Chemogenetic Manipulation of Microglia: Effects on neuronal structure and function

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Marie-Luise Brehme

Hamburg, Germany

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The following evaluators recommend the admission of the dissertation:

Thesis:

Prof. Dr. Thomas Oertner Institute of Synaptic Neuroscience The Center of Molecular Neurobiology Hamburg Fakultät für Mathematik, Informatik und Naturwissenschaften

Prof. Dr. med. Manuel A. Friese Institute for Neuroimmunology and Multiple Sclerosis The Center of Molecular Neurobiology Hamburg

Oral defense: 12.05.2025

Prof. Dr. Thomas Oertner Institute of Synaptic Neuroscience The Center of Molecular Neurobiology Hamburg Fakultät für Mathematik, Informatik und Naturwissenschaften

Prof. Dr. Matthias Kneussel Institute for Molecular Neurogenetics The Center of Molecular Neurobiology Hamburg

Priv.-Doz. Dr. med. Mathias Gelderblom Kopf-und Neurozentrum Universitätsklinikum Hamburg-Eppendorf

Prof. Dr. Baris Tursun Molekulare Zellbiologie der Tiere Fakultät für Mathematik, Informatik und Naturwissenschaften

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Summary

Microglia, the resident immune cells of the central nervous system (CNS), play a critical role in maintaining homeostasis, immune surveillance, and modulating neuronal function. They are highly dynamic cells capable of responding to environmental changes by altering their morphology and function. Recent research has highlighted that microglia are not only involved in immune responses but also play a pivotal role in synaptic plasticity, synaptic pruning, and overall cognitive processes. Activation of microglia, whether by inflammatory processes or chronic diseases, has profound consequences on neurons and synapses, leading to alterations in synaptic strength, connectivity, and ultimately cognitive functions. However, the detailed mechanisms underlying microglia-neuron communication remain under active investigation.

Traditional methods to activate microglia, such as pharmacological agents like lipopolysaccharide (LPS) or genetic approaches including global gene knockout models, can be non-specific and affect multiple cell types, making it difficult to isolate the direct role of microglia in neuronal modulation. These methods also typically lack precise timing, further complicating our understanding of microglia's direct influence. To address these challenges, I took a more targeted approach by directly activating microglia using a chemogenetic tool to target the Gq signaling pathway.

In this dissertation, I employed a chemogenetic strategy with the tamoxifen-inducible Cre-ER loxP system to selectively express Gq-DREADD (Designer Receptors Exclusively Activated by Designer Drugs) in microglia. This approach allowed us to activate microglia in a controlled and targeted way, avoiding unwanted effects on other brain cells. The activation of the Gq pathway led to changes in microglial morphology, specifically in reduced branching complexity and increased intracellular calcium levels suggesting a shift in their functional state. Additionally, the chronic Gq-DREADD activation induced a decrease in the number of excitatory synapses on CA1 pyramidal neurons., which was found to be dependent on brain-derived neurotrophic factor (BDNF) signaling. As well as a reduction in long-term potentiation (LTP) in the hippocampus, which is a key mechanism for synaptic plasticity and memory.

Despite these significant changes at the synaptic level, the mice with activated microglia initially performed well in learning and memory tasks, such as locating a hidden platform in the Morris water maze. However, in the following days, their ability to retain spatial information declined compared to controls, indicating that while learning was unaffected, the stability of the memories formed was compromised.

Our findings show that selective activation of microglia can negatively impact synaptic plasticity and memory retention without inducing other overt symptoms of sickness. This method offers a valuable tool for studying the effects of microglial activation on neuronal function with high specificity and precise timing.

Zusammenfassung

Mikroglia, sind die Immunzellen des zentralen Nervensystems (ZNS) und spielen eine entscheidende Rolle bei der Aufrechterhaltung der Homöostase, der Immunüberwachung und der Modulation der neuronalen Funktion. Sie sind hochdynamische Zellen, die in der Lage sind, auf Veränderungen im ZNS zu reagieren, indem sie ihre Morphologie und Funktion anpassen. Jüngste Forschungen haben gezeigt, dass Mikroglia nicht nur an Immunreaktionen beteiligt sind, sondern auch eine zentrale Rolle bei der synaptischen Plastizität, dem synaptischen "Pruning" und bei kognitiven Prozessen spielen. Die Aktivierung von Mikroglia, sei es durch entzündliche Prozesse oder chronische Erkrankungen, hat tiefgreifende Auswirkungen auf Neuronen und Synapsen, was zu Veränderungen in der synaptischen Stärke, der Konnektivität und letztlich der kognitiven Funktionen führt. Die genauen Mechanismen der Mikroglia-Neuron-Kommunikation sind jedoch noch Gegenstand aktiver Forschung.

Traditionelle Methoden zur Aktivierung von Mikroglia, wie der Einsatz von pharmakologischen Wirkstoffen wie Lipopolysaccharide oder genetische Ansätze wie globale Gen-Knockout-Modelle, können unspezifisch sein und mehrere Zelltypen beeinflussen, was es schwierig macht, die direkte Rolle von Mikroglia bei der neuronalen Modulation zu isolieren. Diese Methoden sind auch oft nicht präzise in Bezug auf den Zeitpunkt der Aktivierung, was unser Verständnis des direkten Einflusses von Mikroglia weiter erschwert. Um diesen Herausforderungen zu begegnen, habe ich einen gezielteren Ansatz gewählt, indem ich Mikroglia direkt mithilfe chemogenetischer Werkzeuge zur Aktivierung des Gq-Signalwegs aktiviert habe.

In dieser Dissertation habe ich eine chemogenetische Strategie mit dem tamoxifeninduzierbaren Cre-ER loxP-System verwendet, um Gq-DREADD (Designer Receptors Exclusively Activated by Designer Drugs) selektiv in Mikroglia zu exprimieren. Dieser Ansatz ermöglichte es uns, Mikroglia auf eine kontrollierte und gezielte Weise zu aktivieren, wodurch unerwünschte Effekte auf andere Gehirnzellen vermieden wurden. Die Aktivierung des Gq-Signalwegs führte zu bemerkenswerten Veränderungen in der Morphologie der Mikroglia, insbesondere zu einer verringerten Verzweigungskomplexität und erhöhten intrazellulären Kalziumspiegeln, was auf eine Veränderung ihres funktionellen Zustands hinweist. Darüber hinaus führte die chronische Gq-DREADD-Aktivierung zu einer Verringerung der Anzahl exzitatorischer Synapsen an CA1-Pyramidenneuronen, was sich als abhängig von der Signalgebung des Gehirn-abgeleiteten neurotrophen Faktors (BDNF) herausstellte. Außerdem kam es zu einer Reduktion der Langzeitpotenzierung (LTP) im Hippocampus, einem Schlüsselmechanismus für die synaptische Plastizität und das Gedächtnis.

Trotz dieser signifikanten Veränderungen auf der synaptischen Ebene zeigten die Mäuse mit aktivierten Mikroglia zunächst gute Leistungen bei Lern- und Gedächtnisaufgaben, wie zum Beispiel beim Auffinden einer versteckten Plattform im Morris-Wasserlabyrinth. In den darauffolgenden Tagen verschlechterte sich jedoch ihre Fähigkeit, räumliche Informationen zu behalten, im Vergleich zu den Kontrolltieren. Dies deutet darauf hin, dass das Lernen zwar unbeeinträchtigt war, die Stabilität der gebildeten Erinnerungen jedoch beeinträchtigt wurde.

Unsere Ergebnisse zeigen, dass die selektive Aktivierung von Mikroglia die synaptische Plastizität und die Gedächtnisstabilität negativ beeinflussen kann, ohne andere offensichtliche Krankheitssymptome auszulösen. Diese Methode bietet ein wertvolles Werkzeug, um die Auswirkungen der Mikroglia-Aktivierung auf die neuronale Funktion mit hoher Spezifität und präzisem Timing zu untersuchen.

1. Introduction

1.1. Synaptic function and plasticity in the hippocampus

1.1.1. The hippocampus

The hippocampus is a brain structure involved in the formation, organization, and storage of memories, as well as spatial navigation and contextual learning (Andersen, 2006). Its unique anatomy and circuitry make it an ideal model for studying synaptic plasticity, a fundamental process for learning and memory. The hippocampus has been studied for over a century, with significant advancements in understanding its function and structure.

Located in the medial temporal lobe of the brain, the hippocampus is part of the limbic system, associated with memory. It includes several interconnected regions: the hippocampus proper, also known as cornu ammonis (CA) areas (CA1, CA2, and CA3), the dentate gyrus (DG), and the subiculum (Figure 1). The different subregions of the hippocampus play distinct roles in memory processes. The DG is primarily involved in pattern separation, which enables distinguishing between similar experiences or environmental context (Leutgeb et al., 2007). The CA3 region acts as an auto-associative network, crucial for enabling pattern completion (Rolls, 2013). The CA1 region is crucial for the encoding of spatial and episodic memories and is extensively involved in in long-term potentiation (LTP), a major form of synaptic plasticity linked to memory formation (Nakazawa et al., 2003). The subiculum serves as the main output structure of the hippocampus, transmitting processed information to other parts of the brain (O'Mara, 2005) All these subregions are organized into a well-defined circuit, known as the trisynaptic pathway, which is critical in memory processing and spatial navigation (Amaral & Witters, 1989; Teyler & Discenna, 1984). The trisynaptic circuit begins with input from the entorhinal cortex, which projects via the perforant path to the granule cells in the DG. From there, mossy fibers transmit signals to the pyramidal neurons in the CA3 region, and subsequently, Schaffer collaterals carry the information to the pyramidal neurons in the CA1 region (Bliss & Lømo, 1973; O'Keefe & Nadel, 1979).



Figure 1: The trisynaptic circuit in the hippocampus. Illustration of the trisynaptic circuit in the hippocampus, showing the Dentate Gyrus (DG), CA3, CA2, CA1, and Subiculum. The DG receives input (red line) from the entorhinal cortex (not shown) and sends excitatory input through mossy fibers (orange) to the CA3 region (blue). CA3 projects to CA1 (green) via the Schaffer collateral pathway. Finally, CA1 projects to the Subiculum, the main output region. Arrows indicate signal flow direction. (Figure created with BioRender.com)

The efficiency of this circuit relies on the precise layering of the different cell types. Granule cells in the DG and pyramidal neurons in the CA regions are organized into distinct layers such as the *stratum oriens*, *stratum pyramidale*, and *stratum radiatum* (Andersen et al., 1971;Amaral & Witters, 1989). Each layer supports a specific aspect of signal processing: the *stratum oriens* contains the basal dendrites of the pyramidal cells, the *stratum pyramidale* houses the cell bodies of the pyramidal neurons, and the *stratum radiatum* contains the apical dendrites, which receive the synaptic input (Amaral & Witters, 1989).

Due to its well-defined anatomy, highly organized circuitry, and robust synaptic connections, the hippocampus is extensively used as a model for studying synaptic transmission and synaptic plasticity. These characteristics make it amenable to precise experimental manipulation and observation, allowing detailed investigation of the mechanism at the synaptic level, which are essential for learning and memory (Andersen, 2006).

1.1.2. The chemical synapse and synaptic transmission

Synapses are specialized neuronal structures responsible for transmitting information between neurons. At the chemical synapse this transmission relies on the release and diffusion of a neurotransmitter from the presynaptic compartment (bouton) to the postsynaptic compartment (dendritic spine). This process begins when an action potential reaches the presynaptic site causing a change in the membrane potential. This change subsequently opens the voltage gated calcium channels, allowing a rapid influx of the Ca²⁺ influx enables the fusion of neurotransmitter-containing synaptic vesicles with the

membrane at the active zone, resulting in the release of neurotransmitters into the synaptic cleft (Südhof, 2012). The neurotransmitter, primarily glutamate and gamma-aminobutyric acid (GABA), diffuses across the synaptic cleft, binds to specific receptors on the membrane of the



Figure 2: Simplified chemical synaptic transmission. The presynaptic terminal releases neurotransmitters in response to an action potential and calcium (Ca²⁺) influx. The neurotransmitters then cross the synaptic cleft and bind to ionotropic and metabotropic receptors on the postsynaptic terminal, leading to changes in membrane potential and activation of biochemical cascades (Figure from Pereda, 2014).

postsynaptic neuron and modulates the electrical state (Fröhlich, 2016). Under typical conditions, glutamate mediates excitatory synaptic transmission, whereas GABA mediates inhibitory synaptic transmission (Figure 2).

This study focuses on excitatory synapses. In these synapses, glutamate is released by the presynaptic compartment and binds to ionotropic glutamate receptor on the postsynaptic membrane. There are three different types of ionotropic receptors for glutamate: the α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), the *N* -methyl-D-aspartate (NMDA) and the kainic acid receptors (Traynelis et al., 2010).

Upon glutamate binding, AMPA receptors mediate fast synaptic transmission by allowing an influx of positively charged ions such as Na⁺, leading to rapid depolarization of the postsynaptic neuron. This initial depolarization subsequently removes the voltage-dependent magnesium (Mg²⁺) block from NMDA receptor, allowing them to be activated by glutamate binding. Once activated, NDMA receptor allows the influx of Na⁺ and Ca²⁺ ions. The Ca²⁺ acts as a secondary messenger, triggering signaling cascades that lead to changes in synaptic strength. This delayed activation allows NMDA receptors to act as "coincidence detectors," meaning they

require simultaneous presynaptic and postsynaptic activity to function, which ensures that synaptic strengthening occurs only at active synapses (Malenka & Bear, 2004).

