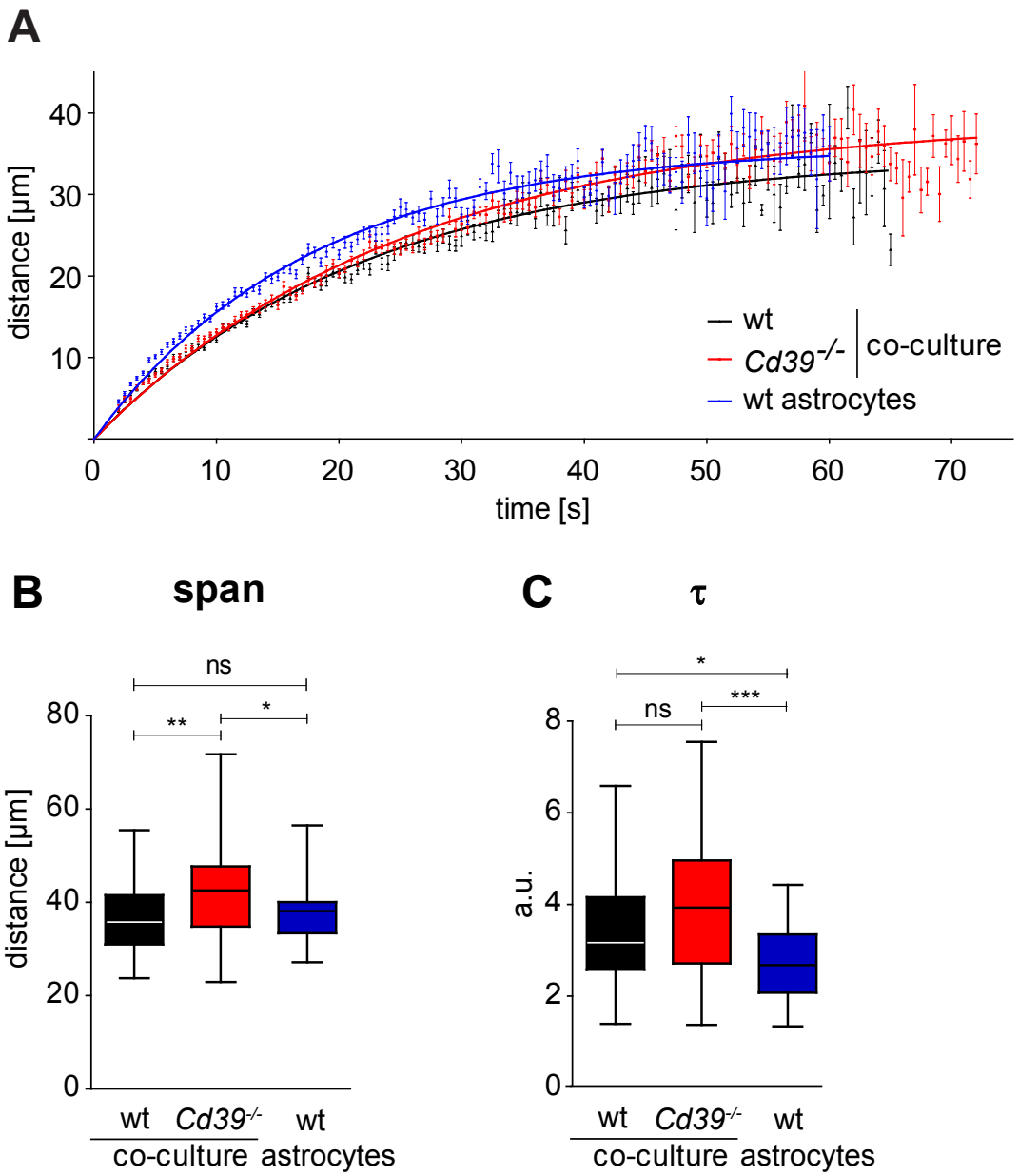


**Figure 3.9: Acceleration and maximal span of astrocytic calcium waves are not different in *Cd39*$^{-/-}$ microglia-astrocyte co-cultures.** Here the results of the single-cell analysis are shown. A: The time [s] versus distance [$\mu$m] plot for each cell participating in the calcium wave for in wildtype, *Cd39*$^{-/-}$ co-cultures and wildtype astrocytes. B: The median of the maximal span of the calcium wave in micrometers for each condition tested, as calculated after fitting every wave to the $\tau$ curve. C: The median $\tau$ parameter for all three conditions tested. Statistical significance is depicted as: *$\leq$0,05,**$\leq$0,01,***$\leq$0,001.

## 3.3 Project 3: Microglial ATP-related properties in a mouse model of schizophrenia

### 3.3.1 Microglia in acute brain slices of adult Poly I:C mice do not respond to ATP with increased phagocytic activity

Microglial phagocytosis of apoptotic neurons is important for proper neurodevelopment and synaptic pruning [Tremblay and Majewska, 2011; Beumer et al., 2012], processes which have been shown to be disturbed in the schizophrenic brain. To study the phagocytic activity in microglia in a mouse model of schizophrenia acute brain slices from the adult offspring of Poly I:C or NaCl injected mothers were prepared and the uptake of fluorescent beads by the microglial cells was quantified. In control slices, the phagocytic index was 22,7±1,53, which did not differ significantly from that of Poly I:C offspring slices (18±1,47, fig.3.10). ATP has been shown to result in an increase in microglial phagocytosis [Bulavina et al., 2013]. Indeed, simultaneous incubation with latex beads and 100 $\mu$M ATP led to an increase in the phagocytic index up to 33,1±2,37 in the control slices ($p \leq 0{,}001$), but not in the Poly I:C slices (20,67±1,88, ns). Taken together, these results show that microglia in Poly I:C offspring acute brain slices have normal phagocytic activity, but do not respond to ATP treatment with an increase in phagocytosis.

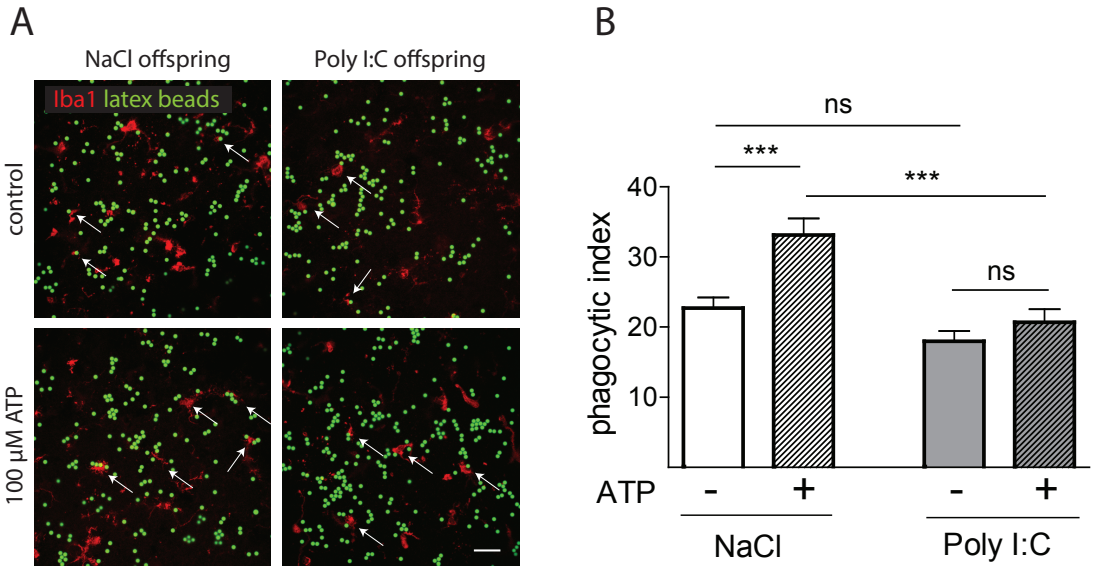


**Figure 3.10: Microglia in acute brain slices of Poly I:C offspring show normal phagocytic activity, which cannot be enhanced by treatment with ATP. A: Representative z-stacks images. Iba1 positive phagocytic cells are marked with arrows. Scale bar: 20 $\mu$m. B: Quantification of the phagocytic index, displayed as mean ±SEM from at least 80 z-stacks from at least 5 independent animals. Statistical significance is shown as ***≤0,001, ns: not significant. Experiments and data analysis were performed by Frank Szulzewsky.**

### 3.3.2 Freshly isolated microglia from adult Poly I:C mice show reduced sensitivity to ATP

Microglia express a set of purinergic receptors, which are activated by nucleotides – in particular ATP – and induce an increase in intracellular calcium. Since ATP-induced microglial phagocytosis is disturbed in Poly I:C adult mice, I next asked whether microglial sensitivity to ATP is affected in general. Microglia were isolated from the whole brains of Poly I:C and NaCl offspring via Percoll gradient for myelin removal and MACS separation with CD11b microbeads for specific microglial purification. Cells were loaded with Fluo-4 AM $Ca^{2+}$ indicator and ATP was applied in concentrations of 0,1 to 30 $\mu$M, which represent the range of ATP concentrations which activate most of the purinergic receptors (table 4.1 on page 67). After a washout of 5 minutes, 300 $\mu$M ATP was applied as a positive control. Only cells which responded to the second ATP application were taken for further analysis. The percentage of cells responding to the first ATP stimulation was decreased in the Poly I:C microglia in comparison to NaCl microglia for 0,3, 1 and 3 $\mu$M ATP with a significant difference for the 3 $\mu$M ATP stimulation (from 87 % to 68 %, p≤0.01)(Fig.3.11, A). To activate 60 % of the Poly I:C microglia nearly the double ATP concentration was needed ($ECF_{60}$): 2,8 $\mu$M (Poly

I:C) compared to 1,476 $\mu$M (NaCl). Altogether, these experiments show that freshly isolated microglial cells from Poly I:C brains display a reduced sensitivity to ATP. The amplitudes of the calcium responses showed an opposite trend: the amplitudes of the Poly I:C microglia were slightly higher than those of NaCl microglia for the 1 $\mu$M and 3 $\mu$M concentrations, and the difference was significant for 1 $\mu$M (0,56±0,26 for Poly I:C versus 0,33±0,25 for NaCl microglia, p≤0,05), Fig.3.11,B).

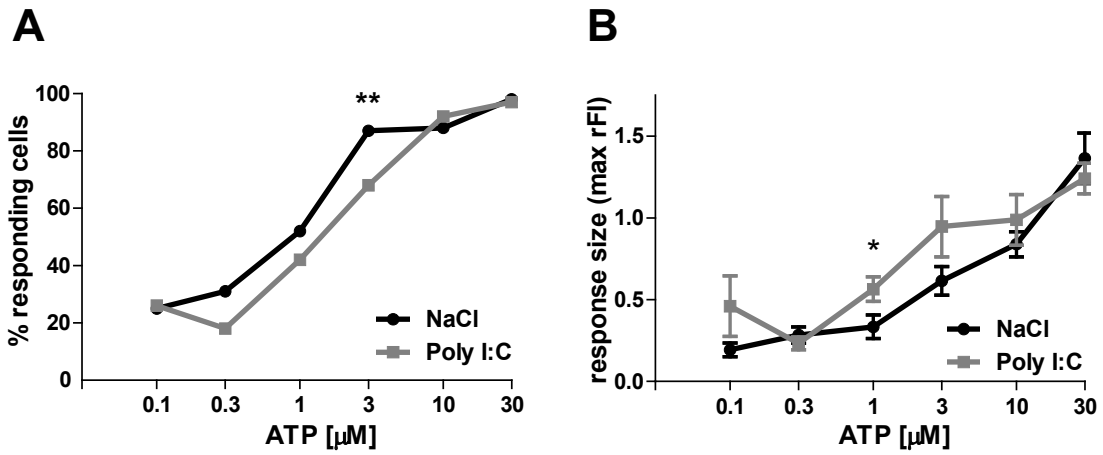


**Figure 3.11: Calcium responses of freshly isolated microglia cells from adult (P60) Poly I:C mice upon application of ATP. A: Percent of cells responding to each application of all experiments performed (at least 12 experiments from at least 3 independent microglial isolations). B: Average response size, maximal relative fluorescence intensity. Statistical significance is shown as: *≤0,05,**≤0,01.**

### 3.3.3 Freshly isolated microglia from adult Poly I:C mice show an altered mRNA expression of *Cd39* and *P2ry12*, but not of *P2ry6*

In the calcium imaging experiments, I observed that freshly isolated microglia derived from the offspring of Poly I:C challenged mothers displayed reduced sensitivity to ATP application. To test whether this can be due to altered expression of *Cd39* or purinergic receptors, I isolated mRNA from these cells and performed qPCR for the genes of interest. I found that *Cd39* expression in Poly I:C microglia was significantly increased to 1,39 fold (p<0,01) of controls and possibly contributes to the decreased sensitivity to ATP of these cells (Fig.3.12, A). Furthermore, mRNA levels of *P2ry12* were significantly (1,2 fold, p<0,01) increased in Poly I:C microglia in comparison to controls (Fig.3.12, B). *P2ry6* and *P2ry13* mRNA levels did not differ between groups.

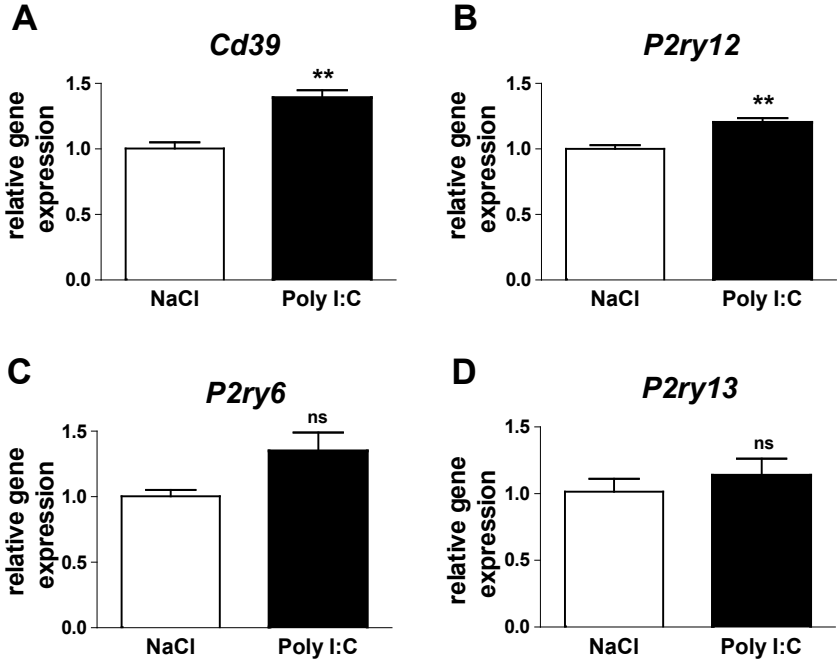


**Figure 3.12: Relative gene expression of A: *Cd39*, B: *P2ry12*, C: *P2ry6* and D: *P2ry13* in freshly isolated microglia from adult Poly I:C mice. Gene expression was normalized to the housekeeping gene $\beta$-*actin* and depicted as $2^{-\Delta\Delta ct}$ values. Average ± SEM gene expression from cells isolated from 4 mice is shown. Statistical significance is shown as **≤0,01.**

### 3.3.4 Freshly isolated microglia from Poly I:C neonatal offspring show increased sensitivity to ATP

Schizophrenia is a disease, which emerges in early adulthood. Since I saw an altered microglial phenotype in the adult Poly I:C offspring, I next asked whether microglial properties also change during development in this model. An early postnatal time-point (P5) was chosen for the following experiments.