In addition to ionotropic receptors, glutamate also binds to metabotropic receptors, which mediate their effects through a G protein and belong to the family of G-protein-coupled receptors (GPCRs). The metabotropic glutamate receptors (mGluRs) are classified into three groups: Group I (mGluR1 and 5), Group II (mGluR2 and 3) and Group III (mGluR4, 6, 7, and 8). These mGluRs are implicated in diverse functions. The mGluR1 and mGluR5 are often located at the postsynapse and mediate the excitation in response to glutamate binding (Ferraguti & Shigemoto, 2006) Besides modulating the electrical state of the postsynaptic neuron, metabotropic receptors also activate signaling pathways that regulates various cellular processes such as synaptic plasticity, gene expression and apoptosis (Ferraguti & Shigemoto, 2006)

Synaptic transmission can be influenced by neural activity, resulting in changes in the strength and efficiency of synapses. This adaptive capability, known as synaptic plasticity, is fundamental to learning and memory.

1.1.3. Synaptic plasticity

Synaptic plasticity is the ability of synapses to change their strength and efficiency in response to neuronal activity and experience. This process was first described by Donald Hebb in 1949 and is fundamental for learning and memory. Hebbian theory, often summarized as "cells that fire together wire together," describes how coordinated activity between neurons strengthens their connections. This concept provides the theoretical foundation for LTP and synaptic plasticity (Hebb, 1949) The first experimental evidence for synaptic plasticity was demonstrated by the work of Bliss and Lømo in 1973, who discovered LTP, an increase in synaptic strength following a high frequency stimulation in the hippocampus of rabbits (Bliss & Lømo, 1973)

Synaptic plasticity encompasses various forms, including short-term plasticity (STP), LTP and long-term depression (LTD). STP refers to transient changes in synaptic strength that occur over milliseconds to minutes and is primarily due to presynaptic mechanism such as changes in neurotransmitter release probability and vesicle depletion (Citri & Malenka, 2008; Zucker & Regehr, 2002). These rapid, reversible changes are crucial for synaptic computation and neural network dynamics but do not involve long-lasting structural changes.

In contrast, long-term plasticity, which includes LTP and LTD leads to persistent changes in synaptic efficacy lasting from hours to days and longer. While LTP is an increase in synaptic

strength, LTD is a prolonged decrease in synaptic efficacy. Both processes involve complex molecular mechanisms and changes at both the pre- and postsynaptic compartments (Citri & Malenka, 2008). LTD can be induced through various mechanisms, such as NMDA receptordependent LTD, which involves moderate calcium influx, or mGluR-dependent LTD, which is mediated by metabotropic glutamate receptors (Collingridge et al., 2010).

At the presynaptic terminal in the hippocampus, LTP involves increased neurotransmitter release, due to a rise in the number of releasable synaptic vesicle. This process is often regulated by a retrograde signaling molecule like nitric oxide (NO) and brain-derived neurotrophic factor (BDNF), which are released from the postsynaptic neuron. (Citri & Malenka, 2008).

The most studied type of LTP in the hippocampus is NMDAR-dependent LTP. Here, a highfrequency stimulation of a synapse leads to the activation of NMDA receptors, resulting in an influx of calcium ions into the postsynaptic neuron. This influx activates several signaling pathways and molecular mechanisms. Calcium ions bind to calmodulin, forming a calcium/calmodulin complex, which activates calcium/calmodulin-dependent protein kinase II (CaMKII) and PKC. CaMKII phosphorylates various target proteins, including AMPA receptors,



Figure 3: NMDAR-dependent longterm potentiation. Glutamate (Glu), released by the presynaptic terminal, binds to NMDA receptors (NMDAR) on the postsynaptic dendrite. This leads to a calcium (Ca²⁺) influx, activating CaMKII and triggering a signaling cascade that results in the AMPA insertion of additional (AMPAR) receptors into the postsynaptic membrane. The increased AMPA receptors enhance synaptic strength, a hallmark of LTP activation. Figure adapted from "NMDAR-dependent long-term potentiation (LTP), by BioRender.com (2024) and based on (Kauer & Malenka, 2007).

enhancing their conductance and promoting their insertion into the postsynaptic membrane (Citri & Malenka, 2008)(Figure 3). This trafficking and insertion of AMPA receptors into the postsynaptic membrane are essential for the expression of LTP.

The increase of AMPA receptors at the synapse enhances synaptic transmission by allowing more sodium ions to enter the postsynaptic neuron during synaptic activity. This process is also mediated by scaffolding proteins, including postsynaptic density protein 95 (PSD95), which anchors AMPA receptors to the postsynaptic density and stabilizes them at synapses (Keith & El-Husseini, 2008). PSD95 clusters glutamate receptors, ion channels, and signaling molecules at the synapse, which is essential for synaptic transmission and plasticity. Due to its specific localization and role in excitatory synaptic function, PSD95 is often used as a marker for excitatory synapses (Keith & El-Husseini, 2008).

Synaptic plasticity provides the cellular foundation for learning and memory, allowing experiences to reshape neuronal circuits. The persistent changes in synaptic strength, such as LTP and LTD, are linked to long-lasting modifications in neuronal function and connectivity (Bliss & Collingridge, 1993; Citri & Malenka, 2008).

1.1.4. Memory

Memory is a cognitive process that allows organisms to encode, store and retrieve information, ultimately shaping behavior, learning and survival. It can be broadly divided into short-term memory (STM) and long-term memory (LTM), each serving different functions (Squire, 2004). One of the most well-known cases in the study of memory is that of Henry Molaison, who underwent bilateral removal of his hippocampi to alleviate epilepsy. This procedure resulted in severe anterograde amnesia, profoundly impairing his ability to form new long-term memories, while sparing his short-term memory. This case offered crucial insights into the role of the hippocampus in memory formation, highlighting the difference between short-term memory and long-term memory (Scoviille & Milner, 1957).

STM serves as a temporary holding system, characterized by limited capacity and short duration, typically lasting seconds to minutes unless actively maintained. One key mechanism underlying STM is the concept of reverberating circuits. These circuits involve sustained activity within a network of interconnected neurons, allowing information to be temporarily retained through feedback loops. (Hebb, 1949; Fuster & Alexander, 1971). This mechanism is particularly important in the prefrontal cortex (Fuster & Alexander, 1971). Additionally, phosphorylation of synaptic proteins and neurotransmitter release such as glutamate and

dopamine contribute to enhancing synaptic strength temporarily, supporting STM (Sweatt, 2010; Goldman-Rakic, 1995).

In contrast, long-term memory involves the stable storage of information over extended periods, from hours to a lifetime and is crucial for learning and behavior adaptation (Squire, 2004). LTM can be divided into declarative and non-declarative memory. Declarative memory involves conscious recall of facts and evens, which both rely heavily on the hippocampus, whereas the non-declarative memory involves skills and conditioned responses and depends on other brain regions such as the striatum and cerebellum (Squire, 2004).

This process of transferring information from STM to LTM is known as consolidation. Consolidation occurs in two phases: synaptic consolidation, which involves changes at the synapses over minutes to hours and system consolidation, which involves the reorganization of memories from the hippocampus to the cortex over extended periods (Dudai, 2004). Synaptic plasticity, especially LTP, is essential for memory consolidation (Bliss & Lømo, 1973) This process relies on gene transcription and protein synthesis to stabilize synaptic changes (Kandel, 2001). Spatial memory is a specific type of LTM, allowing organisms to navigate and understand their environment by remembering the spatial relationship between objects and landmarks. This type of memory is dependent on the hippocampus, which plays a key role in encoding and retrieving spatial information (Bird & Burgess, 2008).

While memory processes largely rely on the function of the hippocampus and synaptic plasticity, other key players in the brain are involved in maintaining and modifying these processes. Especially, microglia, the resident immune cells of the CNS, have been recognized for their influence on synaptic plasticity and, consequently, on learning and memory (M. Ě. Tremblay et al., 2010).

1.2. Microglia in the CNS

1.2.1. The history of microglia

The study of microglia began back in the early 20th century. Pio del Rio-Hortega, a Spanish neuroanatomist, made the initial discovery of these cells. Through the use of silver carbonate staining techniques, he was able to distinguish microglia from other glia cells in the CNS (P. D. Río-Hortega, 1919). In the following years, studies of microglia mainly focused on their morphology and distribution. They were described as small cells with a rod-shaped nuclei and highly ramified processes (P. del Río-Hortega, 1928). The functional role of microglia remained largely speculative during this early phase. The first findings, suggesting that microglia were

active participants in brain immune defense, were published by Wilder Penfield. He demonstrated that microglia proliferate and transform into a phagocytotic cell in response to brain lesions. Using a model of experimentally induced brain injury, Penfield observed that microglia were capable of migrating to the site of injury, undergoing morphological changes from a ramified to an amoeboid state, and engaging in the phagocytosis of cellular debris. This work laid the foundation for understanding microglia as dynamic and responsive cells, capable of adapting their morphology and behavior meet the ever-changing needs of the CNS environment (Penfield W., 1925).

In the latter half of the 20th and early 21st centuries, advancements in molecular and cellular biology techniques transformed the understanding of microglia and their function. Beside their role as immune cells that respond to infection, debris clearing and phagocytosis, microglia have been found to play an active part in the pathogenesis of conditions such as Alzheimer's disease, Parkinson's disease, and Multiple Sclerosis, where they can have both protective and detrimental effects (Heneka et al., 2014; Kettenmann et al., 2011; Streit et al., 1999).

However, several studies have shown that microglia have a wide range of additional functions beyond their traditional immune role, for example that they are involved in synaptic pruning, shaping neural circuits, and influencing neuronal survival and activity through close contact and signaling in the homeostatic brain (Cserép et al., 2020; Hanisch & Kettenmann, 2007; Paolicelli et al., 2011; Schafer et al., 2012). The role of microglia in synaptic plasticity has been shown to be highly context-dependent, with evidence supporting both synapse elimination and synapse formation. During brain development, microglia were shown to be essential for synaptic pruning, helping to eliminate weaker synapses and thereby refine neural circuits (Paolicelli et al., 2011). Other studies have reported that microglia seem to promote synapse formation and enhance connectivity though direct contact with dendrites, indicating a role for microglia in synaptogenesis (Miyamoto et al., 2016).

These seemingly contradictory roles, supporting both synapse elimination and formation highlights the complexity of microglial function and the critical impact of the specific experimental context. For instance, microglial behavior can vary significantly based on the developmental stage, the type of stimulus they receive and the health of the surrounding environment. Experimental settings, such as *in vivo* imaging versus *in vitro* slice cultures, also play a significant role in shaping the observed outcomes. These differences make it challenging to draw broad conclusions about microglial function, and they highlight the need for more standardized approaches to better understand their roles in synaptic remodeling. Consequently, while microglia are commonly recognized as key players in synaptic regulation,

further research is necessary to clarify the conditions under which they contribute to synaptic formation versus elimination and how these processes influence CNS health.

1.2.2. Microglia physiology and function

Microglia are specialized immune cells of the CNS, that originate from primitive macrophages in the yolk sac during early embryonic development. These primitive macrophages infiltrate the brain around embryonic day 8.5 (E8.5) in mice and begin to populate the developing neuroepithelium by E9.5. This early infiltration is crucial for establishing their niche and influencing early brain development (Ginhoux et al., 2010; Matcovitch-Natan et al., 2016).

Upon infiltrating the brain, microglia differentiate through a stepwise developmental program involving three distinct stages: early microglia, pre-microglia and mature microglia (Alliot et al., 1999; Ginhoux et al., 2010; Kierdorf et al., 2013; Matcovitch-Natan et al., 2016). The early microglia phase is characterized by the initial colonization of the CNS and proliferation. During the pre-microglia stage, they begin to exhibit a more ramified morphology and acquire unique gene expression profiles that differentiate them from other macrophages. They reach the mature microglia stage after birth, with the maturation process continuing into the early postnatal weeks. By postnatal day 28 in mice, microglia have fully matured and exhibit the characteristic ramified morphology and gene expression pattern associated with their roles in the adult CNS (Ginhoux et al., 2010; Matcovitch-Natan et al., 2016).

Microglia comprise approximately 5-12% of all cells in the brain, with their density varying across different brain regions (Lawson et al., 1990; Tan et al., 2020). Immunohistochemical staining reveals that microglial density is higher in regions such as the hippocampus, olfactory bulb, basal ganglia, and substantia nigra, whereas it is lower in areas like the fiber tracts, cerebellum, and brainstem (Lawson et al., 1990; Tan et al., 2020). Even within individual brain regions, microglial density varies. For example, in the hippocampus, microglial density appears to be higher in the DG compared to the CA1 and CA3 region (Lawson et al., 1990; Rodriguez et al., 2014). However, these regional differences raise questions about the factors driving such variability and whether they are reflective of distinct functional requirements or simply methodological discrepancies. Furthermore, microglia density also appears to be dynamic in nature. As studies have shown that environmental factors, such as voluntary wheel-running or chronic stress, affect microglial density in specific brain regions (Ehninger, 2003; Tynan et al., 2010). Nevertheless, it is still unknown whether these changes represent a functional adaptation or simply a reactive response with potential consequences for neuronal health. The underlying mechanisms driving these density shifts and the relevance of microglial numbers in

different contexts remain open to debate, warranting further investigation to clarify their functional significance.

Unlike other macrophages, microglia maintain their population through self-renewal by a lowrate proliferation and apoptosis throughout life, without relying on bone marrow-derived monocytes (Ajami et al., 2007; Bruttger et al., 2015; Ginhoux et al., 2010; Hashimoto et al., 2013). Recent research using a multicolor fluorescent mapping system, however, has shown that microglia have variable turnover rates in different regions, with more active division observed in areas like the hippocampus and cerebellum compared to the cortex (Tay et al., 2017).

These findings underscore the complexity of microglial maintenance, showing that their behavior is heavily influenced by local environmental factors and functional needs of each brain region. Despite this adaptability, the precise mechanisms that allow microglia to adjust their density, morphology, and gene expression to align with the unique needs of different CNS environments are still not fully understood. (Masuda et al., 2020; Masuda & Prinz, 2016). This heterogeneity, while potentially allowing microglia to fulfill a wide range of functions, also complicates our ability to clearly define their roles in the CNS (see subsequent chapters).

Microglial morphology and surveillance

The morphology and surveillance capabilities of microglia are intimately connected, enabling these cells to maintain CNS homeostasis effectively. After the morphology of microglia was first described in the early 20th century by Pío del Río-Hortega, he also noted that microglia can drastically change their shape from a ramified form with extensive branching to an amoeboid form in response to environmental cues. (Figure 4, (P. del Río-Hortega, 1928; Penfield W., 1925). While amoeboid formed microglia are commonly associated with their active role in the immune response (P. del Río-Hortega, 1928; Penfield W., 1925), in their ramified state microglia actively surveil the CNS using their highly branched, tree-like processes. These processes, which are constantly extending and retracting to monitor the environment and allows each microglial cell to cover a territory of approximately 50–60 μ m², detecting physiological and pathological changes (Davalos et al., 2005; Nimmerjahn et al., 2005; Kettenmann et al., 2011).



Figure 4: Microglia cells, as described by Pio del Rio-Hortega. (A) Picture of Pio del Rio-Hortega (1882– 1945), who characterized and named microglial cells. (B) Drawing of ramified microglial cells from Hortega. (C) Morphological transformation of microglia to phagocytic macrophage. Panels as lettered in C: A, Microglial cell with modestly thickened processes as compared with ramified microglia; B, microglial cell with short, thick processes and enlarged cell body; C, microglial cell with pseudopodia; D, amoeboid microglial cell; E, amoeboid microglial cell with pseudopodia; F, microglial cell with phagocytosed leukocyte; G, microglial cell with numerous phagocytosed erythrocytes; H, microglial cell with lipid inclusions, also termed "foam cell"; I, microglial cell in mitotic division. Figure from (Ransohoff & Brown, 2012).

Microglia processes are organized hierarchically into larger primary processes, smaller secondary branches and filopodia. Each of these types are equipped with a variety of receptors that allow them to detect these changes and interact with surrounding cells (Petry et al., 2023; Savage et al., 2019; Wake et al., 2009). The lager primary processes are thicker and less mobile, than the smaller once. They are thought to be responsible for broader surveillance, enabling microglia to cover large areas and interacting with astrocytes, neurons and blood vessels. Their movements are suggested to be primarily regulated by purinergic signaling and membrane polarization, although the exact mechanisms remain not fully understood and may

involve additional pathways. Microglia sense ATP and ADP released from damaged cells or neurons, which activates P2Y12 receptor in the microglial membrane. This activation leads to the opening of the two-pore domain halothane-inhibited K+ channel type 1 (THIK-1) potassium channel, causing a hyperpolarization of the membrane. This in turn, is proposed to promote the extension of the primary processes towards injury sites (Dissing-Olesen et al., 2014; Haynes et al., 2006; Madry et al., 2018a). This response mechanism appears to play an important role in guiding microglial processes to areas of inflammation and damage, ensuring that microglia can quickly respond and maintain the homeostasis in the CNS (Davalos et al., 2005).

In contrast, the smaller secondary branches and filopodia are more dynamic and appear to be responsible for detailed environmental surveillance and rapid responses to local changes in the CNS. Their motility is driven by actin polymerization, which is tightly controlled by cAMP signaling. Under "resting" conditions, microglial cAMP levels are kept low, by CX3CR1, a Gi-coupled the fractalkine receptor (Fourgeaud et al., 2016). The intracellular cAMP level rises when norepinephrine binds to Gs-coupled receptors, promoting the assembly of actin filaments. This actin-driven movement allows a quick extension and retraction of secondary branches and filopodia, enabling them to scan their surroundings at a nanoscale (Bernier et al., 2019; Gyoneva et al., 2014). While this localized surveillance has been suggested to be important for regulating synaptic function and maintaining the balance of synaptic activity, it should be noted that the complexity of these interactions and the specific pathways involved are still areas of ongoing research, requiring further experimental validation.

In particular, the bimodal classification of microglia into ramified and amoeboid states may oversimplify their morphological diversity. In the last decade, several studies suggest that microglia morphology appears to exhibit considerable variability, which may have functional implications that are not yet fully understood (Figure 5; Vidal-Itriago et al., 2022). For example, in response to chronic stress and excitotoxicity microglia become hyper-ramified with extensively branched processes. These microglia are thought to play a neuroprotective role by maintaining synaptic integrity and supporting neuronal survival (Schafer et al., 2012b; Wake et al., 2009). Nevertheless, it is important to note that much of the current understanding of microglial morphology is derived from studies using fixed or real-time imaging techniques, which provide detailed structural insights but do not directly reveal functional implications. A recent study compared the transcriptome of isolated amoeboid and ramified microglia and revealed that both have similar levels of cytokines and chemokines, implying that ramified microglia may not differ in their immune properties from the more amoeboid form (Parakalan et al., 2012). This also proves that more advanced techniques, such as comprehensive

sequencing and transcriptomic analyses are necessary to fully understand the functional differences between microglial types.



Figure 5: Diversity of microglial morphology. (A) Ramified microglia are highly branched with multiple primary and secondary processes (often considered surveillant). (B) Amoeboid microglia present with a highly rounded morphology compared to their ramified states (often with a high phagocytic and migratory capacity). (C) Microglia can form ball-and-chain structures at the tip of their processes to phagocytose small amounts of material (such as synapses or apoptotic bodies). (D) Hyper-ramified microglia present with increased branching of their processes (often observed in acute and chronic stress models). (E) Microglia display bulbous budding at the end of some of their processes (considered to be important for ATP sensing). (F) Several microglial cells form a network resembling a honeycomb (reported in response to BBB leakage). (G) Jellyfish morphologies have been reported as a morphological transition of honeycomb microglia after extensive astrocytic death in the glia limitans (in response to TBI). (H) Rod microglia are characterized by an elongated, narrowed soma without planar processes that can form trains of rod microglial cells (in response to injury). Figure and legend from Vidal-Itriago et al., 2022.

Microglial morphological changes appear to be closely tied to their functional capabilities, but the specific roles of each morphology remain debated. The morphological flexibility however allows microglia to effectively surveil their environment, respond to neuronal activity, and manage immune challenges (see Chapter on Microglia–Neuron Interaction and subsequent chapters).

Microglia immune response

Microglia are the resident immune cells of the CNS and act as the first responder to signals of damage and exogenous pathogen-derived signals. Upon activation, microglia shift from a surveillant state into a responsive state, engaging in immune defense mechanisms such as phagocytosis and cytokine release (Hanisch & Kettenmann, 2007). However, the exact mechanisms and consequences of microglial activation remain a topic of debate, particularly regarding how different activation states impact CNS health.

Microglia are highly sensitive to damage-associated molecular patterns (DAMPs), which are endogenous molecules released by injured or dying cells. One well-studied DAMP is ATP, which is released from damaged neurons and binds to purinergic receptors like P2Y12 on microglia. This binding leads to chemotaxis of microglia toward the injury site, driven by phosphatidylinositol 3-kinase (PI3K) signaling (Davalos et al., 2005; Haynes et al., 2006). While ATP signaling is well characterized, the functional implications of microglial recruitment remain elusive, as excessive activation may lead to detrimental inflammation. Beside ATP, microglia recognize other DAMPs including high-mobility group box 1, heat shock proteins and mitochondrial DNA via Toll-like receptors (TLR) (Lehnardt et al., 2003). The binding to TLRs triggers a downstream signaling cascade through the MyD88 adaptor protein, activating nuclear factor kappa B (NF. κ B) and mitogen-activated proteins kinases (MAPKs). These pathways result in the production of pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF- α), interleukin-1 beta (IL-1 β), and interleukin-6 (IL-6), which promote inflammation and attract other immune cells to the site of damage (O'Neill et al., 2013).

Additionally, microglia are activated by pathogen-associated molecular patterns (PAMPs), which are found in various pathogens. Common PAMPs are bacterial lipopolysaccharides (LPS), viral RNA, and fungal cell wall components. Microglia recognize them primarily through pattern recognition receptors such as TLR. LPS, a component of the Gram-negative bacterial cell wall binds to TLR4, which in turn also activates the MyD88-dependent signaling pathway, resulting in a production of the proinflammatory cytokines including TNF- α , IL-1 β , IL-6 and chemokines as CCL2 (Olson & Miller, 2004; O'Neill et al., 2013). It was further shown that LPS triggers the release of reactive oxygen species (ROS) and NO, which are part of the microglial defense against pathogens (Lehnardt et al., 2003). Similarly, bacterial peptidoglycans and fungal cell wall components are recognized by TLR2, triggering MyD88-dependent signaling

(Bsibsi et al., 2002). Viral RNA is detected by microglia through TLR3 and leads to the production of type I interferons (IFN- α and IFN- β), which have antiviral functions. The released interferons activate neighboring cells to enhance their antiviral defense while microglia themselves release pro-inflammatory cytokines (Olson & Miller, 2004).

Once activated by DAMPs or PAMPs, microglia engage in phagocytosis, which is critical for clearing cellular debris, apoptotic cells and pathogens. They recognize "eat me" signals such as phosphatidylserine on apoptotic cells trough receptors like TREM2 (triggering receptor expressed on myeloid cells 2) and Mer tyrosine kinase (Takahashi et al., 2005). These receptors trigger actin cytoskeletal rearrangements, allowing microglia to engulf and degrade debris. This phagocytic interaction often leads to the production of anti-inflammatory cytokines such as IL-10 and transforming growth factor β (TGF- β) to suppress the pro-inflammatory response and facilitate tissue repair (Cherry et al., 2014; Napoli & Neumann, 2009). This transition from pro-inflammatory towards anti-inflammatory phenotype is essential for balancing the microglial immune response, to prevent excessive inflammation.

The immune response of microglia is critical for maintaining the balance between defense and repair in the CNS. Their ability to detect PAMPs and DAMPs allows them to clear cellular debris and resolve inflammation, thereby preserving the brain homeostasis. However, while acute microglial activation is essential for normal immune function, chronic activation can disrupt this balance. Prolonged or excessive activation may lead to sustained neuroinflammation, which has been implicated in the progression of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Multiple Sclerosis (Block et al., 2007; Perry & Teeling, 2013; Ransohoff, 2016). The exact mechanisms that regulate the switch between beneficial and harmful microglial responses remain poorly understood, underscoring the need for further research to elucidate these processes and develop interventions to prevent neuroinflammation-related damage.

1.3. Microglia – neuron interaction

Traditionally, microglia were seen as immune cells, which sole role was to respond to injuries and pathogens. However, recent research has expanded their role far beyond immune surveillance. Through highly dynamic interactions with neurons, microglia apparently influence processes such as synapse formation, synaptic pruning, and synaptic plasticity, thus regulating neural circuit refinement and function. This interaction occurs trough a range of mechanisms, including indirect signaling and direct physical contacts (Hu & Tao, 2024; Wang et al., 2021).

1.3.1. Microglia in synaptogenesis and pruning

Synapses are highly dynamic structures that undergo continuous cycles of formation and elimination, processes critical for neuronal development, synaptic plasticity and the refinement of neural circuits throughout life (Choquet & Triller, 2013; Holtmaat & Svoboda, 2009). Microglia are thought to play an essential role in these processes by detecting and removing excess, dysfunctional, or less active synapses while also promoting the formation of new synapses. This potentially dual role may ensure that only functional synapses persist, thereby supporting healthy and adaptive neural networks. However, the mechanisms behind this selectivity are not entirely clear, and any imbalance in microglial activity could risk unintended synaptic loss. (Kettenmann et al., 2013; Miyamoto et al., 2016; Nebeling et al., 2023; Schafer et al., 2012b; Stevens et al., 2007; M. È. Tremblay et al., 2011; Weinhard, Di Bartolomei, et al., 2018a).

Synaptogenesis during postnatal brain development is closely linked to the activity of microglia. Both *in vivo* and *in vitro* studies have shown that microglia contact with neurons can induce calcium transients and actin accumulation in dendrites, directly promoting the formation of dendritic filopodia (Miyamoto et al., 2016; Weinhard, Di Bartolomei, et al., 2018b). Moreover, targeted photoablation of individual synapses has been found to increase the duration of neuron-microglia contact, significantly enhancing the turnover of dendritic spines and presynaptic boutons while also stimulating the generation of microglia leads to reduced spine density, fewer functional excitatory synapses, and diminished cortical neuron connectivity (Miyamoto et al., 2016; Parkhurst et al., 2013c), highlighting the essential role of microglia in promoting spine turnover and filopodia generation (Cangalaya et al., 2020).