I tested the responsiveness of freshly isolated microglia from Poly I:C P5 pups to low concentrations of ATP. Surprisingly, the percentage of cells responding to stimulation with 0,1 and 0,3 $\mu$M ATP was markedly increased in microglia derived from Poly I:C P5 pups in comparison to controls – 53,6 % vs 21,5 % for 0,1 $\mu$M ATP and 64,3 % vs 26,1 % for 0,3 $\mu$M ATP, respectively (p≤0,001). The responsiveness of the cells from both groups to 1 $\mu$M and 3 $\mu$M is similar, and for 10 $\mu$M ATP stimulation the Poly I:C mice-derived cells are again slightly, but significantly more sensitive – 95,8 % vs 89 % (p≤0,01, Fig.3.13 A). The amplitudes of the calcium responses did not differ significantly between the two groups for any of the ATP concentrations tested (Fig.3.13 B). In summary, freshly isolated microglia from Poly I:C P5 pups are more sensitive to very low concentrations of ATP.

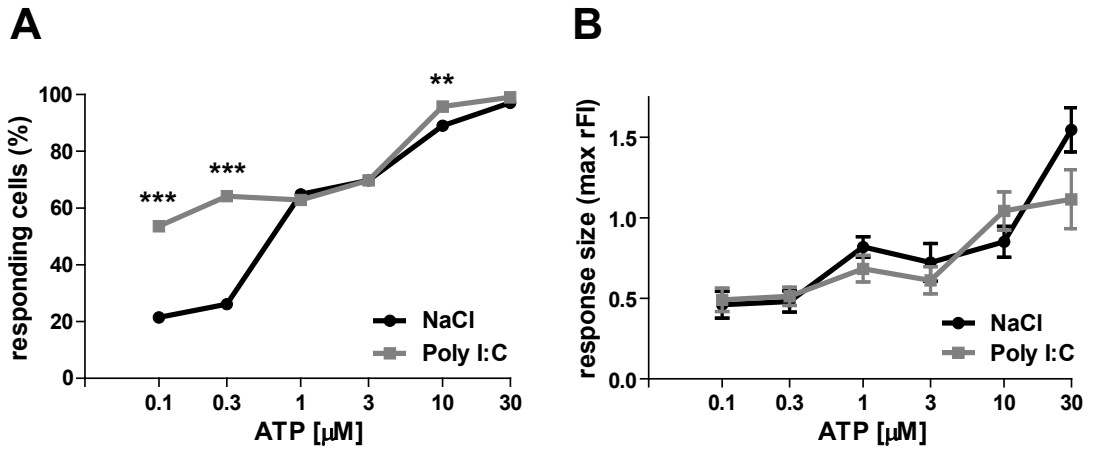


**Figure 3.13: Calcium responses of freshly isolated microglia cells from neonatal Poly I:C mice upon application of ATP. A: Percent of cells responding to each application of all experiments performed (at least 12 experiments from at least 3 independent microglial isolations). B: Average response size, maximal relative fluorescence intensity. Statistical significance is shown as: **≤0,01, ***≤0,001.**

### 3.3.5 Freshly isolated microglia from neonatal Poly I:C mice show a reduced mRNA expression of *P2ry6*

I also tested the expression of *Cd39* and purinergic receptors in the freshly isolated cells from P5 Poly I:C pups. In the Poly I:C offspring the expression of the *P2ry6* was significantly reduced to 47,8% of controls (Fig.3.14 C), whereas *Cd39*, *P2ry12* and *P2ry13* expression remained unchanged (Fig.3.14 A, B and D).

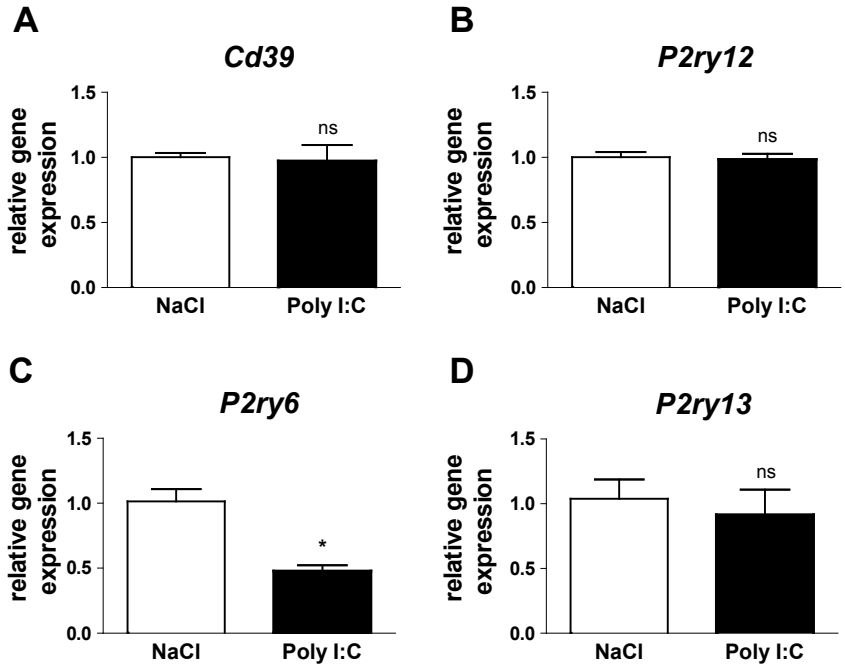


**Figure 3.14: Relative gene expression of A: *Cd39*, B: *P2ry12*, C: *P2ry6* and D: *P2ry13* genes in freshly isolated microglia from neonatal Poly I:C mice. Gene expression was normalized to the housekeeping gene $\beta$-*actin* and depicted as $2^{-\Delta\Delta ct}$ values. Average ± SEM gene expression from cells isolated from 4 mice is shown. Statistical significance is shown as *≤0,05.**

### 3.3.6 Comparison of *Cd39* and *P2* gene expression in P5 and P60 Poly I:C mice

In both postnatal and young adult mice, I found changes in microglial gene expression of *Cd39*, *P2ry6*, and *P2ry12* in Poly I:C offspring (fig.3.12 and fig.3.14). However, it is also of interest to compare the gene expression between postnatal and adult mice. Fig.3.15 shows how the gene expression changes with development. Interestingly, expression of *Cd39*, *P2ry12* and *P2ry13* increases significantly with age, both in NaCl and in Poly I:C animals. In control animals, *Cd39* was increased 2,9±0,13 fold (p≤0,001), *P2ry12* expression was increased 4±0,11 fold (p≤0,001), and *P2ry13* 4,46±0,43 fold (p≤0,001) in adults compared to pups. Expression of *P2ry6* was the only one which did not increase in adulthood in control microglia (1,22±0,06 fold,ns), however, the reduced expression in the Poly I:C pups relative to control pups was recovered in the Poly I:C adults (from 0,48±0,05 to 1,64±0,17 of controls, p≤0,001). In summary, microglial gene expression of *Cd39* and three purinergic receptors is regulated not only in the schizophrenia model, but also increased in development.

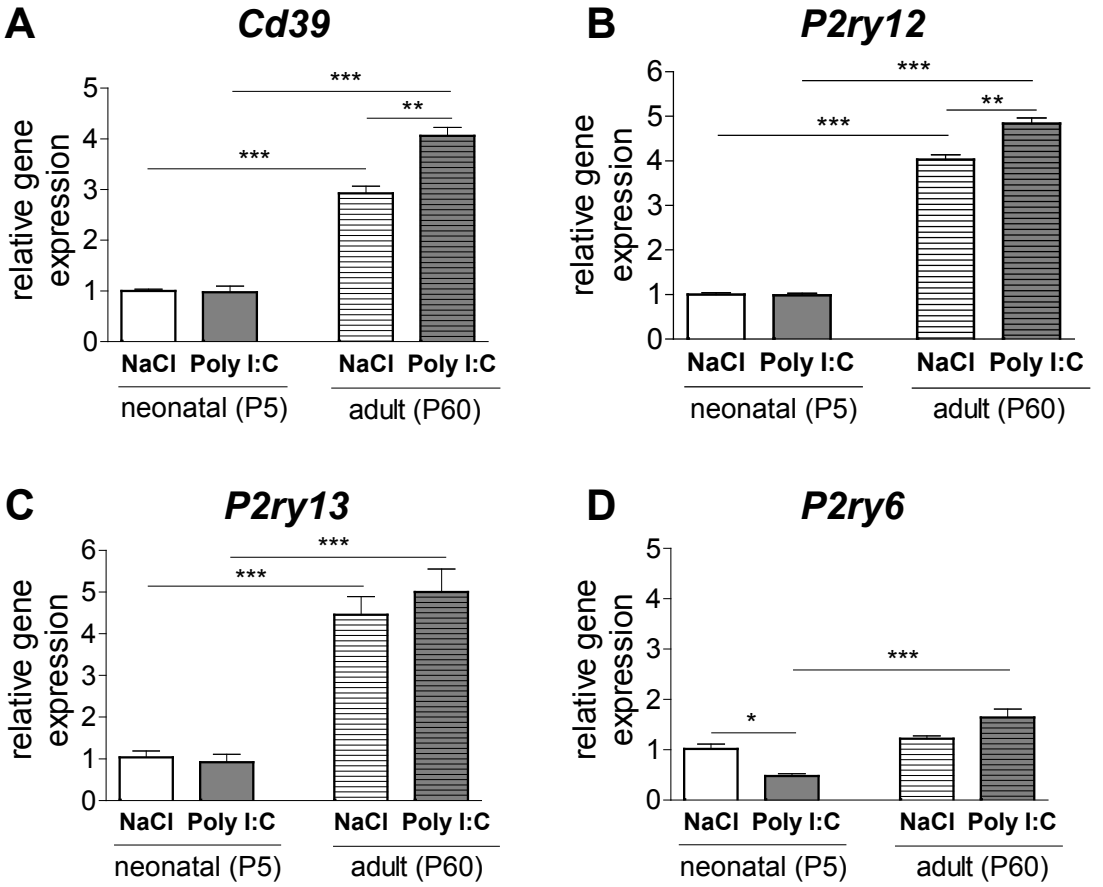


**Figure 3.15: Microglial *Cd39*, *P2ry12* and *P2ry13* expression are upregulated in early adulthood: a direct comparison of the gene expression data shown in fig.3.12 and fig.3.14 reveals, that gene expression changes in development. Relative gene expression of A: *Cd39*, B: *P2ry12*, C: *P2ry13* and D: *P2ry6* receptors in freshly isolated microglia from neonatal and adult Poly I:C mice. Gene expression was normalized to the housekeeping gene *$\beta$-actin* and depicted as $2^{-\Delta\Delta ct}$ values. Average $\pm$ SEM gene expression from cells isolated from 4 mice is shown. Statistical significance is shown as *$\leq$0,05, **$\leq$0,01, ***$\leq$0,001.**

# 4. Discussion

## 4.1 Relevance of the work

Microglia are the resident macrophages of the central nervous system and are responsible for the protection of the brain against damage, bacterial and viral infections. These surveying cells constantly scan their environment for changes: damaged cells and pathogenic exogenous factors or endogenous signals. Such events can lead to activation of the microglial cells and induce both morphological changes towards an amoeboid form and functional changes toward a migrational and/ or phagocytic phenotype as well as cytokine release.

Purinergic signaling is one of the most ubiquituous signaling systems in the body, as nucleotides and their receptors are found in virtually all tissues. For microglial cells, purinergic signaling is one of the most important paracrine and autocrine communication systems, which enables them to respond adequately and effectively to signals in a very time- and space-specific manner. For instance, ATP acts as a danger signal when released from damaged cells and induces migration and phagocytosis in microglia, but it can also be rapidly metabolized to adenosine, which has the opposite effect - it limits inflammation and counteracts the action of ATP. Microglia are equipped with a very efficient enzyme - CD39, which enables them to prevent self-overactivation, as well as overactivation of neighboring cells. Since CD39 is a very potent enzyme, it is not only relevant for microglial cells - the only cell type which express it in the CNS - but also for the effective removal of ATP in the extracellular space of the CNS. Another ATP-dependent process in the CNS is the major communication system of astrocytes, the calcium wave. This is why it is important to study how microglial CD39 affects the purinergic signaling in both microglia and astrocytes.

Since proper purinergic signaling in the brain is a prerequisite for normal brain function, it is no surprise that a disturbed purinergic balance has been connected to different diseases of the nervous system. Schizophrenia, a debilitating psychiatric disorder, is characterized by a disbalance in both the dopaminergic and glutamatergic systems. Only recently has a new hypothesis for the pathophysiology of schizophrenia been proposed. This hypothesis bases on adenosine, a neurotransmitter which tightly controls other neurotransmitter systems. Since adenosine has been shown to affect microglial function and many studies have focused on microglial involvement in schizophrenia, it

is necessary to study how a possible purinergic disbalance in the schizophrenic brain would affect normal microglial function.

## 4.2 Project 1: Distinct *Cd39* gene expression and enzymatic activity in different activation states of microglia

CD39 is an ecto-enzyme, responsible for the rapid removal of ATP from the extracellular space, which is expressed on microglial cells. Since ATP is an important danger signal for microglia, CD39 is also crucial for removal of the signal and thus for controlling the activation of the cells. Microglia can be activated by different exogenous components and endogenous molecules, which induce phenotypic and functional changes in the cells. *In vitro*, three main activation states of microglia have been described: classical activation, acquired deactivation and alternative activation. I treated primary neonatal microglia with different agents to induce their activation and studied how *Cd39* mRNA expression and protein activity change after 6, 24, or 48 hours incubation of the cells. I found that both *Cd39* mRNA expression and protein activity were modulated in a time- and dose-dependent fashion in different activation states. Treatment of the cells with activating agents usually induced a change in gene expression, followed by a change in the activity of the protein. This suggests that microglial regulation of protein translation of CD39 occurs via regulation of the gene expression.
The most striking results were observed after treatment with pro-inflammatory components such as LPS and Poly I:C, which dramatically reduced both gene expression and activity of CD39 after 6 and 24 hours and 24 and 48 hours, respectively. In contrast, treatment with IFN$\gamma$ slightly increased both *Cd39* expression and enzymatic activity. Since IFN$\gamma$ is a cytokine, released from Th1-cells upon activation of TLR receptors [Hanisch, 2002], and is often employed together with LPS for induction of a robust classical activation effect, these contrasting results are rather surprising and would infer that *Cd39* regulation is not a general effect of the activation state of the microglia, but rather a specific response to a certain agent. The alternative activation cytokines IL4 and IL13, which are also usually employed in combination when treating cells *in vitro*, also elicited rather different results - IL4 reduced *Cd39* expression and protein activity, while IL13 had little effect with a tendency to enhance CD39 activity after 6 hours.
The finding that LPS treatment dramatically reduces *Cd39* expression and protein activity is in agreement with the observation of Zanin et al., who found that M1-activated peritoneal macrophages isolated from mice show reduced ATPase activity as well as mRNA expression and protein levels of NTPDase1 [Zanin et al., 2012]. On the

other hand, they saw upregulation of ATPase activity in M2-polarized macrophages treated with IL4, which is in contrast with my results in microglia. Zanin et al. drew a general conclusion that M1 activation reduces and M2 activation enhances activity and expression of CD39 in macrophages. Considering the fact that microglia may share many common properties with peritoneal macrophages, but also display many differences, this discrepancy is not surprising - and in addition, Zanin et al. studied only two activation paradigms - induced by LPS and IL4, while in my work I tested 7 different components at three different time-points, which enables us to provide a more detailed picture. Kittel et al., who describe a transient decrease of CD39 activity on the luminal side of endothelial cells upon rat i.p. LPS treatment, argue that this decrease might be attributed to LPS-induced changes in the structure of cell membranes, which affect CD39 transmembrane domain formation and its functionality [Kittel et al., 2007]. However, since in my experiments LPS-induced reduction of CD39 activity was preceded by a decrease in *Cd39* mRNA expression, I would rather regard the membrane structure hypothesis as a complementary effect and not as the main mechanism, responsible for the alteration of CD39 activity.

What is the possible physiological relevance of the observed microglial reduction of *Cd39* upon LPS or Poly I:C stimulation? Microglia, stimulated with LPS, release small amounts of ATP [Pascual et al., 2011]. Reduction of *Cd39* expression and enzymatic activity on the cell surface during inflammation would lead to accumulation of extracellular ATP, which would support ATP-mediated pathogen clearance and the activation of P2 receptors, an important component of inflammation.

## 4.3 Project 2: CD39 on microglia is an important regulator of autocrine and paracrine purinergic signaling

### 4.3.1 *Cd39*$^{-/-}$ microglia are more sensitive to low concentrations of ATP

Microglial cells can sense ATP via multiple purinergic receptors expressed on their surface (for a review, see [Färber and Kettenmann, 2006]). Binding to metabotropic P2Y receptors leads to an activation of $G_q$-dependent pathway and phospholipase C which mobilizes intracellular $Ca^{2+}$, while binding of ATP to P2X ionotropic receptors leads to opening of the channel pore and increased permeability to $Ca^{2+}$ and $Na^{+}$ [Bucheimer and Linden, 2004]. Each receptor exhibits a different affinity to ATP [Jacobson, 2010], for an overview, see table 4.1. Thus, depending on the concentration of ATP applied to the cells, a different array of P2 receptors becomes activated, a different amount of $Ca^{2+}$ is released in the cytoplasm, and a calcium signal is detectable or not. I studied how deletion of *Cd39* would modify the sensitivity of microglial cells to application of

ATP. Applying ATP in concentrations ranging between 100 nM and 100 $\mu$M, I found that 10 $\mu$M ATP were sufficient to activate almost all microglia cells and applications of 1 and 3 $\mu$M ATP also led to their activation. These findings are similar to what Moller et al. described in their experiments with microglial cells *in vitro* [Moller et al., 2000] and when regarding the dose-response curve of the signal size, my results closely match those published by our lab previously. Interestingly, when I performed the same experiments with *Cd39*$^{-/-}$ microglia, I found that in these cells the percentage of cells responding to the ATP stimulation was significantly increased in comparison to wildtype cells. This was to some extent reflected in the response sizes of the responding cells. Since the mRNA expression of purinergic receptors in *Cd39*$^{-/-}$ cells was not different from that of wildtype cells, this altered sensitivity to ATP might be attributed directly to the lack of CD39. It has been shown, that CD39 "competes" with purinergic receptors for nucleotide binding and can even form heterodimers with P2 receptors [Schicker et al., 2009]. Since CD39 is an extremely fast enzyme, it is plausible to assume that for concentrations of ATP, which are so low that not even all cells are activated, CD39 can prevent activation of P2 receptors before ATP has reached them by degrading ATP to AMP and phosphate. When CD39 is missing, all ATP reaches the P2 receptors and more cells are activated. Applications of 10 $\mu$M of ATP are high enough to circumvent the immediate action of CD39 so there is no detectable difference between wildtype and knockout cells anymore. Thus, for low concentrations of ATP, CD39 has a regulatory effect on microglia and can prevent activation of the cells.

### 4.3.2 *Cd39*$^{-/-}$ microglia release more ATP and degrade it slower

Cells can release ATP upon stress or in pathophysiological conditions. ATP release in the CNS was traditionally attributed to astrocytes [Guthrie et al., 1999; Anderson et al., 2004] and neurons mainly [Robertson and Edwards, 1998; Jo and Role, 2002], but recent reports have shown that microglia can release ATP as well. Imura et al. found that upon activation with the $Ca^{2+}$ ionophore ionomycin, microglia release small amounts of ATP via a vesicular nucleotide transporter (VNUT) -dependent mechanism [Imura et al., 2013]. Another research group has proposed a lysosomal exocytosis mechanism for ATP-induced ATP release [Dou et al., 2012]. In my work, I induced ATP release by microglia via medium change and measured the ATP present in the supernatant over the course of 1 hour. After medium change with ATP-free medium, wildtype microglia released some small amounts of ATP, comparable to the published data of Imura et al. Whenever ATP was present in the new medium, wildtype cells rapidly metabolized it. In the supernatants of microglia, devoid of CD39, ATP levels after medium change were significantly higher and added ATP was removed much slower. Similar observations were made when CD39 function was blocked pharmacologically with ARL67156. Interestingly, when ATP concentrations in the supernatants

of the cells were measured 24 hours after medium change, I found equal levels of ATP in the supernatants of wildtype and *Cd39*$^{-/-}$ microglia. Thus, upon medium change, *Cd39*$^{-/-}$ cells release more ATP, but over time, the balance in extracellular ATP is restored. Thus, CD39 is crucial for preventing autocrine over-activation of microglia in acute stress situations.

Since ATP concentrations upon medium change were measured at certain time points, of course, what cannot be stated from these experiments is whether the difference in ATP concentrations in the supernatants of wildtype and *Cd39*$^{-/-}$ cells are due to increased release or decreased metabolization of ATP in absence of CD39. The first time point studied was 1 minute after medium change and it might well be that wildtype cells release similar amounts of ATP as *Cd39*$^{-/-}$, but hydrolyze it immediately. This hypothesis is supported by the notion that CD39 is an extremely efficient enzyme with a very high Michaelis constant [Kukulski and Komoszyski, 2003]. On the other hand, the possibility that *Cd39*$^{-/-}$ cells release more ATP upon medium change cannot be excluded - since these cells are also more sensitive to ATP applications, it could be that they have a general higher sensitivity to external stimulation. Further experiments are required to gain more insights into this matter.

The lowest ATP concentration necessary for activation of P2 receptors is 50 nM ATP (the ec50 value of $P2X_1R$), according to the literature (see table 4.1). According to my results, in the presence of CD39 ATP levels are kept below this threshold of activation. When ATP is added to the cells, concentrations lower than 10 $\mu$M are removed within 1 minute, while addition of 10 $\mu$M takes a longer time for removal. These observations are consistent with the calcium imaging experiments, which showed that 10 $\mu$M ATP is sufficient to activate almost all cells, and concentrations below 10 $\mu$M only activated a fraction of the cells. In the absence of CD39, however, cells are not protected anymore and even low concentrations of ATP, which cannot be removed efficiently, are sufficient to activate the cells, which is reflected in the increased percentage of *Cd39*$^{-/-}$ cells, which responded to ATP application. Thus, CD39 is instrumental in preventing paracrine and autocrine over-activation of microglia.

| Receptor | ec50 [$\mu$M] ATP |
|---|---|
| $P2X_1$ | 0,05 |
| $P2X_2$ | 1,25 |
| $P2X_3$ | 0,32 |
| $P2X_4$ | 0,50 |
| $P2X_5$ | 1 |
| $P2X_6$ | unknown |
| $P2X_7$ | 100 |
| $P2Y_1$ | unknown |
| $P2Y_2$ | 0,08 |
| $P2Y_4$ | 1,99 (rat) |
| $P2Y_6$ | unknown |
| $P2Y_{11}$ | 15,84 |
| $P2Y_{12}$ | unknown |
| $P2Y_{13}$ | 0,25 |
| $P2Y_{14}$ | unknown |

**Table 4.1: Receptor affinities of the human P2R homologs (unless stated otherwise) to ATP.** ***Data from Jacobson [2010].***

### 4.3.3 ATP is mainly produced by astrocytes and removed by microglia

Astrocytes release many neurotransmitters, such as glutamate and D-serine, and recent studies have shown that they also release ATP [Haydon and Carmignoto, 2006]. Astrocytic ATP release is an important component of the astrocytic calcium wave propagation, driven both by extracellular ATP which activates purinergic receptors on cells in the vicinity and by $IP_3$ diffusion via gap junctions to neighboring astrocytes [Haydon, 2001]. Zhang et al. proposed a lysosome exocytosis mechanism for astrocytic ATP release, since lysosome lysis abolishes both the release of ATP and the calcium wave propagation [Zhang et al., 2007]. Since microglia are also capable of ATP release, in my work I compared the ATP found in the supernatants of cultured microglia, cultured astrocytes and astrocytes with few microglia on top after medium change. I found that 1 minute after medium change, the levels of ATP in the astrocytic supernatants were dramatically increased, which was not the case in the microglial cultures or in the astrocytes in presence of microglia. This trend and significant difference between astrocytic and microglial cultures remained over the 60 minutes observed and was independent of the cell volume and cell numbers in the different cultures. My results show that in an astrocyte-microglia co-culture, astrocytes are the main source of ATP, while microglia are responsible for its fast and effective removal via CD39, which is expressed on microglia, but not on astrocytes [Braun et al., 2000].

### 4.3.4 Microglial CD39 controls the spread, but not the velocity, of astrocytic calcium waves

Astrocytic calcium waves are concentrically spreading increases of intracellular calcium in an astrocytic network, which can be induced both *in vitro* and *in situ* by mechanical, electrical or chemical stimulation [Scemes and Giaume, 2006]. The spread of the wave depends on two components: the intracellular communication via gap junctions which allows diffusion of $Ca^{2+}$-mobilizing second messengers to neighboring cells and the extracellular ATP-driven pathway. Both components work in conjunction and are important for the proper spread of the wave. In my work, I found that astrocyte-derived ATP is rapidly removed by microglial CD39 and studied the contribution of microglial CD39 to the spread and velocity of astrocytic calcium waves *in vitro*.
In microglia-astrocyte co-cultures prepared from mice lacking CD39, as well as in wildtype astrocytic cultures depleted of microglia, the spread of the wave was larger than in wildtype co-cultures. The maximal span of the wave spread was also higher in the $Cd39^{-/-}$ cultures compared to wildtype, but not in the microglia-depleted cultures. Considering the parameter $\tau$, which describes the acceleration of the wave, $Cd39^{-/-}$ was not different from wildtype, however $\tau$ of microglia-depleted cultures was significantly lower than that of both wildtype and $Cd39^{-/-}$ co-cultures.
This work presents the results of two different analysis approaches of the calcium wave recordings. In the first approach, each wave was regarded as a separate data set, while the second approach provides insights into the data from the single-cell perspective. Both approaches led to the conclusion that the spread of the calcium wave is a time- and space-restricted event, which has also been reported in other publications [Scemes and Giaume, 2006]. Two different models for the spread of the calcium waves have been proposed: the regenerative mechanism, in which the spread of the wave requires *de novo* formation of $IP_3$ and ATP in the activated cells, and a non-regenerative mechanism, relying on the diffusion of these factors released from a single cell upon activation. The non-regenerative mechanism would provide an intuitive explanation for the spatial restriction of the wave propagation, whereas the regenerative mechanism is supported by the hypothesis that limiting factors of intracellular calcium signaling would set a threshold for activation of cells at the edge of the wave, which would then eventually stop the propagation [Giaume and Venance, 1998]. My findings, that the spread of the wave depends on the presence of microglial CD39, support the regenerative model. In the wildtype cultures, ATP is released by activated astrocytes [Anderson et al., 2004], which acts in an auto- and paracrine fashion to activate both astrocytes and microglia [Schipke et al., 2001], but is also rapidly metabolized by CD39 on microglia. This rapid turnover of ATP leads to the spread of the wave until the concentration of ATP is too low to activate the astrocytes anymore which is when the wave stops expanding.

Removal of CD39 leads to accumulation of ATP, which diffuses further and activates more cells, leading to a larger spread of the wave. This model is supported by the observation that the concentration of ATP declines ten times over a distance of 100 $\mu$m [Newman, 2001]. On the other hand, the speed of the calcium wave is not affected by deletion of *Cd39*, since the limiting component of the speed is the rate of diffusion of ATP, which might not be affected by changes in the concentration. Of course, in my work I only focus on the extracellular component of calcium wave propagation, the ATP-mediated one. Even though the gap-junctional coupling and the diffusion of intracellular second messengers over gap junctions is not disturbed by deletion of *Cd39*, a significant effect on the wave spread could be observed, which underlines the importance of the ATP-driven pathway and the contribution of CD39 in this aspect.
All the work on this project was performed in cultured cells, which reflect the situation *in vivo* only to a certain extent [Lange et al., 2012]. However, purinergic signaling is a universal signaling system and its properties may not be fundamentally different in the simplified model of astrocyte-microglia co-culture, which allows more precise insights into the mechanisms of communication between these two cell types. Moreover, previous work done in our lab has shown that *Cd39* deletion affects the spread of the astrocytic calcium waves in acute brain slices in a similar fashion [Rocha, 2014, PhD thesis].
What is the physiological importance of astrocytic calcium waves? In recent years, a lot of research has been carried out on the reciprocal communication between neurons and astrocytes: neuronal activity in the hippocampus can trigger astrocytic calcium waves [Dani et al., 1992], while astrocytic calcium waves modulate neuronal activity via release of gliotransmitters [Wang et al., 2012] and are important for the proper functioning of the tripartite synapse [Tanaka et al., 2013]. It has been proposed that, similarly to neuronal action potential, astrocytic calcium signaling is important for modulation of brain function, but on a different, much slower time-scale [Haydon, 2001]. Since ATP has been recognized as one of the major neurotransmitters released by astrocytes and acting on neurons, this work provides new insights in the involvement of microglia through the ectonucleotidase CD39 in the complex astrocyte-neuron signaling relationship.