Microglia also play a crucial role in synaptic pruning. Several molecular mechanisms are involved in identifying and eliminating synapses, with key pathways including the complement system, fractalkine signaling, purinergic receptors and TREM2. One of the primary mechanisms is the classical complement cascade, a component of the innate immune system, is integral to microglial synaptic pruning (Stevens et al., 2007). During neural development and circuit formation, the complement-tagged synapses are recognized by microglial complement receptor 3 (CR3) and C1qR, initiating engulfment and removal of these synapses (Presumey et al., 2017; Schafer et al., 2012b; Stevens et al., 2007). Less active or weaker synapses exhibit increased deposition of C1q and C3, suggesting an activity-dependent mechanism where neuronal activity inversely correlates with complement tagging (Stevens et al., 2007). The

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interaction between microglial CR3 and the complement fragments on synapses triggers intracellular signaling pathways that lead to cytoskeletal rearrangement and engulfment of synaptic material (Schafer et al., 2012b). Impairments in this pathway is associated with disrupted synaptic pruning, resulting in excessive synaptic connection and abnormal neuronal circuitry (Shi et al., 2015).

Another crucial pathway for regulating synaptic pruning is the fractalkine (CX3CL1)-CX3CR1 signaling pathway. Fractalkine is a chemokine expressed by neurons, while its receptor CX3CR1 is exclusively expressed on microglia in the CNS (Jung et al., 2000; Nishiyori et al., 1998). CX3CR1 is essential for regulating microglial activation and inflammatory responses within the CNS. By interacting with fractalkine, CX3CR1 helps maintain microglia in a surveillant, non-inflammatory state, thereby preventing excessive neuroinflammation (Paolicelli et al., 2014). Additionally, it influences microglial phagocytic activity by promoting the clearance of apoptotic cells and debris, contributing to neural homeostasis and neuroprotection (Fuhrmann et al., 2010). However, during development, the fractalkine-CX3CR1 interaction is required for synaptic engulfment. Paolicelli et al. (2011) demonstrated that mice lacking the CX3CR1 receptor exhibit a significant impairment in synapse elimination, leading to a higher density of immature synapses and weaker synaptic transmission and delay the maturation of hippocampal circuits. Fractalkine signaling also plays a role in maintaining functional connectivity in the adult brain. The impaired synaptic elimination in CX3CR1 knockout mice, leads to disruption in LTP, as well as cognitive deficits in tasks such as novel object recognition and contextual fear conditions (Basilico et al., 2019; Rogers et al., 2011).

Purinergic signaling through microglial P2Y receptors also plays a significant role in synaptic pruning. The P2Y6 receptor, a Gq-coupled receptor expressed on microglia, responds to nucleotides like UDP released from damaged or stressed neurons (Koizumi et al., 2007). Activation of P2Y6 enhances microglial phagocytic activity, leading to the engulfment of synaptic elements and contributing to spine loss (Koizumi et al., 2007). This mechanism highlights how microglial activation via Gq-coupled receptors can drive spine elimination. Additionally, the P2Y12 receptor is essential for controlling microglial motility and chemotaxis (Haynes et al., 2006; Madry et al., 2018b). But it is also involved in activity-dependent synaptic pruning (Sipe et al., 2016). It was demonstrated that P2Y12 is necessary for microglia-mediated synaptic pruning in the visual cortex during ocular dominance plasticity, a developmental process where inactive synapses are pruned to enhance visual acuity. In P2Y12 knockout mice, microglia fail to effectively remove inactive synapses (Sipe et al., 2016).

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Several studies support the association between microglia activation and increased spine loss, which can disrupt neuronal circuits and impair cognitive functions. Through the release of proinflammatory cytokines, microglia can alter synaptic function and promote spine elimination (Bolós et al., 2017; J. Zhang et al., 2014). Additionally, excessive production of reactive oxygen species (ROS) by activated microglia can damage synaptic proteins and membranes, leading to spine loss (Block & Hong, 2005). Overactivation of phagocytic pathways, including those involving Gq-coupled P2Y6 receptors, may result in the removal of healthy synapses, contributing to neural dysfunction (Koizumi et al., 2007).

The dysregulation of microglial activation and synaptic pruning are implicated in various neurological disorders. In Alzheimer's disease, excessive complement activation leads to overpruning of synapses, exacerbating synaptic dysfunction and cognitive decline (Hong et al., 2016). Impaired TREM2 function affects microglial responses to amyloid-beta plaques, further contributing to synaptic loss (Ulrich et al., 2014). In Parkinson's disease, microglial activation contributes to dopaminergic neuron degeneration and synaptic loss, influencing disease progression (Tansey & Goldberg, 2010). Traumatic brain injury can induce microglial activation that leads to prolonged synaptic loss and cognitive impairments due to excessive synaptic elimination (Norris et al., 2018).

In summary, microglia-mediated synaptic pruning is a highly dynamic and tightly regulated process essential for brain development and synaptic plasticity. While microglia play crucial roles in synaptogenesis and pruning, the outcomes of these activities can vary widely depending on context. Under normal physiological conditions, these processes help refine neural circuits and ensure synaptic health. However, chronic or excessive activation of microglia, often mediated by pathways like the complement cascade or purinergic signaling, can lead to detrimental outcomes, including excessive synaptic loss, impaired cognitive functions, and neurodegeneration. The exact triggers and mechanisms responsible for the dysregulation of microglial activities remain unclear, underscoring significant gaps in knowledge.

1.3.2. Microglial modulation of neuronal function

While synaptic pruning by microglia is essential for the proper development of neural circuits, as detailed in the previous chapter, recent research has revealed that microglia also play a significant role in modulating synaptic function and plasticity in the adult brain (Kettenmann et al., 2013; Salter & Stevens, 2017). They regulate synaptic plasticity through a combination of direct interaction with the neurons and the release of soluble factors that modulate the neuronal function (Cornell et al., 2022; Cserép et al., 2021).

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Under physiological conditions, microglia maintain dynamic interactions with neurons, by extending their processes to contact various neuronal compartments, including soma, axons and synapses (Cserép et al., 2021). While these interactions allow microglia to sense and respond to changes in neuronal activity, the outcomes are not always straightforward. When neuronal activity increases, microglia extend their processes towards active neurons, enhancing contact time and potentially protecting neurons from excitotoxic damage. This respond appears to be largely mediated by P2Y12 receptors, guiding microglial process towards ATP, released by active neurons. Once at the neuron, microglia degrade ATP into adenosine using the enzymes CD39 and CD73. The adenosine then acts on A1 receptors on neurons, inhibiting excessive neuronal firing and preventing hyperactivation. This negative feedback loop is crucial for maintaining neuronal activity within optimal ranges, protecting neurons from damage associated with overexcitability, such as neuronal death and synaptic loss (Badimon et al., 2020; Cserép et al., 2020; Umpierre & Wu, 2021). In contrast, microglia also extend their processes, when neurons are in a hypoactive state, such as during anesthesia with isoflurane. They respond to the changes of neuronal norepinephrine signaling, that affect microglial β2-adrenoreceptor. During hypoactivity microglia shield GABAergic inputs in the synaptic cleft, to modulate neuronal inhibition and facilitate recovery after anesthesia (Haruwaka et al., 2024; Y. U. Liu et al., 2019; Stowell et al., 2019).

The ability of microglia to sense and modulate neuronal activity through direct contact and soluble factors sets the stage for their crucial role in synaptic plasticity. However, this role is highly context-dependent, and while microglia contribute to the structural and functional remodeling of synapses, their influence can also lead to destabilization and potential synaptic dysfunction in certain scenarios.

Physical interactions involve microglial processes making brief contacts with presynaptic boutons and postsynaptic spines, which can induce spine enlargement during contact (M. Ě. Tremblay et al., 2010). The frequency of microglial contact is influenced by neuronal activity, with increased activity and LTP promoting more frequent microglial contact with active dendritic spines (Pfeiffer et al., 2016). While these interactions can enhance synaptic activity and local network synchronization (Akiyoshi et al., 2018), they have also been linked to reduced spine stability in the hippocampus (Nebeling et al., 2023). This dual effect suggests that microglial interactions can both support synaptic function and potentially undermine it, depending on the specific context.

Fractalkine signaling has been identified as a key mechanism mediating synaptic changes during microglial contact. The interaction between the microglial chemokine receptor CX3CR1

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and its ligand, neuronal CX3CL1, allows precise communication between these neurons and microglia, influencing synaptic plasticity. Studies show that applying CX3CL1 to brain slices induces synaptic depression through adenosine A3 receptors, whereas chronically reduced CX3CL1-CX3CR1 signaling elevates IL-1β levels, impairing LTP in the brain (Ragozzino et al., 2006; Rogers et al., 2011). On the other hand, another study demonstrated that blocking the factralkine/CX3CR1 interaction increased LTP (Maggi et al., 2011). These conflicting findings imply a complex, context-dependent role of fractalkine signaling, where it can act to either enhance or suppress plasticity based on specific regulatory mechanisms, timing, or environmental factors in the brain.

In addition to direct physical interactions, microglia release soluble factors such as brainderived neurotrophic factor (BDNF), TNF α and other cytokines to influence synaptic plasticity. BDNF released by microglia plays a role in learning-related synapse formation, enhancing synaptic transmission, and promoting LTP (Parkhurst et al., 2013a; Zhou et al., 2019). TNF α regulates the phosphorylation of sleep-related synaptic proteins, linking microglia to synaptic plasticity in the context of sleep (Pinto et al., 2023). TNF α can also indirectly modulate synaptic connectivity through different mechanisms, depending on the specific brain region, such as in the spinal cord versus the hippocampus (Lewitus et al., 2014; Y. Liu et al., 2017).

Micorglial activation during immune responses further demonstrates their capacity to influence synaptic plasticity. Immune challenges such as infection or injury transition microglia from their homeostatic surveillance state to an activated phenotype characterized by morphological changes and the release of pro-inflammatory cytokines (see previous chapter). During immune activation, microglial TNF α release induces synaptic scaling, a homeostatic mechanism that adjusts synaptic strength to maintain stability in neural networks (Stellwagen & Malenka, 2006). However, prolonged exposure to inflammatory signals can impair synaptic plasticity, as increased levels of IL-1 β during chronic inflammation have been shown to reduce LTP, negatively affecting learning and memory processes (Rogers et al., 2011).

In summary, microglia are vital modulators of neuronal activity and synaptic plasticity, adapting their response to protect neurons and shape connections. While microglia support brain health and influence learning, memory, and forgetting, the precise mechanisms by which they balance their protective versus potentially harmful roles are not fully understood. Further research is necessary to determine how context and signaling pathways influence microglial actions, particularly in chronic disease states where their involvement may shift from protective to maladaptive, contributing to neurodegeneration and cognitive decline.

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1.3.3. Microglia mediates memory and forgetting

Memory is a cognitive process that enables organisms to encode, store and retrieve information (Squire, 2009). During the formation of memories, synaptic connections between neurons undergo structural and functional changes that encode memories (Josselyn & Tonegawa, 2020). Recent research has highlighted the role of microglia in modulating synaptic strength, connectivity and pruning (as described in previous chapter), thereby contributing to memory formation and forgetting (Wang et al., 2021). Traditionally, microglia have been associated with a negative role in cognitive functions, particularly under conditions of inflammation and neurodegeneration (Mohammadi et al., 2020; D. Zhang et al., 2021). However, it is now indicated that microglia play both a positive and negatively role in memory formation processes.

Microglia have been shown to influence the quality and specificity of memory through interaction with the extracellular matrix (ECM) and signaling pathways like IL-33. It was reported that microglia regulate memory precision via IL-33 signaling, which is expressed in neurons in an experience-dependent manner. This signaling causes microglia to engulf ECM components thereby promoting synaptic plasticity and enhancing the precision of contextual fear memories (Nguyen et al., 2020). While these findings demonstrate a beneficial aspect of microglia in memory formation, the involvement of microglia in ECM degradation may also raise questions about potential long-term impacts on the structural stability of synapses.

Another process linked to learning and forgetting is hippocampal adult neurogenesis, which is indicated to be also mediated by microglia through the phagocytosis of apoptotic newborn neurons. Chronic deficiencies in microglial phagocytic function have been linked to reduced neurogenesis(Diaz-Aparicio et al., 2020; Wang et al., 2020). However, it remains unclear how variations in microglial activity influence the balance between promoting neurogenesis and potentially exacerbating synaptic dysfunction. Recent evidence also highlights microglia's role in memory forgetting, which appears to involve synaptic pruning through complement-mediated pathways and neurogenesis-dependent mechanism (Wang et al., 2020).

Inhibition and elimination of microglia have been shown to yield divers effects on memory and forgetting. It was reported that microglia depletion improves cognitive functions (Acharya et al., 2016; Elmore et al., 2018) and prevents forgetting in contextual fear conditions (Wang et al., 2021). Controversy, the inhibition or depletion of microglia has also been associated with impairments in learning task and memory (Parkhurst et al., 2013b). Interestingly, repopulation after a transient depletion of microglia enhances short-term memory and improves performance in learning tasks (De Luca et al., 2020). This dual role in both supporting learning

and facilitating forgetting further complicates our understanding of how microglia modulate memory.