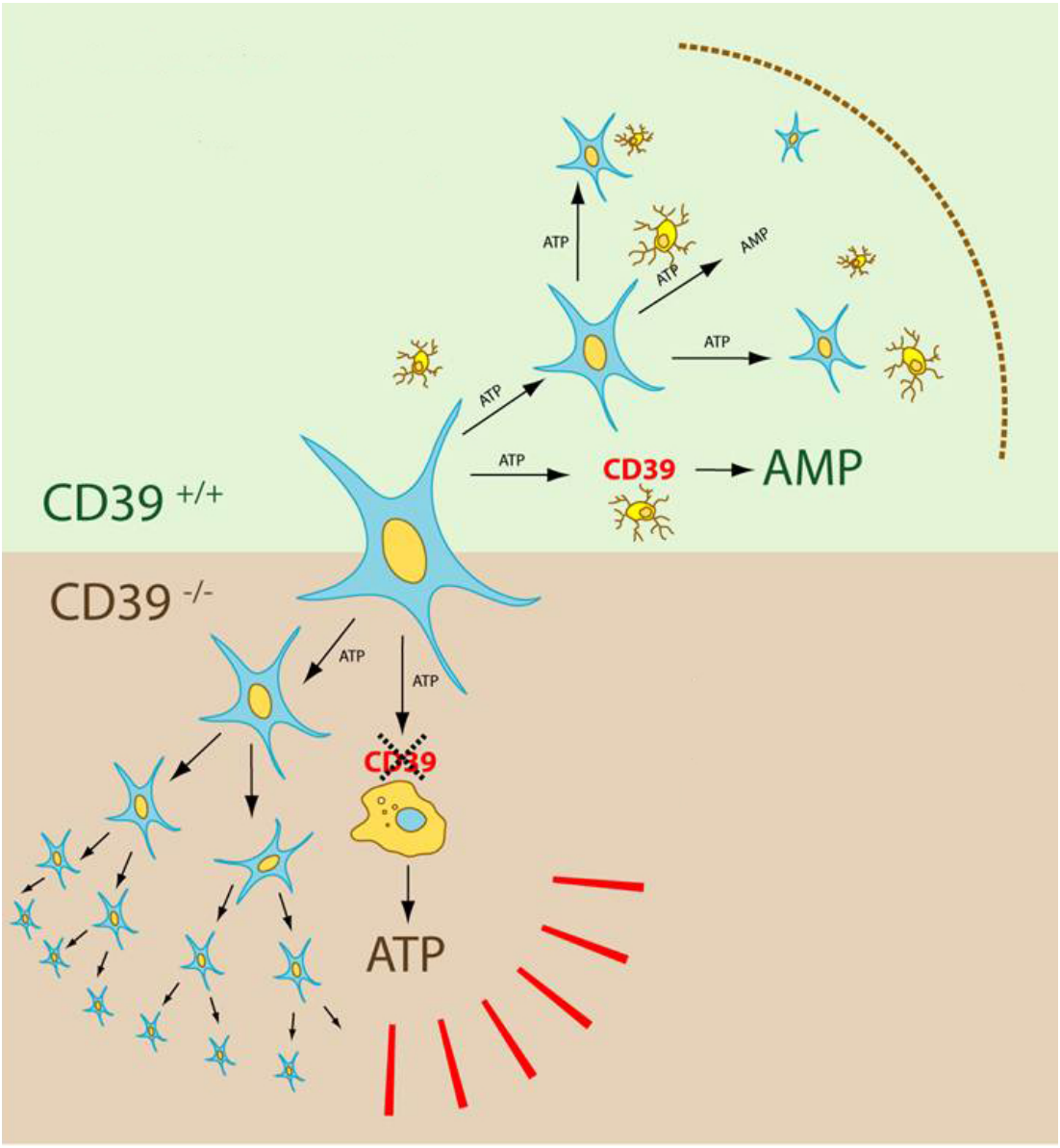


**Figure 4.1: Proposed model for the regulation of autocrine and paracrine purinergic signaling by microglial CD39. In the wildtype brain (upper half), *Cd39* is expressed by microglia (yellow) and rapidly metabolizes ATP, released during calcium waves, to AMP. If CD39 is missing (lower half), ATP is not metabolized and accumulates, activating more astrocytes (blue) and leading to a larger spread of the astrocytic calcium waves. *Illustration: courtesy of Larisa Bulavina.***

## 4.4 Project 3: Purinergic-related microglial functions are disturbed in the Poly I:C mouse model

Microglial involvement in schizophrenia has been addressed in many recent studies, which focused mainly on the quantification of the microglial cells in different brain

areas and/or investigated the activation status of the cells via different antibodies or tracing experiments (for a review, see [Bernstein et al., 2014]). Regarding the inflammatory aspects of the disease, microglia have been connected to the increased levels of pro-inflammatory cytokines in the brain of adult Poly I:C offspring [Garay et al., 2013]. Indeed, mRNA expression of *IL1β* and *TNFαR* is increased in microglia derived from Poly I:C adult mice, indicating a chronical activation of the cells, which is reversible upon treatment with the anti-inflammatory antibiotic minocycline [Mattei et al., 2014]. Microglial phagocytosis is important for removal of apoptotic neurons during development [Tanaka et al., 2013] and in the adult hippocampus [Sierra et al., 2010] and for synaptic pruning [Beumer et al., 2012]. Although recent reviews underline the putative importance of microglial phagocytosis in schizophrenia [Bernstein et al., 2014], no study has so far directly addressed this question.

In acute brain slices from adult Poly I:C mice microglial phagocytosis was comparable to that of control mice, but in contrast to the controls, ATP did not increase phagocytosis in Poly I:C murine microglia. This reduced sensitivity to ATP was also reflected in the calcium imaging experiments, performed with freshly isolated microglia. Furthermore I observed an increased expression of *Cd39* and *P2ry12* in these cells. Increased expression of *Cd39* might account for the faster removal of ATP in the extracellular space which would provide an explanation for the decreased sensitivity to ATP in the microglial cells. Taken together, these results indicate a disturbed purinergic balance in the adult Poly I:C mouse brain which affects microglial properties. Several reviews have suggested a shift in the adenosine/ATP balance in the schizophrenic brain, leading to less adenosine [Boison et al., 2012]. Less adenosine would suggest increased levels of ATP, because ATP is the main source of adenosine in the extracellular space [Linden, 2005]. Moreover, systemic inflammation has been shown to induce ATP release in the CNS *in vivo* [Gourine et al., 2007]. The finding that *Cd39* expression is increased in microglia isolated from adult Poly I:C brains might point toward a compensatory mechanism, necessary for rapid removal of excess ATP in the vicinity of the cells to prevent their overactivation.

Microglial phagocytosis has been studied in many diseases of the CNS [Sierra et al., 2013; Fu et al., 2014]. Generally, functional microglial phagocytosis is considered beneficial [Nagata et al., 2010] and has been found to be disturbed in mouse models of Alzheimer's disease [Krabbe et al., 2013], stroke [Faustino et al., 2011] and prion disease [Park et al., 2008]. Although the presence of pro-inflammatory cytokines, characteristic for the Poly I:C brain, can negatively affect phagocytosis in microglia [Faustino et al., 2011], microglia in acute slices of Poly I:C adult mice display normal phagocytic activity. However, ATP did not enhance phagocytosis in these cells. ATP together with UDP is released in the extracellular space when necrotic cells burst and spill the cell contents, for example by mechanical damage, and induces phagocytosis by activat-

ing the $P2Y_6$ receptor expressed on microglia [Koizumi et al., 2007]. In the Poly I:C context, microglia seem to be less sensitive to ATP, which might lead to a disturbed clearance of necrotic tissue. Moreover, the microglial response to laser lesion, another ATP-mediated microglial function, essential for repair of brain damage, is reduced in acute brain slices from Poly I:C adult mice (Vitali Matyash, unpublished observations). Altogether, microglia in the brains of adult Poly I:C mice display reduced tissue repair functions.

Schizophrenia is a neurodevelopmental disease, which emerges in humans in early adulthood, and the study of a single time-point cannot give proper insights into the processes involved. This is why as a next step I asked whether the observations in adult Poly I:C brains are specific for the age or persist throughout development. To study this, I investigated the microglia in very young mice at an early postnatal time point – P5. Surprisingly, I found a different picture in the pups compared to the adults: the microglial sensitivity to ATP in Poly I:C pups was increased in comparison to controls and mRNA expression of *P2ry6* was strongly reduced, while *Cd39* and *P2ry12* expression were unchanged. *P2ry6* expression on microglia is connected to the phagocytic activity of the cells [Koizumi et al., 2007; Inoue et al., 2009; Koizumi et al., 2013], but whether phagocytosis in P5 Poly I:C offspring is disturbed remains to be investigated. Abnormal microglial phagocytosis of apoptotic neurons at an early postnatal age could be a factor contributing to the aberrant neurodevelopment in schizophrenia.

In addition, I found that expression of *Cd39*, *P2ry12* and *P2ry13* is increased in adults compared to very young pups. Increased *Cd39* expression with age is in consistence with the observations, that $Cd39^{-/-}$ microglia have constitutively increased phagocytosis in adults slices, but not in young ones (P6-P9) [Bulavina, 2013, PhD thesis]. Bulavina speculated, that CD39 might not be important for phagocytosis in very young animals. My results show that young animals have around 3 times lower *Cd39* expression in microglia than adults, which means that in young animals *Cd39* expression is so low that it is much closer to the knockout levels, which is probably the reason why no knockout-specific phagocytic phenotype can be detected. Further work in our lab has provided more evidence for the hypothesis that CD39 is more important in adults than in young animals [Rocha, 2014, PhD thesis].

In conclusion, in this study I have found that the microglial purinergic system is disbalanced in the young and adult Poly I:C offspring, which underlines the importance of microglia in the pathophysiology of schizophrenia.

## 4.5 Outlook

*Cd39* gene expression and protein activity is modulated in microglia in different activation states. Pro-inflammatory conditions strongly reduced both gene expression

and enzymatic activity of *Cd39*, however, the mechanism behind this is still unclear. Reduced levels of *Cd39* would lead to accumulation of ATP in the vicinity of the cells which might be an important part of the pro-inflammatory response. Further research is necessary to elucidate these aspects. On the other hand, the involvement of microglial CD39 in neuroinflammation in diseases like multiple sclerosis also needs to be investigated.

Microglial CD39 is important for the proper spread of astrocytic calcium waves, which are believed to support neuronal communication. Mice lacking *Cd39* have been shown to have prolonged bleeding times and partial alopecia [Enjyoji et al., 1999], but so far no research has investigated whether they exhibit any cognitive abnormalities. Behavioral tests would be required to assess whether lack of CD39 would affect brain function in these mice.

In this work, I have shown that microglial purinergic related properties are altered in a different way in both young and adult Poly I:C offspring, a mouse model of schizophrenia. However, what is yet unclear, is whether microglia are intrinsically different in this model, for example due to epigenetic changes, occurring *in utero* upon the immune challenge of the mother, or is their phenotype challenged by an altered composition of nucleotides in the extracellular space. More research is necessary to answer this question, for example by investigating the epigenetic profile of microglia at early and late time-points of development and also by measuring the ATP and adenosine levels in the brain of Poly I:C mice. More experiments should also be performed to decipher why microglia in the adult Poly I:C offspring are less sensitive to ATP, for example whether phagocytosis can still be induced by UDP or other factors.

Altogether, microglial purinergic signaling is a research area which has been gaining increasing attention in recent years and more work is needed to elucidate its role both in physiological processes and in diseases of the central nervous system.

# 5. Summary

Microglia are the immune competent cells of the central nervous system (CNS). In response to many exogenous and endogenous molecules and in pathologies they can become activated, migrate to the location of the stimuli, release cytokines and become phagocytic. One of the most important molecules, released from other cell types, is adenosine triphosphate (ATP), which is sensed by microglia via purinergic receptors and modulates their activity and function. ATP is degraded on the cell surface by CD39, which is expressed in the CNS only by microglia.

In the first part of my thesis, I studied how *Cd39* deletion affects purinergic signaling in cultured microglia. I found that microglia devoid of CD39 are more sensitive to low concentrations of ATP, but do not show changes in the expression of purinergic receptors. Absence of CD39 leads to higher amounts of ATP found in the supernatants of microglia upon medium change. In astrocyte-microglia co-cultures, astrocytes are the main source of ATP, while microglia are responsible for its rapid and effective removal. Lack of microglial CD39 enzyme also leads to a larger spread of the ATP-mediated astrocytic calcium waves. Taken together, I could show that microglial CD39 is an important regulator of autocrine and paracrine ATP signaling both in microglia and astrocytes. Moreover I found that both *Cd39* gene expression and protein activity are strongly reduced in a pro-inflammatory context, which led me to the second part of my thesis.

Here I investigated ATP-related microglia function in a maternal immune activation (MIA) mouse model of schizophrenia. In adult MIA offspring ATP-induced microglial phagocytosis was impaired. Calcium imaging experiments with freshly isolated microglia confirmed that the cells from MIA offspring exhibit reduced sensitivity to ATP. In addition, mRNA expression of *Cd39* and *P2ry12* was increased in microglia from MIA offspring. In summary, microglia in this mouse model of schizophrenia exhibit an altered ATP-related phenotype, which points toward a purinergic disbalance in the brain of these animals.

# 6. Zusammenfassung

Mikroglia sind die immunkompetenten Zellen im zentralen Nervensystem (ZNS). Sie können durch die Anwesenheit von verschiedenen exogenen und endogenen Signalen aktiviert werden, zum Beispiel in verschiedenen Krankheiten des ZNS, zu der Quelle der Signalen migrieren, Zytokine freisetzen und phagozytische Aktivität aufweisen. Eines der wichtigsten Gefahrensignale ist Adenosintriphosphat (ATP), welches von Zellen freigesetzt werden kann. Es wirkt über verschiedene purinerge Rezeptoren, welche von Mikroglia exprimiert werden, und kann die Aktivität und Funktion von Mikroglia beeinflussen. ATP wird an der Zelloberfläche von CD39 abgebaut, einem Enzym, welches im ZNS nur von Mikroglia produziert wird.

Im ersten Teil meiner Arbeit untersuchte ich wie die Deletion von *Cd39* die purinerge Signalgebung in Kulturmikroglia verändert. Ich konnte feststellen, dass die *Cd39* Expression und Proteinaktivität in einem pro-inflammatorischen Kontext stark reduziert sind. Des Weiteren sind Mikroglia ohne CD39 sensitiver gegenüber niedrigen Konzentrationen von ATP, ohne dabei Änderungen in der Expression von purinergen Rezeptoren aufzuweisen. In Abwesenheit von CD39 konnte ich höhere ATP Konzentrationen im Überstand von Mikrogliakulturen nach Mediumwechsel messen. In Kokulturen von Mikroglia und Astrozyten sind Astrozyten die Hauptquelle von ATP, während Mikroglia für dessen schnellen und effektiven Abbau verantwortlich sind. Das Fehlen von mikroglialem CD39 führt desweiteren zu einer grösseren Ausbreitung von den ATP-abhängigen astrozytischen Kalziumwellen. Zusammengefasst zeigt diese Arbeit, dass CD39 auf Mikroglia ein wichtiger Regulierungsfaktor der autokrinen und parakrinen ATP-Signalgebung von Mikroglia, sowie von Astrozyten ist.

Im zweiten Teil meiner Arbeit untersuchte ich die ATP-bedingten Mikrogliafunktionen in einem Mausmodell für Schizophrenie, welches auf einer Immunaktivierung der Muttertiere während der Schwangerschaft beruht. In adulten Nachkommen von Poly I:C behandelten Muttertieren war die Mikrogliaphagozytose in akuten Hirnschnitten ähnlich zu der von Kontrolltieren. Allerdings war die Phagozytose nach Zugabe von ATP im Unterschied zu den Kontrollschnitten nicht erhöht. Kalziumimagingexperimente mit frisch isolierten Mikroglia bestätigten, dass diese Zellen eine reduzierte Sensitivität zu ATP besitzen. Zusätzlich waren die mRNA Expression von *Cd39* und von

*P2ry12* erhöht. Zusammenfassend kann gesagt werden, dass Mikroglia in diesem Mausmodell für Schizophrenie einen veränderten ATP-bedingten Phänotyp zeigen. Dies gibt einen Hinweis auf ein gestörtes purinerges Gleichgewicht in den Gehirnen dieser Tiere.

# Eidesstattliche Erklärung

Ich, Petya Georgieva, erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit mit dem Titel "Microglial purinergic signaling in mouse models of CD39 deficiency and schizophrenia" selbstständig verfasst habe und keine weiteren Hilfsmittel als die angegebenen benutzt habe.
Des Weiteren erkläre ich, dass ich die vorliegende Arbeit nie in dieser oder einer anderen Form bei einem Promotionsverfahren eingereicht habe.

.
Petya Georgieva

Berlin, den 16.12.2014

# References


M. P. Abbracchio, G. Burnstock, A. Verkhratsky, and H. Zimmermann. Purinergic signalling in the nervous system: an overview. *Trends in neurosciences*, 32(1):19–29, Jan. 2009. ISSN 0166-2236. doi: 10.1016/j.tins.2008.10.001.

A. Abi-Dargham, J. Rodenhiser, D. Printz, Y. Zea-Ponce, R. Gil, L. S. Kegeles, R. Weiss, T. B. Cooper, J. J. Mann, R. L. Van Heertum, J. M. Gorman, and M. Laruelle. Increased baseline occupancy of D2 receptors by dopamine in schizophrenia. *Proceedings of the National Academy of Sciences of the United States of America*, 97(14):8104–9, July 2000. ISSN 0027-8424.

S. Akhondzadeh, E. Shasavand, H. Jamilian, O. Shabestari, and A. Kamalipour. Dipyridamole in the treatment of schizophrenia: adenosine-dopamine receptor interactions. *Journal of clinical pharmacy and therapeutics*, 25(2):131–7, Apr. 2000. ISSN 0269-4727.

L. Alexopoulou, A. C. Holt, R. Medzhitov, and R. A. Flavell. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature*, 413(6857):732–8, Oct. 2001. ISSN 0028-0836. doi: 10.1038/35099560.

E. Aliagas, I. Villar-Menéndez, J. Sévigny, M. Roca, M. Romeu, I. Ferrer, M. Martín-Satué, and M. Barrachina. Reduced striatal ecto-nucleotidase activity in schizophrenia patients supports the "adenosine hypothesis". *Purinergic signalling*, 9(4):599–608, Dec. 2013. ISSN 1573-9546. doi: 10.1007/s11302-013-9370-7.

C. M. Anderson, J. P. Bergher, and R. A. Swanson. ATP-induced ATP release from astrocytes. *Journal of neurochemistry*, (88):246–256, 2004. doi: 10.1046/j.1471-4159.2003.02204.x.

L. Antonioli, P. Pacher, E. S. Vizi, and G. Haskó. CD39 and CD73 in immunity and inflammation. *Trends in molecular medicine*, 19(6):355–67, June 2013. ISSN 1471-499X. doi: 10.1016/j.molmed.2013.03.005.

D. Attwell, A. M. Buchan, S. Charpak, M. Lauritzen, B. a. Macvicar, and E. a. Newman. Glial and neuronal control of brain blood flow. *Nature*, 468(7321):232–43, Nov. 2010. ISSN 1476-4687. doi: 10.1038/nature09613.

V. Balasingam, T. Tejada-Berges, E. Wright, R. Bouckova, and V. Yong. Reactive Astrogliosis in the Neonatal Mouse Brain and Its Modulation by Cytokines. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 14(2):846 – 856, 1994.

J. T. Bartrup and T. W. Stone. Activation of NMDA receptor-coupled channels suppresses the inhibitory action of adenosine on hippocampal slices. *Brain research*, 530(2):330–4, Oct. 1990. ISSN 0006-8993.

J. Bastid, A. Cottalorda-Regairaz, G. Alberici, N. Bonnefoy, J.-F. Eliaou, and A. Bensussan. ENTPD1/CD39 is a promising therapeutic target in oncology. *Oncogene*, 32(14):1743–51, Apr. 2013. ISSN 1476-5594. doi: 10.1038/onc.2012.269.

A. Bauer, M. H. Holschbach, P. T. Meyer, C. Boy, H. Herzog, R. A. Olsson, H. H. Coenen, and K. Zilles. In vivo imaging of adenosine A1 receptors in the human brain with [18F]CPFPX and positron emission tomography. *NeuroImage*, 19(4):1760–9, Aug. 2003. ISSN 1053-8119.

M. Bélanger, I. Allaman, and P. J. Magistretti. Brain energy metabolism: focus on astrocyte-neuron metabolic cooperation. *Cell metabolism*, 14(6):724–38, Dec. 2011. ISSN 1932-7420. doi: 10.1016/j.cmet.2011.08.016.

Bendich A. Chemistry of purines and pyrimidines. In C. A and Davidson JN, editors, *The Nucleic Acids in Chemistry and Biology*, pages 81–136. Academic press, New York, 3 edition, 1955.

H.-G. Bernstein, J. Steiner, P. C. Guest, H. Dobrowolny, and B. Bogerts. Glial cells as key players in schizophrenia pathology: recent insights and concepts of therapy. *Schizophrenia research*, June 2014. ISSN 1573-2509. doi: 10.1016/j.schres.2014.03.035.

W. Beumer, S. M. Gibney, R. C. Drexhage, L. Pont-Lezica, J. Doorduin, H. C. Klein, J. Steiner, T. J. Connor, A. Harkin, M. A. Versnel, and H. A. Drexhage. The immune theory of psychiatric diseases: a key role for activated microglia and circulating monocytes. *Journal of leukocyte biology*, 92(5): 959–75, Nov. 2012. ISSN 1938-3673. doi: 10.1189/jlb.0212100.

K. Biber, H. Neumann, K. Inoue, and H. W. G. M. Boddeke. Neuronal 'On' and 'Off' signals control microglia. *Trends in neurosciences*, 30(11):596–602, Nov. 2007. ISSN 0166-2236. doi: 10.1016/j.tins.2007.08.007.

F. Blomstrand, N. D. Aberg, P. S. Eriksson, E. Hansson, and L. Rönnbäck. Extent of intercellular calcium wave propagation is related to gap junction permeability and level of connexin-43 expression in astrocytes in primary cultures from four brain regions. *Neuroscience*, 92(1):255–65, Jan. 1999. ISSN 0306-4522.

D. Boison, P. Singer, H.-Y. Shen, J. Feldon, and B. K. Yee. Adenosine hypothesis of schizophrenia–opportunities for pharmacotherapy. *Neuropharmacology*, 62(3):1527–43, Mar. 2012. ISSN 1873-7064. doi: 10.1016/j.neuropharm.2011.01.048.

G. Borsellino, M. Kleinewietfeld, D. Di Mitri, A. Sternjak, A. Diamantini, R. Giometto, S. Höpner, D. Centonze, G. Bernardi, M. L. Dell'Acqua, P. M. Rossini, L. Battistini, O. Rötzschke, and K. Falk. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood*, 110(4):1225–32, Aug. 2007. ISSN 0006-4971. doi: 10.1182/blood-2006-12-064527.

N. Braun, J. Sévigny, S. C. Robson, K. Enjyoji, O. Guckelberger, K. Hammer, F. Di Virgilio, and H. Zimmermann. Assignment of ecto-nucleoside triphosphate diphosphohydrolase-1/cd39 expression to microglia and vasculature of the brain. *The European journal of neuroscience*, 12(12):4357–66, Dec. 2000. ISSN 0953-816X.

A. Brown and E. Derkitis. Prenatal Infection and Schizophrenia: A review of epidemiologic and translational studies. *American Journal of Psychiatry*, 167(3):261–280, 2010. doi: 10.1176/appi.ajp.2009.09030361.Prenatal.

A. S. Brown and E. S. Susser. In utero infection and adult schizophrenia. *Mental retardation and developmental disabilities research reviews*, 8(1):51–7, Jan. 2002. ISSN 1080-4013. doi: 10.1002/mrdd.10004.

A. S. Brown, J. Hooton, C. A. Schaefer, H. Zhang, E. Petkova, V. Babulas, M. Perrin, J. M. Gorman, and E. S. Susser. Elevated Maternal Interleukin-8 Levels and Risk of Schizophrenia in Adult Offspring. Nov. 2004.

M. G. Brunstein, E. S. Ghisolfi, F. L. P. Ramos, and D. R. Lara. A clinical trial of adjuvant allopurinol therapy for moderately refractory schizophrenia. *The Journal of clinical psychiatry*, 66(2):213–9, Feb. 2005. ISSN 0160-6689.

R. E. Bucheimer and J. Linden. Purinergic regulation of epithelial transport. *The Journal of physiology*, 555(Pt 2):311–21, Mar. 2004. ISSN 0022-3751. doi: 10.1113/jphysiol.2003.056697.

L. Bulavina. *Roles of ENTPDase1 and 5'-eNT in the regulation of microglial phagocytosis*. PhD thesis, Charite Berlin, 2013.

L. Bulavina, F. Szulzewsky, A. Rocha, G. Krabbe, S. C. Robson, V. Matyash, and H. Kettenmann. NTPDase1 activity attenuates microglial phagocytosis. *Purinergic signalling*, 9(2):199–205, June 2013. ISSN 1573-9546. doi: 10.1007/s11302-012-9339-y.

G. Burnstock. Physiology and pathophysiology of purinergic neurotransmission. *Physiological reviews*, 87(2):659–797, Apr. 2007. ISSN 0031-9333. doi: 10.1152/physrev.00043.2006.

G. Burnstock, B. B. Fredholm, R. a. North, and A. Verkhratsky. The birth and postnatal development of purinergic signalling. *Acta physiologica (Oxford, England)*, 199(2):93–147, June 2010. ISSN 1748-1716. doi: 10.1111/j.1748-1716.2010.02114.x.

S. Busse, M. Busse, K. Schiltz, H. Bielau, T. Gos, R. Brisch, C. Mawrin, A. Schmitt, W. Jordan, U. J. Müller, H.-G. Bernstein, B. Bogerts, and J. Steiner. Different distribution patterns of lymphocytes and microglia in the hippocampus of patients with residual versus paranoid schizophrenia: further evidence for disease course-related immune alterations? *Brain, behavior, and immunity*, 26(8): 1273–9, Nov. 2012. ISSN 1090-2139. doi: 10.1016/j.bbi.2012.08.005.

W. S. Carbonell, S.-I. Murase, A. F. Horwitz, and J. W. Mandell. Migration of perilesional microglia after focal brain injury and modulation by CC chemokine receptor 5: an in situ time-lapse confocal imaging study. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 25 (30):7040–7, July 2005. ISSN 1529-2401. doi: 10.1523/JNEUROSCI.5171-04.2005.

F. Chalmin, G. Mignot, M. Bruchard, A. Chevriaux, F. Végran, A. Hichami, S. Ladoire, V. Derangère, J. Vincent, D. Masson, S. C. Robson, G. Eberl, J. R. Pallandre, C. Borg, B. Ryffel, L. Apetoh, C. Rébé, and F. Ghiringhelli. Stat3 and Gfi-1 transcription factors control Th17 cell immunosuppressive activity via the regulation of ectonucleotidase expression. *Immunity*, 36(3):362–73, Mar. 2012. ISSN 1097-4180. doi: 10.1016/j.immuni.2011.12.019.

A. Charles. Intercellular calcium waves in glia. *Glia*, 24(1):39–49, Sept. 1998. ISSN 0894-1491.

A. C. Charles, E. R. Dircksen, J. E. Merrill, and M. J. Sanderson. Mechanisms of intercellular calcium signaling in glial cells studied with dantrolene and thapsigargin. *Glia*, 7(2):134–145, 1993.

F. B. Chekeni, M. R. Elliott, J. K. Sandilos, S. F. Walk, M. Kinchen, E. R. Lazarowski, A. J. Armstrong, S. Penuela, W. Laird, G. S. Salvesen, B. E. Isakson, D. A. Bayliss, and S. Kodi. Pannexin 1 channels mediate findme signal release and membrane permeability during apoptosis. *Nature*, 467(7317): 863–867, 2010. doi: 10.1038/nature09413.

Y. Chen, R. Corriden, Y. Inoue, L. Yip, N. Hashiguchi, A. Zinkernagel, V. Nizet, P. a. Insel, and W. G. Junger. ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors. *Science (New York, N.Y.)*, 314(5806):1792–5, Dec. 2006. ISSN 1095-9203. doi: 10.1126/science.1132559.

J. Choi, K. Tanaka, Y. Cao, Y. Qi, J. Qiu, Y. Liang, S. Y. Lee, and G. Stacey. Identification of a plant receptor for extracellular ATP. *Science (New York, N.Y.)*, 343(6168):290–4, Jan. 2014. ISSN 1095-9203. doi: 10.1126/science.343.6168.290.

A. H. Cornell-Bell, S. M. Finkbeiner, M. S. Cooper, and S. J. Smith. Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. *Science*, 247(24):470–473, 1990.

M. L. Cotrina, J. H. Lin, A. Alves-Rodrigues, S. Liu, J. Li, H. Azmi-Ghadimi, J. Kang, C. C. Naus, and M. Nedergaard. Connexins regulate calcium signaling by controlling ATP release. *Proceedings of the National Academy of Sciences of the United States of America*, 95(26):15735–40, Dec. 1998. ISSN 0027-8424.

J. W. Dani, A. Chernjavsky, and S. J. Smith. Neuronal activity triggers calcium waves in hippocampal astrocyte networks. *Neuron*, 8(3):429–40, Mar. 1992. ISSN 0896-6273.

D. Davalos, J. Grutzendler, G. Yang, J. V. Kim, Y. Zuo, S. Jung, D. R. Littman, M. L. Dustin, and W.-B. Gan. ATP mediates rapid microglial response to local brain injury in vivo. *Nature neuroscience*, 8(6):752–8, June 2005. ISSN 1097-6256. doi: 10.1038/nn1472.

R. H. DeKruyff, X. Bu, A. Ballesteros, C. Santiago, Y.-L. E. Chim, H.-H. Lee, P. Karisola, M. Pichavant, G. G. Kaplan, D. T. Umetsu, G. J. Freeman, and J. M. Casasnovas. T cell/transmembrane, Ig, and mucin-3 allelic variants differentially recognize phosphatidylserine and mediate phagocytosis of apoptotic cells. *Journal of immunology (Baltimore, Md. : 1950)*, 184(4):1918–30, Feb. 2010. ISSN 1550-6606. doi: 10.4049/jimmunol.0903059.

P. Del Rio-Hortega. El tercer elemento de los centros nerviosos I La microglia en estado normal II Intervencíon de la microglia en los procesos patológicos III Naturaleza probable de la microglia. *Bol de la soc esp de biol*, 9:69–120, 1919.

P. Del Rio-Hortega. Microglia. In W. Penfield, editor, *Cytology and Cellular Pathology of the Nervous System*. New York, 1932.

J. Doorduin, E. F. J. de Vries, A. T. M. Willemsen, J. C. de Groot, R. A. Dierckx, and H. C. Klein. Neuroinflammation in schizophrenia-related psychosis: a PET study. *Journal of nuclear medicine*, 50(11):1801–7, Nov. 2009. ISSN 1535-5667. doi: 10.2967/jnumed.109.066647.