Overall, microglia modulate memory and forgetting through complex mechanisms involving synaptic remodeling, neurogenesis and interaction with neuronal signaling pathways. Dysregulation of these mechanisms, often due to excessive microglial activation, leads to increased dendritic spine loss and contributes to the pathogenesis of neurodegenerative and neuroinflammatory diseases (Hong et al., 2016; Norris et al., 2018; Ulrich et al., 2014). Importantly, while microglia can play beneficial roles in maintaining memory precision and synaptic health, their involvement in memory processes is not fully understood.

1.3.4. Unresolved mechanisms of microglial-neuron interactions

Microglia are emerging as crucial regulators of brain health, influencing synaptic plasticity, connectivity and ultimately the functional organization of neural circuits (as described in previous chapters). Despite our growing understanding of their multifaceted functions, there is still much to uncover about the precise mechanism by which microglia interact with neurons and influences brain function. Key aspects include the specific signaling pathways that microglia use to modulate synaptic plasticity, the molecular cues that decide whether microglia support synapse formation or elimination, how microglia distinguish between healthy and dysfunctional synapses, and how microglia regulate neuronal activity. The complexity of microglial activity, which varies depending on the context and stage of development, emphasizes the need for further investigation. Employing advanced methods to investigate the nuanced interactions between microglia and neurons, such as chemogenetics will provide deeper insights into their role in maintaining and adapting the brain's intricate network.

1.4. Manipulation of microglia

1.4.1. State of art and limitations

Traditional methods for manipulating microglia in neuroscience research involve a range of pharmacological, genetic, immunological, and physical approaches, applied both *in vivo* and *in vitro*. Despite their contributions to advancing our understanding of microglial functions, these techniques face challenges such as issues with specificity, timing, and potential side effects. Moreover, variability in experimental outcomes is often influenced by differences in model organisms, developmental stages, or experimental conditions, emphasizing the need for standardized methods to gain more consistent insights into microglial dynamics.

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In vivo and *in vitro* models

To investigate the complex function of microglia, researchers use a variety of *in vitro* and *in vivo* models. *In vitro* models, such as primary microglial cultures provided a controlled setting to study microglial biology without the complexities of the *in vivo* environment. However, cultured microglia do not fully recapitulate the *in vivo* phenotype and can undergo phenotypic changes over time in cultures (Pesti et al., 2024). A more advanced technique involves the use of organotypic slice cultures, which maintain a more physiologically relevant environment compared to dissociated cell cultures, while still offering a controlled and easy to manipulate settings (Delbridge et al., 2020; Stoppini et al., 1991). Even though *in vitro* models offer a high level of experimental control and reproducibility, they often fail to capture the full spectrum of microglial interaction and the dynamic environment of the living brain. This could lead to discrepancies between *in vitro* observation and *in vivo* realities (Delbridge et al., 2020).

Conversely, *in vivo* models, such as mice or rats, allow researchers to investigate microglia functions within the intact CNS, including their interactions with other cell types and the blood brain barrier (Eme-Scolan & Dando, 2020). These models are indispensable for studying the influence of microglia function and the behavioral outcomes following microglial manipulation. Nevertheless, *in vivo* studies are often time-consuming and technically demanding, with inherent variability such as sex, age and hormones that can complicate data interpretation (Eme-Scolan & Dando, 2020; Prinz & Priller, 2014a).

Thus, both *in vivo* and *in vitro* models are invaluable for studying microglia functions, provided that their inherent limitations are carefully considered during experimental design and data interpretation.

Pharmacological and genetic approaches

Pharmacological approaches involve the use of drugs or chemical agents to activate or inhibit microglial function. These approaches are widely used due to their relative ease of application but often lack cell-type specificity. Chemical agents such as LPS and Minocycline are commonly used to activate and inhibit microglia, respectively. However, both approaches lack cell-specificity and activate multiple signaling pathways in several cell types, which makes it challenging to attribute observed effects solely to microglia (Möller et al., 2016; Pena-Ortega, 2017).

A more specific method involves the genetic manipulation of microglia. By using gene knockout models, such as *Cx3cr1* knockout mice, researchers can investigate the role of specific genes

in microglial function. However, global knockout often lacks precise temporal control because the gene is absent from the earliest stage of development. Furthermore, the absence of the gene during development can lead to compensatory mechanisms, where other genes or pathways adjust to mitigate the effects of the knockout (Eme-Scolan & Dando, 2020; Ginhoux et al., 2010; Prinz & Priller, 2014b; Salter & Stevens, 2017).

A more temporally controlled approach is the inducible version of the Cre-loxP System, which allows a cell-type specific gene manipulation with a more precise timing. The original Cre-loxP system involves the Cre recombinase and the specific loxP DNA sequence. The loxP sites are engineered to flox a gene of interest. When Cre recombinase is expressed in a cell, it recognizes these loxP sites and either excises (removes) or inverts the DNA segment between them, depending on the orientation of the loxP site (Sauer, 1998). In the inducible version of the Cre-loxP system, such as the tamoxifen-inducible Cre-ER, the Cre recombinase is fused to a modified estrogen receptor (ER) and remains inactive in the cytoplasm until tamoxifen is administered. Upon tamoxifen treatment Cre recombinase translocates to the nucleus and mediates the recombination at loxP sites (Metzger & Chambon, 2001). These inducible systems provide precise spatial and temporal regulation, making them powerful tools for dissecting gene function in specific tissues at desired time points.

Despite their widespread use and valuable contributions to the understanding of microglial functions, pharmacological and genetic approaches are limited by the mentioned issues. These limitations highlight the necessity for more precise and controllable methods, leading researchers to adopt optogenetic and chemogenetic techniques that offer enhanced specificity and temporal regulation in microglia studies.

1.4.2. Optogenetic manipulation of microglia

Optogenetic is a method that enables precise manipulation of neuronal activity through lightsensitive proteins. This technique involves the genetic modification of targeted neurons to express specific light-sensitive ion channels, such as channelrhodopsin, which can be activated or inhibited by exposure to specific wavelength of light. This allows researchers to modulate neuronal circuits with exceptional temporal and spatial precision (Boyden et al., 2005; Deisseroth, 2015).

So far only a few studies have used optogenetic approaches in microglia to explore their functional roles in the CNS(Parusel et al., 2023). One study used the red activated channelrhodopsin in microglia to induce a depolarization of spinal microglia, leading to a calcium-dependent release of pro-inflammatory cytokines (Yi, Liu, Umpierre, et al., 2021).
Additionally, our lab used a modified channelrhodopsin, ChETA to demonstrate that the optogenetic depolarization of microglia slowed their chemotactic response to tissue damage, highlighting the role of membrane potential in microglial motility (Laprell et al., 2021).

These studies showed that optogenetics are effective tools for investigating microglial function. In addition to optogenetics, chemogenetic approaches provide a powerful means to manipulate microglia, offering unique insights into their role in the CNS.

1.4.3. Chemogenetic manipulation of microglia

G Protein-protein coupled receptors

G Protein-protein coupled receptors (GPCRs) are a large family of membrane proteins, which play essential roles in cellular signal transduction. They respond to a wide range of ligands, including hormones, neurotransmitters and sensory stimuli. GPCRs are classified into several families based on their sequence homology and structural characteristics, with the major families including rhodopsin, secretin, glutamate, adhesion, and frizzled/taste-2 receptors (Fredriksson et al., 2003). Signaling pathways mediated by GPCRs are diverse and affect various physiological functions such as neurotransmission, immune responses, and cellular metabolism (Hauser et al., 2017).

GCPR are characterized by their conserved structure, comprising seven transmembrane alpha-helices (7TM), an extracellular N-terminus and an intracellular C-terminus. The extracellular regions are involved in ligand recognition and binding, whereas the intracellular loops interact with G proteins to transmit signals inside the cell (Palczewski et al., 2000). Upon ligand binding, the 7TM structure undergoes conformational changes, allowing the receptor to activate heterotrimeric G proteins composed of three subunits: G α , G β , and G γ . Activation involves the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the G α subunit, leading to its dissociation from the G $\beta\gamma$ dimer. Both G α and G $\beta\gamma$ subunits can then regulate various downstream effectors, thereby modulating diverse cellular responses (Lambright et al., 1994; Sprang, 1997).

Among the different types of G α subunits, the G α q family plays a pivotal role in mediating the effect of Gq-coupled receptors. Upon activation, G α q stimulates phospholipase C β (PLC β), which generates inositol trisphosphate (IP₃) and diacylglycerol (DAG), leading to the release of intracellular calcium and activation of protein kinase C (PKC). IP₃ diffuses through the cytosol and bind to IP₃ receptor on the endoplasmic reticulum, triggering the release of calcium ions into the cytoplasm, leading to the activation of various calcium-dependent proteins and

signaling pathways such as gene expression, metabolism and secretion (Berridge, 1993). DAG remains in the plasma membrane and activates PKC, which phosphorylates target proteins to regulate cell proliferation, differentiation and other cellular functions (Newton, 1995). In the nervous system, Gq-coupled receptors modulate neuronal excitability and synaptic plasticity, impacting learning and memory (Leung & Wong, 2017).

Other $G\alpha$ subunits include $G\alpha$ s and $G\alpha$ i, which mediate signaling pathways that differ from those of $G\alpha q$. The $G\alpha$ s subunit activates adenylate cyclase, increasing cAMP, a secondary messenger that activates protein kinase A (PKA). PKA phosphorylates various target protein, regulating processes such as gene transcription and cell growth (Taylor et al., 2013). Conversely, $G\alpha$ i subunits inhibit adenylate cyclase, leading to decreased cAMP levels and reduced PKA activity, influencing ion channel activity and neurotransmitter release (Neves et al., 2002).

The signaling cascade of GPCRs are tightly regulated and terminated by several mechanism. The Gas subunit has an intrinsic guanosine triphosphatase (GTPase) activity, which hydrolyzes GTP to GDP, leading to the reassociation of the Ga subunit with the G $\beta\gamma$ dimer and returning the G protein to its inactive state (Siderovski & Willard, 2005). Additionally, GPCR signaling is modulated by receptor desensitization, wherein the activated receptor is phosphorylated by GPCR kinases (GRKs). This phosphorylation promotes the binding of β -arrestin, which not only prevent further G protein coupling but also mediate receptor internalization and facilitate signaling through alternative pathways, such as the mitogen-activated protein kinase (MAPK) cascade. The MAPK pathway is particularly significant as it transduces signals from the cell surface to the nucleus, regulating gene expression and playing a crucial role in cell proliferation, differentiation, and survival (Lefkowitz, 1998; Shenoy & Lefkowitz, 2011).

Muscarinic acetylcholine receptors are a well-characterized subset of GPCRs, that bind the neurotransmitter acetylcholine. Among these, the M3 muscarinic receptor primarily couples with Gq proteins, leading to cellular activation, while the M4 muscarinic receptor couples with Gi proteins, resulting in inhibitory effects (Fredriksson et al., 2003; Simon et al., 1991). This specific coupling has been utilized to develop Designer Receptors Exclusively Activated by Designer Drugs (DREADDs). By engineering muscarinic receptors to respond selectively to synthetic ligands, DREADDs allow precise manipulation of cellular activity (Armbruster et al., 2007a; Roth, 2016). The following chapter will explore the design, functionality, and applications of DREADDs, highlighting their pivotal role in advancing neuroscience research.

DREADD: a chemogenetic tool

DREADDs are a powerful tool in neuroscience, enabling precise control over neuronal activity and offering new insights into brain function and behavior. These engineered receptors are modified GPCRs that respond exclusively to synthetic ligands, thereby overcoming limitations of traditional GPCR manipulation methods, such as lack of specificity and potential off-target effects (Roth, 2016).

Traditional methods for manipulating GPCRs were limited by the lack of specific and the potential for off-target effects, as endogenous ligands can activate multiple receptor subtypes. To overcome these limitations, researchers engineered GPCRs, specifically muscarinic acetylcholine receptors, to respond only to synthetic ligands, such as clozapine-N-oxid (CNO) (Armbruster et al., 2007a). This was achieved by introducing mutation into the ligand-binding domain of the receptor until a variant was identified that could be activated by the synthetic ligand but not by endogenous acetylcholine.

The first successful DREADD was hM3Dq, a Gq-coupled receptor that increases intracellular calcium levels upon activation by CNO, leading to enhanced neuronal excitability. Followed by the development of Gi-coupled DREADDs such as hM4Di, which reduce cAMP levels and inhibit neuronal activity. Both are among the most commonly used chemogentic tools in neuroscience, each activating distinct intracellular pathways, as detailed in the previous chapter on GPCRs (Figure 6) (Alexander et al., 2009; Armbruster et al., 2007b; Roth, 2016).

These DREADDs offer exceptional precision in controlling neuronal activity through several key mechanisms. Firstly, their selective ligand activation ensures that DREADDs respond exclusively to synthetic ligands like CNO, which do not interact with endogenous GPCRs. This specificity eliminates off-target effects and enhances specificity in neuronal modulation (Armbruster et al., 2007). Additionally, DREADDs enable temporal control by allowing researchers to administer synthetic ligands at specific times, facilitating the precise activation or inhibition of neuronal populations in a controlled manner. This temporal precision is crucial for examining dynamic neural processes and behaviors. The effects of DREADD activation are reversible, allowing repeated and controlled modulation of neuronal activity over time. Furthermore, in combination with genetic targeting techniques such as Cre-Lox systems, DREADDs can be expressed in specific neuronal populations or distinct brain regions. This spatial targeting allows researchers to dissect the functional roles of discrete neural pathways in both normal and pathological conditions (Alexander et al., 2009; Roth, 2016; Stachniak et al., 2014; Teissier et al., 2015; Zhu et al., 2016).

Due to their precise control, Gq-coupled DREADDs have been extensively utilized to study behaviors such as social interaction and memory by enhancing neuronal excitability in targeted circuits (Alexander et al., 2009; Krashes et al., 2011). In contrast, Gi-coupled DREADDs (e.g., hM4Di) lead to reduced neuronal excitability by inhibiting adenylate cyclase and decreasing cAMP levels. This inhibitory mechanism allows for the silencing of specific neuronal populations, facilitating the investigation of neural circuits underlying anxiety and seizure activity (Ferguson et al., 2011; Stachniak et al., 2014).

Overall, DREADDs have significantly impacted neuroscience by enabling precise and flexible control over neuronal activity. The ability to selectively modulate specific neural circuits has led to important discoveries in behavior, cognition, and neurological disorders. Although initially designed for use in neurons, researchers recognized the potential of DREADDs in other cell types, particularly in glia cells like microglia.



Figure 6: Effects of activation of hM3Dq (Gq-DREADD) and hM4Di (Gi-DREADD) with CNO in neurons. The activation of Gq-DREADD leads to a depolarization of the neuron, whereas the activation of Gi-DREADDs leads to a hyperpolarization. Figure from Mueller et al., 2022.

DREADDs in microglia

The application of DREADDs in microglia represents a significant advancement in the field of neurobiology. Due to its precise control over microglial activity, DREADDs provide a powerful tool for dissecting the complex role of microglia in the CNS.

Gi-DREADD activation in microglia

Gi-coupled DREADDs are designed to inhibit cellular activity through the reduction of cAMP levels upon activation by CNO. Gi-DREADD activation in microglia has predominantly been associated with anti-inflammatory effects. For instance, studies have shown that Gi-DREADDs in microglia significantly reduce the production of pro-inflammatory cytokines, such as IL-1 β and TNF α , in models of neuropathic pain. This reduction is accompanied by a decrease in microglial proliferation. This process is critical for the maintenance of chronic neuroinflammation and pain sensitization (Grace et al., 2016, 2018; Yi, Liu, Liu, et al., 2021).

Furthermore, Dheer et al. 2024 used Gi-DREADDs in an epilepsy model, to explore the impact of microglia inhibition on seizure activity. They found that acute Gi-DREADD activation reduced seizure severity by enhancing microglia-neuron interactions and decreasing neuronal hyperactivity. However, prolonged Gi-DREADD activation led to increased neuronal loss. This suggests that while short-term Gi-DREADD activation may be neuroprotective, long-term inhibition of microglial activity could impair their ability to support neuronal health, highlighting the complexity of microglial roles in the CNS (Dheer et al., 2024).

Gq-DREADD activation in microglia

Conversely, Gq-DREADDs activate signaling pathways that increase intracellular calcium levels, leading to the activation of various downstream pathways associated with proinflammatory functions. Acute Gq-DREADD activation has been shown to heighten neuroinflammatory responses, resulting in increased pain sensitivity and elevated mRNA expression levels of pro-inflammatory cytokines such as IL-1 β , TNF α , and IL-6 (Saika et al., 2021). However, chronic activation of Gq-DREADD in a model of sustained neuroinflammation resulted in a decrease mRNA expression level of pro-inflammatory cytokine, accompanied by improvements in social behavior. These findings suggest that chronic Gq-DREADD activation may induce adaptive changes in microglial function, shifting their phenotype from a pro-inflammatory to a more regulatory or anti-inflammatory state over time (Binning et al., 2020). Furthermore, Klawonn et al. (2021) investigated the effect of Gq-DREADD activation on affective behavior. They demonstrated that Gq-DREADD activation in the striatum resulted in negative affective states in mice, mediated through prostaglandin signaling. This study underscores the broad influence of microglial activity on CNS functions beyond inflammation, implicating microglia in the modulation of mood and behavior (Klawonn et al., 2021).

These studies collectively demonstrate that the application of DREADDs in microglial research has provided valuable insights into the complex and multifaceted roles of microglia in the CNS. Unlike traditional methods such as LPS administration, which activate multiple immune pathways and can lead to widespread inflammation, DREADDs offer targeted and reversible control over microglial activity. Despite the valuable insights gained from Gq-DREADD research, significant gaps remain in understanding the long-term implications of chronic microglial activation, particularly in specific brain regions like the hippocampus, which is critically involved in memory processes. However, these findings also emphasize the importance of considering the timing, duration, and context of microglial activation when interpreting results and planning further research, as the effects of microglial modulation can vary significantly based on these factors.

2. Aim of this Thesis

The primary aim of my thesis is to investigate the interaction between microglia and neurons in the hippocampus, with a specific focus on how selective activation of microglia influences neuronal structure, synaptic plasticity, and cognitive function. To achieve this, I utilize the chemogenetic approach combined with the tamoxifen-inducible Cre-ER loxP system to express Gq-DREADD exclusively in microglia to selectively activate them and assess whether this tool can effectively induce measurable effects on neurons. Unlike traditional methods of microglia activation, which can be non-specific and lead to off-target effects, Gq DREADD provides a more and controllable approach. This allows me to investigate the effect of Gq-DREADD-mediated microglial activation on hippocampal neurons in both in vitro and in vivo, offering greater insight into the specific role of microglia in the CNS.

Chronic activation of microglia is often associated with neuroinflammation, which has been linked to synaptic dysfunction and neurodegenerative processes (Heneka et al., 2014; Salter & Stevens, 2017). Understanding how selective microglial activation affects neuronal health is crucial for unraveling the mechanisms underlying neuroinflammation and its impact on the brain.

The first objective of my study is to determine whether chronic or acute Gq-DREADD activation in microglia induces morphological and functional changes in these cells, which subsequently affect neuron-microglia interactions in hippocampal slices cultures. Building on this, the second objective of this work is to examine the influence of selective microglia activation on dendritic spine density in CA1 pyramidal neuron in hippocampal slices cultures. The third objective is to explore the effect of selective microglial activation on synaptic plasticity, specifically on LTP, and its implications for learning and memory *in vivo*. By examining the impact of microglial activation on LTP, we aim to understand whether and how microglia modulate synaptic strength and contribute to cognitive functions.

By integrating both *in vitro* and *in vivo* approaches, this thesis aims to provide new insights into the relationship between microglia and neurons, particularly regarding how selective microglial activation shapes neuronal structure and synaptic function in the hippocampus. The use of Gq-DREADD technology as a targeted and precise tool sets this study apart from traditional methods, enabling a more detailed understanding of microglial contributions to neuronal health, synaptic plasticity, and cognitive processes.

3. Material & Methods

3.1. Animals

3.1.1. Transgenic mouselines

Gq-DREADD expressing mice were generated by crossing B6.129P2(C) – Cx3cr1^{tm2.1/cre/ERT2)Jung}/J Gt(ROSA)26-Sor^{tm9(CAG-tdTomato)Hze} (JAX: 020940; JAX: 007909), which carry a tamoxifen-inducible Cre-recombinase in microglia and a floxed fluorescent marker tdTomato, with mice heterozygously carrying the floxed Tg^{(CAG-CHRM3,-mCitrine)1Ute}/J (JAX: 026220) allele. This cross generated offspings expressing Gq-DREADD (CHRM3) and tdTomato in microglia along with littermate controls without Gq-DREADD. These mice (both sex) were used for *in vivo* experiments and for organotypic hippocampal slice cultures.

For expressing the a calcium-indicator (GCaMP6s) in microglia, mice carrying the Gt(ROSA)26-Sor^{tm96(CAG-CGaMP6s)Hze}/J (JAX: 024106) allele were crossed with B6.129P2(C) – Cx3cr1^{tm2.1/cre/ERT2)Jung}/J Tg^{(CAG-CHRM3,-mCitrine)1Ute}/J (JAX: 026220) (JAX: 020940, JAX: 026220). This cross produced offspring that express GCaMP6s and Gq-DREADD in microglia. These animals were used for organotypic hippocampal slice cultures.

All mice were housed and bred at the University Medical Center Hamburg-Eppendorf with a 12-hour light/dark cycle and had water and food ad libitum. All procedures were performed in compliance with German law and the guidelines of Directive 2010/63/EU. This study was approved by the local authorities (Amt für Verbraucherschutz,Lebensmittelsicherheit und Veterinärwesen, Hamburg; permission # 42/17, #49/18 and #103/22).

3.1.2. Genotyping

To identify the genotype of the various mice, tail biopsies were collected by animal caretakers at postnatal day 3-4. These tail biopsies were lysed in 75 µl lysis buffer containing: 25 mM NaOH, 0.2 mM EDTA-H₂O, H₂O for 60 min at 95 °C. Subsequently, 75 µl neutralization buffer (40 mM Tris-HCL, pH 5.5) was added. The lysed samples were then used for a polymerase chain reaction (PCR) based genotyping using AmpliTaq GoldTm 360 Master Mix (Applied BiosystemsTM) and different primer combination (see Table 1). Animals with a suitable genotype were used either for slice culture preparation or in vivo experiments.

Table 1: Primer List for Genotyping

Moucolino	Cono of Interact	Forward Primer	Revers Primer
Wouseinie	Gene of Interest	Sequence 5'-3'	Sequence 5'-3'
B6.129P2(C) – Cx3cr1 ^{tm2.1/cre/ERT2)Jung} /J	Cre-knock in	-AAG ACT CAC GTG GAC CTG CTT ACT GCA TG-	-AAA CGT TGA TGC CGG TGA ACG TGC-
B6.129P2(C) – Cx3cr1 ^{tm2.1/cre/ERT2)Jung} /J	Cre-wildtype	-AAG ACT CAC GTG GAC CTG CTT ACT GCA TG-	-CCA ATG AAG AAG AAG GCA GTC GTG AGC T-
Gt(ROSA)26- Sor ^{tm9(CAG-tdTomato)Hze}	R26-tdTomato-knock in	-CTC CAA GGC GTA CGT GAA GC-	-ACT GTT CCA CGA TGG TGT AGT C-
Tg ^{(CAG-CHRM3,-} ^{mCitrine)1Ute} /J	R26 CHRM3 – Knock in	-GCT GGG GAT GGT GGA CT-	-TCA CAC TGG CAC AGC AGC-
Gt(ROSA)26- Sor ^{tm96(CAG-CGaMP6s)Hze} /J	R26-GCaMP6s – Knock in	-CTG GTC GAG CTG GAC GGC GAC G-	-GTA GGT CAG GGT GGT CAC GAG-
Gt(ROSA)26	ROSA Locus 26 - wildtyp	- AAG GGA GCT GCA GTG GAG TA-	-CCG AAA ATC TGT GGG AAG TC-

3.2. Slice culture experiments

3.2.1. Preparation of hippocampal slice cultures

Organotypic slice cultures were prepared from mice at P4-7 as described in (Gee et al., 2017). In brief, newborn mice were anesthesized with 80 % CO₂ 20 %O₂ and subsequently decapitated. Brains were quickly extracted and kept in an ice-cold dissection medium comprising: 1 mM CaCl₂, 5 mM MgCl₂, 10 mM D-glucose, 4 mM KCl, 26 mM NaHCO₃, 0.001% phenol red, and 2 mM kynurenic acid. The hippocampi were dissected and sliced into 410 μ m sections using a tissue chopper. These slices were then placed on porous membranes (Millicell CM, Millipore). Cultures were maintained at 37 ° C in a 95% O2 5% CO2 atmosphere in a slice culture medium containing 80 % Minimum Essential Medium (MEM, Sigma M7278) and 20 % heat-inactivated horse serum (Sigma H1138) and supplemented with 1 mM L-glutamine, 0.00125 % ascorbic acid, 0.01 mg/mL insulin, 1.44 mM CaCl₂, 2 mM MgSO₄, and 13 mM D-glucose. For the first 48 h after preparation, 1 μ M (Z)-4-hydroxy-tamoxifen (SigmaH7904) was

added to the slice medium to induce Cre-activation. The slice medium was completely changed after 48 h of incubation. Subsequently, the medium was changed twice a week.

Slice cultures were used for experiments between 13 and 25 days in vitro (DIV).

3.2.2. Characterization of Gq-DREADD in microglia

Gq-DREADD activation *in vitro*

For the treatment of organotypic slice cultures (13-21 DIV), including slices expressing Gq-DREADD (GqDREADD+) and control slices without Gq-DREADD (GqDREADD-) fresh medium was added to each group, supplemented with one of the following: 1 μ M Clozapine N-oxide dihydrochloride (CNO, Tocris 6329) or 1 μ l water as control. After three days of incubation, all slices were fixed for 30 min in 500 μ l 4 % paraformaldehyde (PFA), followed by three consecutive washing steps with phosphate buffered saline (PBS) for 10 min at room temperature (RT) and stored at 4 °C until further use.