Y. Dou, H.-j. Wu, H.-q. Li, S. Qin, Y.-e. Wang, J. Li, H.-f. Lou, Z. Chen, X.-m. Li, Q.-m. Luo, and S. Duan. Microglial migration mediated by ATP-induced ATP release from lysosomes. *Cell research*, 22(6):1022–33, June 2012. ISSN 1748-7838. doi: 10.1038/cr.2012.10.

T. V. Dunwiddie and S. A. Masino. The role and regulation of adenosine in the central nervous system. *Annual review of neuroscience*, 24:31–55, Jan. 2001. ISSN 0147-006X. doi: 10.1146/annurev.neuro.24.1.31.

K. Enjyoji, J. Sévigny, Y. Lin, and P. Frenette. Targeted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation. *Nature medicine*, 5(9), 1999.

V. A. Fadok, D. R. Voelker, P. A. Campbell, J. J. Cohen, D. L. Bratton, and P. M. Henson. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *Journal of immunology (Baltimore, Md. : 1950)*, 148(7):2207–16, Apr. 1992. ISSN 0022-1767.

V. A. Fadok, de Cathelineau A, D. L. Daleke, P. M. Henson, and D. L. Bratton. Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts. *The Journal of biological chemistry*, 276(2):1071–7, Jan. 2001. ISSN 0021-9258. doi: 10.1074/jbc.M003649200.

K. Färber and H. Kettenmann. Purinergic signaling and microglia. *Pflügers Archiv : European journal of physiology*, 452(5):615–21, Aug. 2006. ISSN 0031-6768. doi: 10.1007/s00424-006-0064-7.

K. Farber, S. Markworth, U. Pannasch, C. Nolte, V. Prinz, G. Kronenberg, K. Gertz, M. Endres, I. Bechmann, K. Enjyoji, S. C. Robson, and H. Kettenmann. The Ectonucleotidase cd39 / ENTPDase1 Modulates Purinergic-Mediated Microglial Migration. *Glia*, 341(December 2007):331–341, 2008. doi: 10.1002/glia.

J. V. Faustino, X. Wang, C. E. Johnson, A. Klibanov, N. Derugin, M. F. Wendland, and Z. S. Vexler. Microglial cells contribute to endogenous brain defenses after acute neonatal focal stroke. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 31(36):12992–3001, Oct. 2011. ISSN 1529-2401. doi: 10.1523/JNEUROSCI.2102-11.2011.

S. Ferré. Adenosine-dopamine interactions in the ventral striatum. Implications for the treatment of schizophrenia. *Psychopharmacology*, 133(2):107–20, Sept. 1997. ISSN 0033-3158.

S. G. Fillman, N. Cloonan, V. S. Catts, L. C. Miller, J. Wong, T. McCrossin, M. Cairns, and C. S. Weickert. Increased inflammatory markers identified in the dorsolateral prefrontal cortex of individuals with schizophrenia. *Molecular psychiatry*, 18(2):206–14, Feb. 2013. ISSN 1476-5578. doi: 10.1038/mp.2012.110.

E. Fischer. *Untersuchungen in der Puringruppe*. J. Springer, Berlin, 1907.

B. B. Fredholm, J.-F. Chen, R. A. Cunha, P. Svenningsson, and J.-M. Vaugeois. Adenosine and brain function. *International review of neurobiology*, 63:191–270, Jan. 2005. ISSN 0074-7742. doi: 10.1016/S0074-7742(05)63007-3.

R. Fu, Q. Shen, P. Xu, J. J. Luo, and Y. Tang. Phagocytosis of microglia in the central nervous system diseases. *Molecular neurobiology*, 49(3):1422–34, June 2014. ISSN 1559-1182. doi: 10.1007/s12035-013-8620-6.

M. Fumagalli, R. Brambilla, N. D'Ambrosi, C. Volonté, M. Matteoli, C. Verderio, and M. P. Abbracchio. Nucleotide-mediated calcium signaling in rat cortical astrocytes: Role of P2X and P2Y receptors. *Glia*, 43(3):218–03, Sept. 2003. ISSN 0894-1491. doi: 10.1002/glia.10248.

P. a. Garay, E. Y. Hsiao, P. H. Patterson, and a. K. McAllister. Maternal immune activation causes age- and region-specific changes in brain cytokines in offspring throughout development. *Brain, behavior, and immunity*, 31:54–68, July 2013. ISSN 1090-2139. doi: 10.1016/j.bbi.2012.07.008.

L. Garey. When cortical development goes wrong: schizophrenia as a neurodevelopmental disease of microcircuits. *Journal of anatomy*, 217(4):324–33, Oct. 2010. ISSN 1469-7580. doi: 10.1111/j.1469-7580.2010.01231.x.

C. Giaume and L. Venance. Intercellular calcium waves and Gap Junctional Communication in Astrocytes. *Glia*, 64(November 1997):50–64, 1998.

F. Ginhoux, M. Greter, M. Leboeuf, S. Nandi, P. See, S. Gokhan, M. F. Mehler, S. J. Conway, L. G. Ng, E. R. Stanley, I. M. Samokhvalov, and M. Merad. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science (New York, N.Y.)*, 330(6005):841–5, Nov. 2010. ISSN 1095-9203. doi: 10.1126/science.1194637.

J. W. Goding. Ecto-enzymes : physiology meets pathology. *Journal of leukocyte biology*, 67(March), 2000.

D. Gómez-Nicola, N. L. Fransen, S. Suzzi, and V. H. Perry. Regulation of microglial proliferation during chronic neurodegeneration. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 33(6):2481–93, Feb. 2013. ISSN 1529-2401. doi: 10.1523/JNEUROSCI.4440-12.2013.

L. Gotoh, H. Mitsuyasu, Y. Kobayashi, N. Oribe, A. Takata, H. Ninomiya, V. P. Stanton, G. M. Springett, H. Kawasaki, and S. Kanba. Association analysis of adenosine A1 receptor gene (ADORA1) polymorphisms with schizophrenia in a Japanese population. *Psychiatric genetics*, 19 (6):328–35, Dec. 2009. ISSN 1473-5873. doi: 10.1097/YPG.0b013e3283328e26.

A. Goudriaan, C. de Leeuw, S. Ripke, C. M. Hultman, P. Sklar, P. F. Sullivan, A. B. Smit, D. Posthuma, and M. H. G. Verheijen. Specific glial functions contribute to schizophrenia susceptibility. *Schizophrenia bulletin*, 40(4):925–35, July 2014. ISSN 1745-1701. doi: 10.1093/schbul/sbt109.

A. V. Gourine, N. Dale, E. Llaudet, D. M. Poputnikov, K. M. Spyer, and V. N. Gourine. Release of ATP in the central nervous system during systemic inflammation: real-time measurement in the hypothalamus of conscious rabbits. *The Journal of physiology*, 585(Pt 1):305–16, Nov. 2007. ISSN 0022-3751. doi: 10.1113/jphysiol.2007.143933.

A. Grinthal and G. Guidotti. Dynamic motions of CD39 transmembrane domains regulate and are regulated by the enzymatic active site. *Biochemistry*, 43(43):13849–58, Nov. 2004. ISSN 0006-2960. doi: 10.1021/bi048644x.

A. Grinthal and G. Guidotti. CD39, NTPDase 1, is attached to the plasma membrane by two transmembrane domains. Why? *Purinergic signalling*, 2(2):391–8, June 2006. ISSN 1573-9538. doi: 10.1007/s11302-005-5907-8.

D. R. Gude, S. E. Alvarez, S. W. Paugh, P. Mitra, J. Yu, R. Griffiths, S. E. Barbour, S. Milstien, and S. Spiegel. Apoptosis induces expression of sphingosine kinase 1 to release sphingosine-1-phosphate as a "come-and-get-me" signal. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 22(8):2629–38, Aug. 2008. ISSN 1530-6860. doi: 10.1096/fj.08-107169.

P. B. Guthrie, J. Knappenberger, M. Segal, M. V. Bennett, a. C. Charles, and S. B. Kater. ATP released from astrocytes mediates glial calcium waves. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 19(2):520–8, Jan. 1999. ISSN 0270-6474.

R. Hanayama, M. Tanaka, K. Miwa, A. Shinohara, A. Iwamatsu, and S. Nagata. Identification of a factor that links apoptotic cells to phagocytes. *Nature*, 417(6885):182–7, May 2002. ISSN 0028-0836. doi: 10.1038/417182a.

U.-K. Hanisch. Microglia as a source and target of cytokines. *Glia*, 40(2):140–55, Nov. 2002. ISSN 0894-1491. doi: 10.1002/glia.10161.

U.-k. Hanisch. Proteins in Microglial Activation - Inputs and Outputs by Subsets. *Current Protein and Peptide Science*, 14:3–15, 2013.

U.-K. Hanisch and H. Kettenmann. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nature neuroscience*, 10(11):1387–94, Nov. 2007. ISSN 1097-6256. doi: 10.1038/nn1997.

T. D. Hassinger, P. B. Guthrie, P. B. Atkinson, M. V. Bennett, and S. B. Kater. An extracellular signaling component in propagation of astrocytic calcium waves. *Proceedings of the National Academy of Sciences of the United States of America*, 93(23):13268–73, Nov. 1996. ISSN 0027-8424.

P. G. Haydon. GLIA: listening and talking to the synapse. *Nature reviews. Neuroscience*, 2(3):185–93, Mar. 2001. ISSN 1471-003X. doi: 10.1038/35058528.

P. G. Haydon and G. Carmignoto. Astrocyte control of synaptic transmission and neurovascular coupling. *Physiological reviews*, 86(3):1009–31, July 2006. ISSN 0031-9333. doi: 10.1152/physrev.00049.2005.

S. E. Haynes, G. Hollopeter, G. Yang, D. Kurpius, M. E. Dailey, W.-B. Gan, and D. Julius. The P2Y12 receptor regulates microglial activation by extracellular nucleotides. *Nature neuroscience*, 9 (12):1512–9, Dec. 2006. ISSN 1097-6256. doi: 10.1038/nn1805.

D. J. Hines, R. M. Hines, S. J. Mulligan, and B. a. Macvicar. Microglia processes block the spread of damage in the brain and require functional chloride channels. *Glia*, 57(15):1610–8, Nov. 2009. ISSN 1098-1136. doi: 10.1002/glia.20874.

S. Honda, Y. Sasaki, K. Ohsawa, Y. Imai, Y. Nakamura, K. Inoue, and S. Kohsaka. 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):1975–82, Mar. 2001. ISSN 1529-2401.

Y. Imura, Y. Morizawa, R. Komatsu, K. Shibata, Y. Shinozaki, H. Kasai, K. Moriishi, Y. Moriyama, and S. Koizumi. Microglia release ATP by exocytosis. *Glia*, 61(8):1320–30, Aug. 2013. ISSN 1098-1136. doi: 10.1002/glia.22517.

K. Inoue, S. Koizumi, A. Kataoka, H. Tozaki-Saitoh, and M. Tsuda. P2Y(6)-Evoked Microglial Phagocytosis. *International review of neurobiology*, 85:159–63, Jan. 2009. ISSN 0074-7742. doi: 10.1016/S0074-7742(09)85012-5.

T. Ishibashi, K. a. Dakin, B. Stevens, P. R. Lee, S. V. Kozlov, C. L. Stewart, and R. D. Fields. Astrocytes promote myelination in response to electrical impulses. *Neuron*, 49(6):823–32, Mar. 2006. ISSN 0896-6273. doi: 10.1016/j.neuron.2006.02.006.

K. A. Jacobson. P2X and P2Y Receptors. Technical report, Tocris Bioscience, 2010.

Y.-H. Jo and L. W. Role. Coordinate release of ATP and GABA at in vitro synapses of lateral hypothalamic neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 22 (12):4794–804, June 2002. ISSN 1529-2401.

G. Juckel, M. P. Manitz, M. Brüne, A. Friebe, M. T. Heneka, and R. J. Wolf. Microglial activation in a neuroinflammational animal model of schizophrenia–a pilot study. *Schizophrenia research*, 131 (1-3):96–100, Sept. 2011. ISSN 1573-2509. doi: 10.1016/j.schres.2011.06.018.

E. Kaczmarek, K. Koziak, J. Sevigny, J. B. Siegel, J. Anrather, A. R. Beaudoin, F. H. Bach, and C. Robson. Identification and Characterization of Identification and Characterization of CD39 / Vascular ATP Diphosphohydrolase. *The Journal of biological chemistry*, 1996.

G. S. Kansas, G. S. Wood, and T. F. Tedder. Expression, distribution, and biochemistry of human CD39. Role in activation-associated homotypic adhesion of lymphocytes. *Journal of immunology (Baltimore, Md. : 1950)*, 146(7):2235–44, Apr. 1991. ISSN 0022-1767.

H. Kettenmann and B. Ransom, editors. *Neuroglia*. Oxford University Press, New York, 3 edition, 2013.

H. Kettenmann, F. Kirchhoff, and A. Verkhratsky. Microglia: new roles for the synaptic stripper. *Neuron*, 77(1):10–8, Jan. 2013. ISSN 1097-4199. doi: 10.1016/j.neuron.2012.12.023.

M. Kimura, L. A. Toth, H. Agostini, A. B. Cady, J. A. Majde, and J. M. Krueger. Comparison of acute phase responses induced in rabbits by lipopolysaccharide and double-stranded RNA. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, 267(6 36-6), 1994. ISSN 03636119.

B. Kirkpatrick. Understanding the physiology of schizophrenia. *The Journal of clinical psychiatry*, 74 (3):e05, Mar. 2013. ISSN 1555-2101. doi: 10.4088/JCP.12045tx3c.

A. Kittel, B. Sperlágh, J. Pelletier, J. Sévigny, and T. L. Kirley. Transient changes in the localization and activity of ecto-nucleotidases in rat hippocampus following lipopolysaccharide treatment. *International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience*, 25(5):275–82, Aug. 2007. ISSN 0736-5748. doi: 10.1016/j.ijdevneu.2007.05.007.

N. Kobayashi, P. Karisola, V. Peña Cruz, D. M. Dorfman, M. Jinushi, S. E. Umetsu, M. J. Butte, H. Nagumo, I. Chernova, B. Zhu, A. H. Sharpe, S. Ito, G. Dranoff, G. G. Kaplan, J. M. Casasnovas, D. T. Umetsu, R. H. Dekruyff, and G. J. Freeman. TIM-1 and TIM-4 glycoproteins bind phosphatidylserine and mediate uptake of apoptotic cells. *Immunity*, 27(6):927–40, Dec. 2007. ISSN 1074-7613. doi: 10.1016/j.immuni.2007.11.011.

S. Koizumi, Y. Shigemoto-Mogami, K. Nasu-Tada, Y. Shinozaki, K. Ohsawa, M. Tsuda, B. V. Joshi, K. a. Jacobson, S. Kohsaka, and K. Inoue. UDP acting at P2Y6 receptors is a mediator of microglial phagocytosis. *Nature*, 446(7139):1091–5, Apr. 2007. ISSN 1476-4687. doi: 10.1038/nature05704.

S. Koizumi, K. Ohsawa, K. Inoue, and S. Kohsaka. Purinergic receptors in microglia: functional modal shifts of microglia mediated by P2 and P1 receptors. *Glia*, 61(1):47–54, Jan. 2013. ISSN 1098-1136. doi: 10.1002/glia.22358.