Immunohistochemistry

Fixed cultures were blocked for 2 h at RT in a blocking solution containing: 10 % donor goat serum, 0.2 % Boverin Serum Albumin, 0.3 % Triton[™]X-100 in 1x PBS. Subsequently, the slices were incubated in carrier solution (1 % Donor goat serum, 0.2 % Boverin Serum Albumin, 0.3 % Triton[™]X-100 in 1 x PBS) with primary antibodies at 4°C. After 24 hours of incubation, slices were washed 3 times in PBS and incubated in carrier solution with secondary antibodies for 2 h (Antibody List and concentration are listed in Table 2). Before mounting, slices were washed 3 times, and incubated with DAPI for 10 min. Slices were mounted with Shandon Immuno-Mount under the glass coverslip and left to dry in darkness before storing them at 4°C until further use.

Immunogen	Host	Label	Producer Code	Dilution
IBA1	Rabbit	None	FujiFllm WAKO 019-19741	1:1000
IBA1/AIF-1	Rabbit	None	Cell Signaling 17198	1:200
GFP	Chicken	None	Invitrogen A10262	1:1000
HA	Rabbit	None	Cell Signaling C29F4	1:500
Rabbit	Goat	Alexa Flour 647	Cell Signaling 4414	1:1000
Rabbit	Goat	Alexa Flour 647	Invitrogen A21245	1:1000
Rabbit	Goat	Alexa Flour 488	Lifetechnologies A11008	1:1000
Chicken	Goat	Alexa Flour 488	Invitrogen A11039	1:1000

Table 2: Antibody List and Concentration

Confocal imaging

Imaging of immunostained hippocampal slice cultures was performed using a confocal laser scanning microscope (Zeiss LSM 900) equipped with a Zeiss Plan-Apochromat 20 x/0.8 NA M27 objective. For each culture, a stack was acquired with a step size of 1 µm and a resolution of 1024 x 1024 pixels. Depending on the specific antibody combinations used, fluorescence excitation was achieved using laser lines at 405 nm, 488 nm, 568 nm or 647 nm with a pixel dwell time of 2.06 µs per pixel. To prevent spectral bleed through between the channels, imaging was conducted sequentially. The imaging parameters were maintained consistently across all imaging sessions to ensure reproducibility and comparability between datasets.

Sholl Analysis

Microglia morphology was analyzed using semi-automated filament tracing in IMARIS[™]. Prior the filament tracing, a median filter (3x3x1) was applied to reduce noise and improve the quality of the microglia structure. Only microglia that were completely in the field of view (FOV) were selected for analysis. After the initial filament tracing, any inaccurately detected filaments were manually removed to ensure accuracy. The resulting filaments were then used for Sholl analysis to compare the number of Sholl intersections between GqDREADD+ and GqDREADD-.

The statistical analysis of the Sholl intersections was performed using GraphPad Prism. The Area under the curve (AUC) of each microglia's Sholl intersection graph was calculated and compared between the two groups to assess differences in microglial morphology.

Calcium imaging in microglia

Organotypic slice cultures, expressing the calcium-indicator GCaMP6s and Gq-DREADD in microglia were placed in a chamber of a costume built two-photon microscope and superfused with HEPES containing 140 mM KCl, 10 mM HEPES, 1 mM MgCl₂, and 0.1 mM CaCl₂. To avoid drift during imaging cultures were incubated for 30 min before imaging. The pulsed Ti:Sapphire laser (MaiTai Deep See, Spectra Physics) was tuned to 960 nm to excite GCaMP6s in microglia. The emitted photons were collected through a water immersion objective (Leica 25 x/0.95 W VISIR) and the oil immersion condenser (1.4 NA, Olympus) and collected by two pairs of photomultiplier tubes (PMTs H7422P-40, Hamamatsu). 300 Frames from 3-4 microglia in the field of view were acquired with a resolution of 256 x 256 pixels and a frame rate of 1Hz every 10 min. After two aquisitions of baseline, 1 μ M CNO or water was washed in into the chamber and the responses were acquired for another four times.

To quantify the calcium response in microglia, I analyzed the fluorescence transients using Fiji. A region of interest (ROI) was assigned to the microglia soma, and the fluorescence intensity (F) was measured for each frame. The change in fluorescence was calculated as Δ F/F0, where F0 represents the baseline fluorescence prior to CNO treatment (Δ F = F - F0 / F0). These Δ F/F0 values were then used to calculate the area under the curve (AUC) of each calcium transient, both before and after CNO treatment.

3.2.3. Spine density experiments

Single cell electroporation

After 16 DIV, organotypic slice cultures expressing Gq-DREADD were transfected using singlecell electroporation. The slice cultures were placed in a sterile microscope chamber filled with prewarmed HEPES buffer (140 mM KCl, 10 mM HEPES, 1 mM MgCl₂, and 0.1 mM CaCl₂, adjusted to pH 7.3). Thin-wall glass pipettes with a resistance of 12-14 M Ω were filled with 1 µL of the desired DNA plasmid (listed in Table 3) diluted in an intracellular solution containing 135 mM K-gluconate, 4 mM MgCl₂, 4 mM Na₂-ATP, 0.4 mM Na-GTP, 10 mM Na₂phosphocreatine, 3 mM ascorbate, and 10 mM HEPES (pH 7.2). The following combinations of plasmids were used in the experiment: ElpoMix 1 contained pAAV-Syn-PSD95-eGFP-Sacl CCR5TC and pCI-Syn-mCerulean, while ElpoMix 2 contained P-DNAbinding-Syn-Xph20eGFP-CCR5TC and pCI-Syn-tDimer. The pipettes were carefully positioned near the cell membrane of CA1 pyramidal neurons using a micromanipulator. Upon reaching the cell membrane, short electrical pulses were applied to facilitate the entry of the plasmid into the cells (Wiegert et al., 2017). Following electroporation, the cultures were incubated in fresh culture medium at 37°C to recover. The expression period of the construct varied between 3 to 7 days.

Table 3: Plasmid list

Plasmid	Internal Number	Concentration	
pAAV-Syn-PSD95eGFP-Sacl CCR5TC	#168	15 ng/ml	
pCI-Syn-mCerulean	E19-150	5 ng/ml	
P-DNAbinding-Syn-Xph20- eGFP-CCR5TC	#312	10 ng/ml	
pCI-Syn-tDimer	E22-231	30 ng/ml	

Two-photon microscopy and PSD95 imaging

Single-cell electroporated hippocampal slice cultures were placed in a freshly cleaned chamber of a custom-built two-photon laser scanning microscope based on an Olympus BX51WI microscope. The chamber was filled with sterile filtered prewarmed HEPES buffer containing (in mM): 145 NaCl, 10 HEPES, 12.5 D-Glucose, 1.25 NaH₂PO₄, 2.5 KCl, 1MgCl₂, 2 CaCl₂ (pH 7.4). The pulsed Ti:Sapphire laser (MaiTai Deep See, Spectra Physics) was tuned to 940 nm to simultaneously excite both the expressed PSD95eGFP-SacI-CCR5TC or Xph20eGFP-CCR5TC in the CA1 pyramidal neurons and the cytoplasmic tdTomato within the microglia. The red and green fluorescence were detected through a water immersion objective (Leica 25 x/0.95 W VISIR) and the oil immersion condenser (1.4 NA, Olympus) and collected by two pairs of photomultiplier tubes (PMTs H7422P-40, Hamamatsu). 560 DXCR dichroic mirrors and 525/50 and 607/70 emission filters (Chroma) were used to separate green and red fluorescence. The image acquisition was controlled by a customized version of the opensource software package ScanImage (Pologruto et al., 2003). For each slice culture, a single electroporated CA1 pyramidal cell was imaged at a resolution if 1024 x 1024 pixels, with a frame rate 0.23 Hz, and a Z-position stepsize of 0.75 µm. Following imaging, cultures were incubated in fresh culture medium supplemented with one of the following: 1 µM Clozapine N-

oxide dihydrochloride (CNO, Tocris 6329), 300 ng TrkB Fc (R&D Systems 688-TK), a combination of 1 μ M CNO and 300 ng TrkB Fc, or 1 μ I water, all at 37 °C. After three days of incubation, each cell was imaged again using the same parameters. Subsequently, the slices were fixed in 4 % PFA and stored at 4 °C until further use.

Spot detection and filament tracing

The raw image files acquired with the two-photon microscope contained two interleaved channels corresponding to detected red (tdTomato) and green (GFP) fluorescence. To enable further analysis, these files were preprocessed using FIJI (ImageJ). This process involved deinterleaving the green and red channels and subsequently merging them into a single stack. The final images were saved in TIFF format and used for further analysis.

Preprocessed TIFF files were further analyzed using the image analysis software IMARIS[™]. Therefore, the TIFF files were converted into IMARIS format (ims). As a preliminary step, a median filter (3x3x1) was applied to each file to reduce noise and enhance image quality, thereby optimizing the data for further analysis.

Analysis of pyramidal cells with ElpoMix1 in IMARIS

To optimize the spot detection from the GFP signal in the spines, it was important to minimize the presence of autofluorescent objects and microglia in the green channel, as both emit in the green and red spectra. To exclude these unwanted objects from the green channel, the surface function in IMARIS was used. A surface was created on the red channel, encompassing all microglia and autofluorescent objects. This surface was then subtracted from the green channel, resulting in a new green channel that displayed only the imaged pyramidal cell, with the green signal confined to the spines.

To quantify the total dendritic length of the imaged pyramidal cell, a semi-automatic reconstruction of the cell in the processed green channel using the Filament Tracing Tool in IMARIS[™]. This allows us to compare the structure of the neuron on both time points.

To detect the spines, the Spot Detection tool in IMARISTM was utilized. For automatic detection, the spot size diameter was set to 0.75 μ m. In the subsequent step, the Xtension tool "Spots Close to Filament" was applied to differentiate spots based on their distance from the filament center, separating those closer than 2 μ m from those further away. Only the spots within 2 μ m of the filament were retained for further analysis. Finally, any incorrectly detected spots were manually removed to ensure accuracy.

For image, excel files including all necessary parameter (e.g. filament length, spot size, spot number) were exported from IMARIS[™] and used for statistical analysis.

Analysis of pyramidal cells with ElpoMix2 in IMARIS

By expressing tDimer in the pyramidal neuron, the semi-automatic reconstruction was performed in the red channel. The generated filament was then used as a surface mask to isolate the pyramidal neuron in the green channel, removing any unwanted objects (as described in the previous chapter). This process allowed us to perform spot detection on the neuron as described above.

Statistical analysis

To assess changes in spine density over time, spine density was quantified by dividing the total number of detected spines by the total filament length (μ m) at each time point. The percentage change in the spine density between before and after CNO treatment (T1 and T2) was calculated by normalizing the spine density at T2 to T1 using the formula (Spine density T2/Spine Density T1*100) and expressing it as a percentage of the baseline (T1).

3.3. In vivo Gq-DREADD activation

3.3.1. Activation of Cre-ER recombinase by tamoxifen injection

(Z) 4 -Hydroxy-Tamoxifen (Sigma H7904) was dissolved in 99 % ethanol (1 mg/50 μ l) and then diluted with sterile corn oil (1 mg/100 μ l). After adding the corn oil, the solution was placed in a vacuum centrifuge to evaporate the ethanol. The solution was continuously protected from light until injection. To activate Cre recombinase and induce recombination, each mouse 8-10 weeks old (either sex) received an intraperitoneal injection (i.p.) of (Z) 4-hydroxy-tamoxifen at a dosage of 1 mg per 20 g of body weight. Following the injection, mice were monitored and weighed twice daily for the next three days. To ensure only microglia express the construct, we waited for at least 3 weeks until further experiments.

3.3.2. Electrophysiology in acute slices after Gq-DREADD activation

Activation of Gq-DREADD in vivo by CNO feeding

CNO (Tocris 6329) was dissolved in sterile water and diluted to a final concentration of 2 mM. Previously (Z) 4-Hydroxy-Tamoxifen -injected mice (aged 13-20 weeks) were fed with wet food containing CNO (1-1.2 mg/kg) to activate Gq-DREADD or water as a control, for a period of three days. All animals were housed in a single cage to ensure consistent CNO uptake over their food Following this treatment, the mice were utilized for acute slice preparation.

Acute hippocampal slice preparation and electrophysiology

Previously treated animals were anesthetized with 80 % CO₂ 20 % O₂ and decapitated. The brains were rapidly removed from the skull and cut along the midline. A thin cortical slice, approximal 12° off-parallel from the midline was removed. Both hemispheres were glued to the support of a Compresstome. Slices (350 µm) were cut in ice-cold artificial cerebrospinal fluid (ACSF 1/2.4) containing (in mM): 124 NaCl, 26 NaHCO₃, 10 D-Glucose, 1 MgSO₄, 1 NaH₂PO₄, 4 KCl, 2.4 CaCl₂.Subsequently, slices were left to recover at 33 °C for 15 min, followed by an incubation at 30 °C for 45 min in carbonated ACSF 1/2.4.

To measure field excitatory postsynaptic potentials (fEPSPs) in the CA1 region of the acute hippocampal slices, two concentric stimulation electrodes were positioned in the stratum radiatum. A bipolar recording electrode was then placed in the stratum radiatum, adjacent to the stratum pyramidale. The placement of these electrodes was optimized to achieve fEPSPs with an amplitude of approximately 2 mV, using a stimulation pulse of 200 µs and a current of approx. 800 µA. Subsequently, input-output curves were generated to calibrate the stimulation intensity, ensuring the evocation of fEPSPs at half-maximum amplitude. For baseline recordings, stimulation pulses of 200 µs were applied every 30 seconds to each electrode, with a 1-second delay between the two electrodes. After establishing a stable baseline for 30 minutes, the Schaffer collaterals were stimulated using a single theta-burst stimulation (TBS). The TBS protocol consisted of 10 bursts, each containing 5 pulses of 200 µs delivered at a frequency of 100 Hz every 200 ms. Following the TBS, fEPSPs were recorded for 1 hour. Analysis of the recorded potentials was performed using proprietary SynchroSlice software. The slope of the currents was calculated from 30 % to 70 % of the response amplitude. Recordings exhibiting baseline instability, defined as a drift exceeding 25 %, were excluded from further analysis.