G. Krabbe, A. Halle, V. Matyash, J. L. Rinnenthal, G. D. Eom, U. Bernhardt, K. R. Miller, S. Prokop, H. Kettenmann, and F. L. Heppner. Functional impairment of microglia coincides with Beta-amyloid deposition in mice with Alzheimer-like pathology. *PloS one*, 8(4):e60921, Jan. 2013. ISSN 1932-6203. doi: 10.1371/journal.pone.0060921.

M.-C. Ku, S. a. Wolf, D. Respondek, V. Matyash, A. Pohlmann, S. Waiczies, H. Waiczies, T. Niendorf, M. Synowitz, R. Glass, and H. Kettenmann. GDNF mediates glioblastoma-induced microglia attraction but not astrogliosis. *Acta neuropathologica*, 125(4):609–20, Apr. 2013. ISSN 1432-0533. doi: 10.1007/s00401-013-1079-8.

K. V. Kuchibhotla, C. R. Lattarulo, B. T. Hyman, and B. J. Bacskai. Synchronous Hyperactivity and Intercellular Calcium Waves in Astrocytes in Alzheimer Mice. *Science*, 323(February):1211–1215, 2009.

F. Kukulski and M. Komoszyski. Purification and characterization of NTPDase1 (ecto-apyrase) and NTPDase2 (ecto-ATPase) from porcine brain cortex synaptosomes. *European journal of biochemistry / FEBS*, 270(16):3447–54, Aug. 2003. ISSN 0014-2956.

F. Kukulski, S. a. Lévesque, E. G. Lavoie, J. Lecka, F. Bigonnesse, a. F. Knowles, S. C. Robson, T. L. Kirley, and J. Sévigny. Comparative hydrolysis of P2 receptor agonists by NTPDases 1, 2, 3 and 8. *Purinergic signalling*, 1(2):193–204, June 2005. ISSN 1573-9538. doi: 10.1007/s11302-005-6217-x.

A. Kurumaji and M. Toru. An increase in [3H] CGS21680 binding in the striatum of postmortem brains of chronic schizophrenics. *Brain research*, 808(2):320–3, Oct. 1998. ISSN 0006-8993.

R. Ladeby, M. Wirenfeldt, I. Dalmau, R. Gregersen, D. García-Ovejero, A. Babcock, T. Owens, and B. Finsen. Proliferating resident microglia express the stem cell antigen CD34 in response to acute neural injury. *Glia*, 50(2):121–31, Apr. 2005a. ISSN 0894-1491. doi: 10.1002/glia.20159.

R. Ladeby, M. Wirenfeldt, D. Garcia-Ovejero, C. Fenger, L. Dissing-Olesen, I. Dalmau, and B. Finsen. Microglial cell population dynamics in the injured adult central nervous system. *Brain research. Brain research reviews*, 48(2):196–206, Apr. 2005b. ISSN 01650173. doi: 10.1016/j.brainresrev.2004.12.009.

S. C. Lange, L. K. Bak, H. S. Waagepetersen, A. Schousboe, and M. D. Norenberg. Primary cultures of astrocytes: their value in understanding astrocytes in health and disease. *Neurochemical research*, 37(11):2569–88, Nov. 2012. ISSN 1573-6903. doi: 10.1007/s11064-012-0868-0.

D. R. Lara and D. O. Souza. Schizophrenia: a purinergic hypothesis. *Medical hypotheses*, 54(2):157–66, Feb. 2000. ISSN 0306-9877. doi: 10.1054/mehy.1999.0003.

D. R. Lara, O. Dall'Igna, E. S. Ghisolfi, and M. Brunstein. Involvement of adenosine in neurobiology of schizophrenia and its therapeutic interactions. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, (30):617–629, 2006.

M. Laruelle and A. Abi-Dargham. Dopamine as the wind of the psychotic fire: new evidence from brain imaging studies. *Journal of psychopharmacology (Oxford, England)*, 13(4):358–71, Dec. 1999. ISSN 0269-8811.

K. Lauber, S. G. Blumenthal, M. Waibel, and S. Wesselborg. Clearance of apoptotic cells: getting rid of the corpses. *Molecular cell*, 14(3):277–87, May 2004. ISSN 1097-2765.

S. Leucht, C. Corves, D. Arbter, R. R. Engel, C. Li, and J. M. Davis. Second-generation versus first-generation antipsychotic drugs for schizophrenia: a meta-analysis. *Lancet*, 373(9657):31–41, Jan. 2009. ISSN 1474-547X. doi: 10.1016/S0140-6736(08)61764-X.

J. Linden. Adenosine in tissue protection and tissue regeneration. *Molecular pharmacology*, 67(5): 1385–7, May 2005. ISSN 0026-895X. doi: 10.1124/mol.105.011783.

L. H. Lindström, O. Gefvert, G. Hagberg, T. Lundberg, M. Bergström, P. Hartvig, and B. Lå ngström. Increased dopamine synthesis rate in medial prefrontal cortex and striatum in schizophrenia indicated by L-(beta-11C) DOPA and PET. *Biological psychiatry*, 46(5):681–8, Sept. 1999. ISSN 0006-3223.

K. Lohmann. Über die Pyrophosphatfraktion im Muskel. *Naturwissenschaften*, 17(31):624–625, 1929.

F. O. Martinez, A. Sica, A. Mantovani, and M. Locati. Macrophage activation and polarization. *Frontiers in bioscience (Scholar edition)rontiers in Bioscience*, 13(4):453–461, 2008.

F. O. Martinez, L. Helming, and S. Gordon. Alternative activation of macrophages: an immunologic functional perspective. *Annual review of immunology*, 27:451–83, Jan. 2009. ISSN 0732-0582. doi: 10.1146/annurev.immunol.021908.132532.

D. Mattei, A. Djodari-Irani, R. Hadar, A. Pelz, L. F. de Cossío, T. Goetz, M. Matyash, H. Kettenmann, C. Winter, and S. a. Wolf. Minocycline rescues decrease in neurogenesis, increase in microglia cytokines and deficits in sensorimotor gating in an animal model of schizophrenia. *Brain, behavior, and immunity*, 38:175–84, May 2014. ISSN 1090-2139. doi: 10.1016/j.bbi.2014.01.019.

J. McGrath, S. Saha, D. Chant, and J. Welham. Schizophrenia: a concise overview of incidence, prevalence, and mortality. *Epidemiologic reviews*, 30:67–76, Jan. 2008. ISSN 0193-936X. doi: 10.1093/epirev/mxn001.

U. Meyer. Prenatal poly(i:C) exposure and other developmental immune activation models in rodent systems. *Biological psychiatry*, 75(4):307–15, Feb. 2014. ISSN 1873-2402. doi: 10.1016/j.biopsych.2013.07.011.

M. Miyanishi, K. Tada, M. Koike, Y. Uchiyama, T. Kitamura, and S. Nagata. Identification of Tim4 as a phosphatidylserine receptor. *Nature*, 450(7168):435–9, Nov. 2007. ISSN 1476-4687. doi: 10.1038/nature06307.

T. Moller, O. Kann, A. Verkhratsky, and H. Kettenmann. Activation of mouse microglial cells affects P2 receptor signaling. *Brain research*, pages 49–59, 2000.

D. M. Mosser and J. P. Edwards. Exploring the full spectrum of macrophage activation. *Nature reviews. Immunology*, 8(12):958–69, Dec. 2008. ISSN 1474-1741. doi: 10.1038/nri2448.

S. Nagata, R. Hanayama, and K. Kawane. Autoimmunity and the clearance of dead cells. *Cell*, 140 (5):619–30, Mar. 2010. ISSN 1097-4172. doi: 10.1016/j.cell.2010.02.014.

I. Napoli and H. Neumann. Microglial clearance function in health and disease. *Neuroscience*, 158(3): 1030–8, Feb. 2009. ISSN 0306-4522. doi: 10.1016/j.neuroscience.2008.06.046.

E. A. Newman. Propagation of intercellular calcium waves in retinal astrocytes and muller cells. *Journal of Neuroscience*, 21(7):2215–2223, 2001.

A. Nimmerjahn, F. Kirchhoff, and F. Helmchen. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science (New York, N.Y.)*, 308(5726):1314–8, May 2005. ISSN 1095-9203. doi: 10.1126/science.1110647.

A. Nimmerjahn, E. a. Mukamel, and M. J. Schnitzer. Motor behavior activates Bergmann glial networks. *Neuron*, 62(3):400–12, May 2009. ISSN 1097-4199. doi: 10.1016/j.neuron.2009.03.019.

K. Ohsawa, Y. Irino, T. Sanagi, Y. Nakamura, E. Suzuki, K. Inoue, and S. Kohsaka. P2Y12 receptor-mediated integrin-beta1 activation regulates microglial process extension induced by ATP. *Glia*, 58 (7):790–801, May 2010. ISSN 1098-1136. doi: 10.1002/glia.20963.

A. Ohta, R. Kini, A. Ohta, M. Subramanian, M. Madasu, and M. Sitkovsky. The development and immunosuppressive functions of CD4(+) CD25(+) FoxP3(+) regulatory T cells are under influence of the adenosine-A2A adenosine receptor pathway. *Frontiers in immunology*, 3(July):190, Jan. 2012. ISSN 1664-3224. doi: 10.3389/fimmu.2012.00190.

J. W. Olney and N. B. Farber. Glutamate receptor dysfunction and schizophrenia. *Archives General Psychiatry*, 52(12):998–1007, 1995.

A. G. Orr, A. L. Orr, X.-J. Li, R. E. Gross, and S. F. Traynelis. Adenosine A(2A) receptor mediates microglial process retraction. *Nature neuroscience*, 12(7):872–8, July 2009. ISSN 1546-1726. doi: 10.1038/nn.2341.

M. Pannell, F. Szulzewsky, V. Matyash, S. a. Wolf, and H. Kettenmann. The subpopulation of microglia sensitive to neurotransmitters/neurohormones is modulated by stimulation with LPS, interferon-$\gamma$, and IL-4. *Glia*, Feb. 2014. ISSN 1098-1136. doi: 10.1002/glia.22633.

R. C. Paolicelli, G. Bolasco, F. Pagani, L. Maggi, M. Scianni, P. Panzanelli, M. Giustetto, T. A. Ferreira, E. Guiducci, L. Dumas, D. Ragozzino, and C. T. Gross. Synaptic pruning by microglia is necessary for normal brain development. *Science (New York, N.Y.)*, 333(6048):1456–8, Sept. 2011. ISSN 1095-9203. doi: 10.1126/science.1202529.

D. Park, A.-C. Tosello-Trampont, M. R. Elliott, M. Lu, L. B. Haney, Z. Ma, A. L. Klibanov, J. W. Mandell, and K. S. Ravichandran. BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module. *Nature*, 450(7168):430–4, Nov. 2007. ISSN 1476-4687. doi: 10.1038/nature06329.

S.-Y. Park, M.-Y. Jung, H.-J. Kim, S.-J. Lee, S.-Y. Kim, B.-H. Lee, T.-H. Kwon, R.-W. Park, and I.-S. Kim. Rapid cell corpse clearance by stabilin-2, a membrane phosphatidylserine receptor. *Cell death and differentiation*, 15(1):192–201, Jan. 2008. ISSN 1350-9047. doi: 10.1038/sj.cdd.4402242.

O. Pascual, S. Ben Achour, P. Rostaing, A. Triller, and A. Bessis. Microglia activation triggers astrocyte-mediated modulation of excitatory neurotransmission. *Proceedings of the National Academy of Sciences of the United States of America*, 109:E197–205, 2011. ISSN 10916490. doi: 10.1073/pnas.1111098109.

K. S. Ravichandran. Beginnings of a good apoptotic meal: the find-me and eat-me signaling pathways. *Immunity*, 35(4):445–455, 2011. doi: 10.1016/j.immuni.2011.09.004.Beginnings.

N. Rebola, R. Lujan, R. A. Cunha, and C. Mulle. Adenosine A2A receptors are essential for long-term potentiation of NMDA-EPSCs at hippocampal mossy fiber synapses. *Neuron*, 57(1):121–34, Jan. 2008. ISSN 0896-6273. doi: 10.1016/j.neuron.2007.11.023.

T. Regen, D. van Rossum, J. Scheffel, M.-E. Kastriti, N. H. Revelo, M. Prinz, W. Brück, and U.-K. Hanisch. CD14 and TRIF govern distinct responsiveness and responses in mouse microglial TLR4 challenges by structural variants of LPS. *Brain, behavior, and immunity*, 25(5):957–70, July 2011. ISSN 1090-2139. doi: 10.1016/j.bbi.2010.10.009.

S. J. Robertson and F. a. Edwards. ATP and glutamate are released from separate neurones in the rat medial habenula nucleus: frequency dependence and adenosine-mediated inhibition of release. *The Journal of Physiology*, 508(3):691–701, May 1998. ISSN 0022-3751. doi: 10.1111/j.1469-7793.1998.691bp.x.

S. C. Robson, J. Sévigny, and H. Zimmermann. The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. *Purinergic signalling*, 2(2):409–30, June 2006. ISSN 1573-9538.

A. Rocha. *E-NTPDase 1 modulation of neuronal and astrocytic activity in the CNS*. PhD thesis, Charite Berlin, 2014.

N. I. Rothwell, G. Luheshi, and S. Toulmond. Cytokines and Their Receptors in the Central Nervous System: Physiology, Pharmacology, and Pathology. *Pharmacology & therapeutics*, 69(2):85–95, 1996.

S. Saha, D. Chant, and J. Mcgrath. A Systematic Review of Mortality in Schizophrenia: Is the differential mortality gap worsening over time? *Archives General Psychiatry*, 64(10):1123–1131, 2007.

E. Scemes and C. Giaume. Astrocyte Calcium waves: what they are and what they do. *Glia*, 54(203): 716–725, 2006. doi: 10.1002/glia.20374.Astrocyte.

V. Scheele. Examen Chemicum Calculi Urinari. *Opuscula*, 2:73, 1776.

J. Scheffel, T. Regen, D. Van Rossum, S. Seifert, S. Ribes, R. Nau, R. Parsa, R. a. Harris, H. W. G. M. Boddeke, H.-N. Chuang, T. Pukrop, J. T. Wessels, T. Jürgens, D. Merkler, W. Brück, M. Schnaars, M. Simons, H. Kettenmann, and U.-K. Hanisch. Toll-like receptor activation reveals developmental reorganization and unmasks responder subsets of microglia. *Glia*, 60(12):1930–43, Dec. 2012. ISSN 1098-1136. doi: 10.1002/glia.22409.

M. R. C. Schetinger, V. M. Morsch, C. D. Bonan, and A. T. S. Wyse. NTPDase and 5'-nucleotidase activities in physiological and disease conditions: new perspectives for human health. *BioFactors (Oxford, England)*, 31(2):77–98, Jan. 2007. ISSN 0951-6433.

K. Schicker, S. Hussl, G. K. Chandaka, K. Kosenburger, J.-W. Yang, M. Waldhoer, H. H. Sitte, and S. Boehm. A membrane network of receptors and enzymes for adenine nucleotides and nucleosides. *Biochimica et biophysica acta*, 1793(2):325–34, Feb. 2009. ISSN 0006-3002. doi: 10.1016/j.bbamcr.2008.09.014.

C. G. Schipke, C. Boucsein, C. Ohlemeyer, F. Kirchhoff, and H. Kettenmann. Astrocyte Ca 2+ waves trigger responses in microglial cells in brain slices. *The FASEB journal*, (6):4–6, 2001.

P. Schuler, M. Harasymczuk, B. Schilling, S. Lang, and T. Whiteside. Separation of human CD4 +CD39 + T cells by magnetic beads reveals two phenotypically and functionally different subsets. *Journal of immunological methods*, 369(1-2):59–68, 2011.

J. Schulte am Esch, J. Sévigny, E. Kaczmarek, J. B. Siegel, M. Imai, K. Koziak, a. R. Beaudoin, and S. C. Robson. Structural elements and limited proteolysis of CD39 influence ATP diphosphohydrolase activity. *Biochemistry*, 38(8):2248–58, Feb. 1999. ISSN 0006-2960. doi: 10.1021/bi982426k.

P. Seeman, J. Schwarz, J. F. Chen, H. Szechtman, M. Perreault, G. S. McKnight, J. C. Roder, R. Quirion, P. Boksa, L. K. Srivastava, K. Yanai, D. Weinshenker, and T. Sumiyoshi. Psychosis pathways converge via D2high dopamine receptors. *Synapse*, 60(4):319–46, 2006.

C.-H. Shieh, A. Heinrich, T. Serchov, D. van Calker, and K. Biber. P2X7-dependent, but differentially regulated release of IL-6, CCL2, and TNF-$\alpha$ in cultured mouse microglia. *Glia*, Jan. 2014. ISSN 1098-1136. doi: 10.1002/glia.22628.

A. Sierra, J. M. Encinas, J. J. P. Deudero, J. H. Chancey, G. Enikolopov, L. S. Overstreet-Wadiche, S. E. Tsirka, and M. Maletic-Savatic. Microglia shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis. *Cell stem cell*, 7(4):483–95, Oct. 2010. ISSN 1875-9777. doi: 10.1016/j.stem.2010.08.014.

A. Sierra, O. Abiega, A. Shahraz, and H. Neumann. Janus-faced microglia: beneficial and detrimental consequences of microglial phagocytosis. *Frontiers in cellular neuroscience*, 7(January):6, Jan. 2013. ISSN 1662-5102. doi: 10.3389/fncel.2013.00006.

J. Steiner, C. Mawrin, A. Ziegeler, H. Bielau, O. Ullrich, H.-G. Bernstein, and B. Bogerts. Distribution of HLA-DR-positive microglia in schizophrenia reflects impaired cerebral lateralization. *Acta neuropathologica*, 112(3):305–16, Sept. 2006. ISSN 0001-6322. doi: 10.1007/s00401-006-0090-8.

W. J. Streit, S. A. Walter, and N. A. Pennell. Reactive microgliosis. *Progress in neurobiology*, 57: 563–581, 1999.

M. Tanaka, P.-Y. Shih, H. Gomi, T. Yoshida, J. Nakai, R. Ando, T. Furuichi, K. Mikoshiba, A. Semyanov, and S. Itohara. Astrocytic Ca2+ signals are required for the functional integrity of tripartite synapses. *Molecular brain*, 6(1):6, Jan. 2013. ISSN 1756-6606. doi: 10.1186/1756-6606-6-6.

A. B. Tonchev, T. Yamashima, L. Zhao, and H. Okano. Differential proliferative response in the postischemic hippocampus, temporal cortex, and olfactory bulb of young adult macaque monkeys. *Glia*, 42(3):209–24, May 2003. ISSN 0894-1491. doi: 10.1002/glia.10209.

M.-E. Tremblay and A. K. Majewska. A role for microglia in synaptic plasticity? *Communicative & integrative biology*, 4(2):220–2, Mar. 2011. ISSN 1942-0889. doi: 10.4161/cib.4.2.14506.

L. A. Truman, C. A. Ford, M. Pasikowska, J. D. Pound, S. J. Wilkinson, I. E. Dumitriu, L. Melville, L. A. Melrose, C. A. Ogden, R. Nibbs, G. Graham, C. Combadiere, and C. D. Gregory. CX3CL1/fractalkine is released from apoptotic lymphocytes to stimulate macrophage chemotaxis. *Blood*, 112(13):5026–36, Dec. 2008. ISSN 1528-0020. doi: 10.1182/blood-2008-06-162404.

G. Tsai, H. Y. Lane, P. Yang, M. Y. Chong, and N. Lange. Glycine transporter I inhibitor, N-methylglycine (sarcosine), added to antipsychotics for the treatment of schizophrenia. *Biological psychiatry*, 55(5):452–6, 2004.

N. A. Uranova, I. S. Zimina, O. V. Vikhreva, N. O. Krukov, V. I. Rachmanova, and D. D. Orlovskaya. Ultrastructural damage of capillaries in the neocortex in schizophrenia. *The world journal of biological psychiatry*, 11(3):567–578, Mar. 2010.

B. N. van Berckel, M. G. Bossong, R. Boellaard, R. Kloet, A. Schuitemaker, E. Caspers, G. Luurtsema, A. D. Windhorst, W. Cahn, A. a. Lammertsma, and R. S. Kahn. Microglia activation in recent-onset schizophrenia: a quantitative (R)-[11C]PK11195 positron emission tomography study. *Biological psychiatry*, 64(9):820–2, Dec. 2008. ISSN 1873-2402. doi: 10.1016/j.biopsych.2008.04.025.

K. Van den Eynde, S. Missault, E. Fransen, L. Raeymaekers, R. Willems, W. Drinkenburg, J.-P. Timmermans, S. Kumar-Singh, and S. Dedeurwaerdere. Hypolocomotive behaviour associated with increased microglia in a prenatal immune activation model with relevance to schizophrenia. *Behavioural brain research*, 258:179–86, Jan. 2014. ISSN 1872-7549. doi: 10.1016/j.bbr.2013.10.005.

J. van Os and S. Kapur. Schizophrenia. *Lancet*, 374(9690):635–45, Aug. 2009. ISSN 1474-547X. doi: 10.1016/S0140-6736(09)60995-8.

R. Ventura and K. M. Harris. Three-dimensional relationships between hippocampal synapses and astrocytes. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 19 (16):6897–906, Aug. 1999. ISSN 1529-2401.

J. P. Vivian, P. Riedmaier, H. Ge, J. Le Nours, F. M. Sansom, M. C. J. Wilce, E. Byres, M. Dias, J. W. Schmidberger, P. J. Cowan, A. J. F. D'Apice, E. L. Hartland, J. Rossjohn, and T. Beddoe. Crystal structure of a Legionella pneumophila ecto -triphosphate diphosphohydrolase, a structural and functional homolog of the eukaryotic NTPDases. *Structure*, 18(2):228–38, Feb. 2010. ISSN 1878-4186. doi: 10.1016/j.str.2009.11.014.

H. Wake, A. J. Moorhouse, S. Jinno, S. Kohsaka, and J. Nabekura. Resting microglia directly monitor the functional state of synapses in vivo and determine the fate of ischemic terminals. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 29(13):3974–80, Apr. 2009. ISSN 1529-2401. doi: 10.1523/JNEUROSCI.4363-08.2009.

W. Walz and L. Hertz. Functional interactions between neurons and astrocytes. *Progress in neurobiology*, 20:133–183, 1983.

F. Wang, N. A. Smith, Q. Xu, T. Fujita, A. Baba, T. Matsuda, T. Takano, L. Bekar, and M. Nedergaard. Astrocytes modulate neural network activity by Ca2+- dependent uptake of extracellular K+. *Science signaling*, 5(218), 2012. doi: 10.1126/scisignal.2002334.Astrocytes.

T. Wang and G. Guidotti. CD39 Is an Ecto-(Ca2+,Mg2+)-apyrase. *Journal of Biological Chemistry*, 271(17):9898–9901, Apr. 1996. ISSN 00219258. doi: 10.1074/jbc.271.17.9898.

C. Winter, T. J. Reutiman, T. D. Folsom, R. Sohr, R. J. Wolf, G. Juckel, and S. H. Fatemi. Dopamine and serotonin levels following prenatal viral infection in mouse–implications for psychiatric disorders such as schizophrenia and autism. *European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology*, 18(10):712–6, Oct. 2008. ISSN 0924-977X. doi: 10.1016/j.euroneuro.2008.06.001.

C. Winter, A. Djodari-Irani, R. Sohr, R. Morgenstern, J. Feldon, G. Juckel, and U. Meyer. Prenatal immune activation leads to multiple changes in basal neurotransmitter levels in the adult brain: implications for brain disorders of neurodevelopmental origin such as schizophrenia. *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum (CINP)*, 12(4):513–24, May 2009. ISSN 1469-5111. doi: 10.1017/S1461145708009206.

J. Xue, S. V. Schmidt, J. Sander, A. Draffehn, W. Krebs, I. Quester, D. De Nardo, T. D. Gohel, M. Emde, L. Schmidleithner, H. Ganesan, A. Nino-Castro, M. R. Mallmann, L. Labzin, H. Theis, M. Kraut, M. Beyer, E. Latz, T. C. Freeman, T. Ulas, and J. L. Schultze. Transcriptome-based network analysis reveals a spectrum model of human macrophage activation. *Immunity*, 40(2): 274–88, Feb. 2014. ISSN 1097-4180. doi: 10.1016/j.immuni.2014.01.006.

B. K. Yee, E. Balic, P. Singer, C. Schwerdel, T. Grampp, L. Gabernet, I. Knuesel, D. Benke, J. Feldon, H. Mohler, and D. Boison. Disruption of glycine transporter 1 restricted to forebrain neurons is associated with a procognitive and antipsychotic phenotypic profile. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 26(12):3169–81, Mar. 2006. ISSN 1529-2401. doi: 10.1523/JNEUROSCI.5120-05.2006.

G. G. Yegutkin. Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. *Biochimica et biophysica acta*, 1783(5):673–94, May 2008. ISSN 0006-3002. doi: 10.1016/j.bbamcr.2008.01.024.

R. F. Zanin, E. Braganhol, L. S. Bergamin, L. F. I. Campesato, A. Z. Filho, J. C. F. Moreira, F. B. Morrone, J. Sévigny, M. R. C. Schetinger, A. T. de Souza Wyse, and A. M. O. Battastini. Differential macrophage activation alters the expression profile of NTPDase and ecto-5'-nucleotidase. *PloS one*, 7(2):e31205, Jan. 2012. ISSN 1932-6203. doi: 10.1371/journal.pone.0031205.

Z. Zhang, G. Chen, W. Zhou, A. Song, T. Xu, Q. Luo, W. Wang, X.-s. Gu, and S. Duan. Regulated ATP release from astrocytes through lysosome exocytosis. *Nature cell biology*, 9(8):945–53, Aug. 2007. ISSN 1465-7392. doi: 10.1038/ncb1620.

Y. Zhou, J. N. Murthy, D. Zeng, L. Belardinelli, and M. R. Blackburn. Alterations in adenosine metabolism and signaling in patients with chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis. *PloS one*, 5(2):e9224, Jan. 2010. ISSN 1932-6203. doi: 10.1371/journal.pone.0009224.

H. Zimmermann, A. R. Beaudoin, M. Bollen, J. W. Goding, G. Guidotti, T. L. Kirley, S. C. Robson, and K. Sano. Proposed nomenclature for two novel nucleotide hydrolyzing enzyme families expressed on the cell surface. In L. Vanduf and R. Lemmens, editors, *Proceedings of the Second International Workshop on Ecto- ATPases and Related Ectonucleotidases, Diepenbeek, Belgium*. Shaker Publishing BV, Maastricht, The Netherlands, 1999.

H. Zimmermann, S. Mishra, V. Shukla, D. Langer, K. Gampe, I. Grimm, J. Delic, and N. Braun. Ecto-nucleotidases, molecular properties and functional impact. *An. R. Acad. Nac. Farm.*, pages 537–566, 2007.

L. Zuckerman, M. Rehavi, R. Nachman, and I. Weiner. Immune activation during pregnancy in rats leads to a postpubertal emergence of disrupted latent inhibition, dopaminergic hyperfunction, and altered limbic morphology in the offspring: a novel neurodevelopmental model of schizophrenia. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, 28(10):1778–89, Oct. 2003. ISSN 0893-133X. doi: 10.1038/sj.npp.1300248.

# Curriculum vitae

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

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

# List of communications

## Publications

***Georgieva PB**, Jordan P, Weinheimer V, Delgado-Martinez I, Robson S, Wolf SA, Matyash V, and Kettenmann H.* E-NTPDase1 is an important regulator of autocrine/paracrine purinergic signalling in microglia (in preparation)

***Georgieva PB**, Szulzewsky F, Hagen A, Wolf SA and Kettenmann H.* Microglia from a mouse model of schizophrenia show an impaired response in ATP dependent functions (in preparation).

*W Schaafsma, X Zhang, KC van Zomeren, S Jacobs,* ***PB Georgieva**, SA Wolf, H Kettenmann, H Janova, U-K Hanisch, PJ van den Elsen, N Brouwer, HWGM Boddeke and BJL Eggen.* Long-lasting inflammatory suppression of microglia by LPS-preconditioning is mediated by RelB-induced epigenetic silencing. (submitted)

*Richter N, Wendt S,* ***Georgieva PB**, Hambardzumyan D, Nolte C, and Kettenmann H, 2014.* Glioma-associated microglia and macrophages/monocytes display distinct electrophysiological properties and do not communicate via gap junctions. Neurosci Let 2014; doi: 10.1016.

*Vinnakota K, Hu F, Ku MC,* ***Georgieva PB**, Szulzewsky F, Pohlmann A, Waiczies S, Waiczies H, Niendorf T, Lehnardt S, Hanisch UK, Synowitz M, Markovic D, Wolf SA, Glass R, Kettenmann H, 2013.* Toll-like receptor 2 mediates microglia/brain macrophage MT1-MMP expression and glioma expansion. Neuro Oncol 2013;15(11):1457-68.

*Raykov Z,* ***Georgieva PB**, Angelova A, Galabov AS and Rommelaere J, 2009.*Anticancer effects of an oncolytic parvovirus combined with non-conventional therapeutics on pancreatic carcinoma cell lines. Acta Virol 2009;53(1):57-60.

*Degen J, Kirbach A, Reiter L, Lehmann K, Norton P, Storms M, Koblofsky M, Sarah,* ***Georgieva PB**, Nguyen H, Chamkhi H, Greggers U and Menzel R.* Bees apply effective exploration strategies during their orientation flights (submitted)

## Poster presentations

***PB Georgieva**, L Bulavina, V Matyash, S Wolf and H Kettenmann.* CD39 expression is down-regulated in activated microglia (2013). 9th International PhD Symposium Berlin Brain Days, Berlin, Germany

***PB Georgieva**, L Bulavina, V Matyash, S Wolf and H Kettenmann.* CD39 expression is down-regulated in activated microglia (2013). 15th MDC/FMP PhD retreat, Kremmen, Germany.

***PB Georgieva**, L Bulavina, A Rocha, S Wolf, V Matyash and H Kettenmann.* Distinct CD39 expression and activity in different activation states in microglia.(2013) 10th Gttingen Meeting of the German Neuroscience Society, Gttingen, Germany.

*Chakroborty NK,* ***Georgieva PB*** *and Menzel R.* Odour coding in projection neurons of Apis mellifera changes under olfactory adaptation: A calcium imaging study. Poster presentation (2010). Proceedings of the International Symposium in Honeybee Neuroscience, Berlin, Germany (pp.79).