3.3.3. Behavioral experiment

Previously, (Z) 4-Hydroxy-Tamoxifen injected mice with and without Gq-DREADD expression (GqDREADD+ and GqDREADD-) were used to investigate spatial learning and memory. To ensure that the behavior of GqDREADD+ mice was not altered (e.g., in terms general activity, exploratory behavior or anxiety levels), an open field experiment was performed beforehand with both groups. All animals were handled for three days before the experiment t to reduce stress during the behavioral task. After the open field experiments all animals were fed wet food containing CNO (1-1.2 mg/kg). To ensure consistent CNO uptake and to maintain identical conditions, all mice were housed in a single cage during the behavioral experiment.

Open Field Experiment for behavioral assessment

To exclude the possibility that potential behavioral differences in the following Morris water maze experiment are based on the two different genotypes (GqDREADD+ and GqDREADD-), an open field experiment was performed. Mice were placed in the center of a white square arena (50 x 50 cm, 20 LUX) for 30 minutes. During this time, mice were allowed to explore the arena freely.

For analysis, the arena was divided into different areas (center, periphery, and corners) to assess spatial preferences. After the 30-minute exploration period, each mouse was returned to its cage.

Pre-training for water maze experiment

All mice were pre-trained for 2 days before their exposure to the Water Maze (WM) arena. Each mouse underwent four training trials (max. 60 seconds) per day in a small rectangular water tank in the dark, in the same room as the WM arena. In the water tank is a 14cm diameter escape platform positioned 1cm below the water surface. The position of the platform was alternated between the left and the right side of the tank between trials, maintaining a distance of 5 cm from the walls to minimize thigmotaxis. Once the mouse found the platform, a grid was presented to climb onto it(Lamothe-Molina et al., 2022). After each trial, each mouse was returned to their home cages. After the first pre-training session, all mice were fed with wet food containing CNO for the next four days.

Morris water maze

The WM arena was a circular tank with a diameter of 1.45 meters, displaying asymmetrical visual landmarks on the walls and filled with water mixed with non-toxic white paint. An escape platform, positioned 1 cm below the water surface, was placed in the center of the east quadrant for all training trials. On the first day, each mouse underwent four training trials (TT), each lasting a maximum of 90 seconds. During each TT, mice were lowered into the tank facing the wall of the west quadrant at randomized positions (excluding the east quadrant) using an opaque, cup-sized chamber. Ten seconds after locating the platform, the mice were retrieved using a plastic grid on a pole and returned to their home cages. If a mouse did not find the platform within 90 seconds, it was guided to the platform using the grid and allowed to remain there for 10 seconds before being returned to its cage. A heat lamp was positioned over the waiting area to prevent hypothermia. On the second day, all mice performed two TTs followed by one probe trial (PT) with a maximum duration of 60 seconds. During the PT, the platform was removed to assess spatial memory, and the mice were introduced into the center of the tank and retrieved after 60 seconds of swim time. This was followed by two additional TTs. On the third day, the protocol included one PT followed by two TTs, another PT, and one final TT (Lamothe-Molina et al., 2022). Throughout all three training days, mice were fed with CNO (1-1.2 mg/kg) incorporated into their wet food. After a seven-day interval without CNO administration and training, spatial memory was assessed again with a single PT. Ninety minutes after the final PT, 12 mice were perfused and their brains extracted for further analysis, while the remaining animals were euthanized, and their brains were extracted and fixed in 4 % PFA for 24 h.

Behavioral Analysis

All behavioral data were recorded and analyzed using EthoVision XT software (Noldus information Technology). The automated video tracking provided by EthoVision ensured consistent detection of the animal movement across all trials. Data were extracted for each trial over the duration of the experiment.

3.4. Statistical Analysis

All statistical analyses were performed using Graphpad Prism software. The statistical results are provided in the figure legends or described in the results. Statistical significance was determined using a p-value of 0.05 and multiple comparisons were conducted where

appropriated. Criteria for inclusion or exclusion of data are specified in the methods section. Blinding was performed for all experiment types. Results

4. Results

4.1. Characterization of Gq-DREADD in microglia

4.1.1. Gq-DREADD expression in microglia

To investigate the impact of microglial Gq signaling on neuronal function, we crossed a transgenic mouse with inducible Cre recombinase under the *Cx3Cr1* promotor carrying additionally a floxed fluorescent marker tdTomato (Cx3cr1^{tm2.1/cre/ERT2)Jung/J Gt(ROSA)26-Sor^{tm9(CAG-tdTomato)Hze}) with a floxed Gq-DREADD mouse (Tg^{(CAG-CHRM3,-mCitrine)1Ute}/J). Tamoxifen administration induced Cre-mediated excision of an upstream floxed-STOP cassette, resulting in the expression of HA-tagged Gq-DREADD and tdTomato specifically in Cx3Cr1-positive cells. This crossing enabled specific labeling of microglial cells and precise manipulation of the Gq signaling pathway within these cells (Figure 7 A).}

Before assessing the impact of Gq-DREADD mediated microglia activation on neurons, we initially characterized the expression and function of Gq-DREADD in microglia. Immunostaining for the HA tag of the Gq-DREADD confirmed that the chemogenetic tool was specifically expressed in microglia within organotypic slice cultures (Fig. 1 B). Co-expression of HA and tdTomato was detected in microglia from GqDREADD+ slice cultures, but not in microglia from the GqDREADD- control group. These observations are consistent with previous reports showing that Gq-DREADD expression driven by the Cx3Cr1-CreERT2 system is restricted to microglia (Binning et al., 2020; Jung et al., 2000; Saika et al., 2021).

4.1.2. Chronic Gq-DREADD activation leads to morphological changes

The morphology of microglia is dynamically linked to their functional state in the CNS, including their roles in surveillance, immune response, and interactions with other cells (see Introduction). To investigate the effects of Gq-DREADD activation on microglial morphology in organotypic slice cultures, we compared the microglial morphology between the GqDREADD+ and GqDREADD- groups. Cultures from both groups were incubated in a medium containing 1 μ M CNO for three days.

Post-incubation, the slices were fixed with 4% PFA, stained against the microglia marker lonized Calcium-Binding Adapter Molecule 1 (Iba1), and imaged using a confocal microscope. Iba1 staining allowed for clear visualization of microglial cell bodies and processes (Figure 7

C). To quantify the complexity and branching patterns of the microglia, a semi-automatic Sholl analysis was performed.

Microglia in the GqDREADD+ group have fewer intersections at distances beyond approximately 20 µm from the soma compared to the control (GqDREADD-) group. The area under the curve (AUC) of the Sholl analysis, a cumulative measure of the number of intersections across all distances, was significantly lower in the GqDREADD+ group compared to the GqDREADD—control group (Figure 7D), indicating a reduction in microglial process complexity due to chronic Gq-DREADD activation.

4.1.3. Acute Gq-DREADD activation increases the frequency of spontaneous calcium transients in microglia

Previous reports indicated that Gq-DREADD activation increases intracellular Ca²⁺ levels (Armbruster et al., 2007a). To confirm that Gq-DREADD activation elevates intracellular Ca²⁺ in microglia, we bred tamoxifen-dependent Cx3Cr1 driver mice with floxed Gq-DREADD and floxed GCaMP6s (Gt(ROSA)26-Sor^{tm96(CAG-CGaMP6s)Hze}/J, (Madisen et al., 2015) mouse lines (Fig. 7E). This breeding strategy resulted in offspring expressing both GCaMP6s and Gq-DREADD specifically in microglia.

The green fluorescent-Calmodulin Protein 6s (GCaMP6s), is a genetically encoded calcium indicator, composed of a GFP fused to a calmodulin and the calmodulin-binding peptide M13. Upon binding with Ca²⁺ ions, GCaMP6s undergoes a conformational change that increases its fluorescence. These changes can be tracked during live imaging (Chen et al., 2013). We used two-photon microscopy to image organotypic slice cultures expressing Gq-DREADD and GCaMP6s in microglia. After recording calcium levels at baseline for 5 min, we washed in CNO to activate Gq-DREADD, with control cultures receiving no CNO treatment.

Following CNO application, a clear increase in fluorescence was observed in microglia, indicating an elevation in intracellular Ca²⁺ levels (Figure 1F). During the baseline period, microglia produced spontaneous calcium transients at a very low rate (Figure 1G). The frequency of these calcium transients greatly increased in the CNO-treated group, with individual transients merging into a plateau of elevated calcium (Figure 7G). To quantify these changes, the AUC of calcium transients was assessed before and after CNO application. The CNO-treated group showed a significant increase in total calcium while the controls did not (Figure 7H). Comparing the two groups after normalization to their respective baselines revealed a significant difference between groups (Figure 7I).

Results

These results demonstrate that acute Gq-DREADD activation through CNO treatment significantly increases intracellular Ca²⁺ levels in microglia. The calcium transients we observed most likely reflect release of calcium from intracellular stores, a process that is triggered by IP₃ receptors downstream of Gq. The morphological changes and increased calcium levels observed upon chronic and acute Gq-DREADD activation suggest that Gq-DREADD activation profoundly impacts microglial function.



Figure 7: Gq activation in microglia. (**A**). Strategy of DREADD delivery and activation in microglia. (**B**) Example confocal images of stained tdTomato and HA tag in organotypic hippocampal slices from GqDREADD+ or GqDREADD- mice. (**C**) Example confocal images of stained lba1 in organotypic hippocampal slices from GqDREADD+ or GqDREADD+ or GqDREADD- mice treated with CNO. (**D**) Relationship between microglial ramification and distance from soma in slices from GqDREADD+ or GqDREADD+ or GqDREADD+ or GqDREADD+ or GqDREADD- mice treated with CNO. (**D**) Relationship between microglial ramification and distance from soma in slices from GqDREADD+ or GqDREADD- mice that were traeted with CNO for three days. Data are shown as mean \pm SEM. (**E**) Area under curve of Sholl interaction curves (C). n = 328 (GqDREADD+), 203 (GqDREADD-) microglia; Unpaired t-test, **** P < 0.0001, t = 6.5, df = 529. (**F**) Strategy of GcaMP6s and DREADD delivery in microglia and example 2 photon images of calcium response before and after CNO wash in. (**G**) Example calcium response changes (Δ F/F0) of GcaMP6s-expressing microglia from GqDREADD+ organotypic slice cultures before and after CNO. Black trace shows calcium responses from microglia with or without CNO application; Sidak's multiple comparison test before – after CNO, ** P = 0.0013, no CNO, P = 0.967. (**I**) Baseline-normalized responses from (**F**) Sidak's multiple comparison test before – after CNO, ** P = 0.0042, no CNO, P = 0.979. Data are shown as mean \pm SD.

4.2. Effects of Gq activation in microglia on CA1 neurons

4.2.1. Chronic Gq-DREADD activation in microglia leads to sex-specific synaptic spine loss

Microglia exhibit a bidirectional function in regulating synaptic density, by promoting both spine loss and spine formation, depending on their functional state. Activation of microglia, through various signaling pathways in responds to neuroinflammation, injuries or neuronal activity can lead to targeted elimination of synapses to maintain synaptic homeostasis in the brain (see Introduction).

To determine whether the chemogenetic activation of Gq-DREADD in microglia would induce synaptic spine loss, we labeled all excitatory synapses on a CA1 pyramidal neuron. We performed single-cell electroporation of a Fibronectin intrabody generated with mRNA display (FingR) that binds to PSD95 (PSD95-FingR(eGFP)) (Figure 8A). The FingR contained additional elements for transcriptional regulation (Zinc finger), linking its expression to the target protein's level without altering its expression pattern or function (Gross et al., 2013). This allowed us to visualize all postsynaptic densities on the transfected neuron, providing insight into the changes in excitatory synapses.

To track synaptic changes over time, each electroporated neuron was imaged twice with a two-photon microscope, once before and once after a three-day incubation period in medium containing either CNO or no CNO (control). Gq-DREADD activation caused a visible reduction in synaptic density without altering the overall morphology of neurons (Figure 8B). To quantify the changes in synaptic density, a semi-automatically spot detection to count PSD95-postitve puncta was performed for both timepoints. Chronic activation of Gq-DREADD in microglia resulted in a significant loss of excitatory synapses compared to the sham-treated control group (Figure 8C). Next, we separate the data by the sex of the pups the slice cultures were produced from. Surprisingly, synapse loss was significant only in cultures derived from male pups, cultures from female pups were not affected (Fig 8 D).



Figure 8: Gq activation in microglia triggers excitatory synapse loss in cultures from male mice. (A) Excitatory synapses of CA1 neurons from GqDREADD+ organotypic slices that expressed PSD95-FingR intrabody and the soluble fluorescent protein Cerulean were imaged using a 2-photon microscope. Inset shows tdTomato labelled microglia and GFPlabeled PSD95 from a CA1 apical dendrite. (B) Example images of PSD95 distribution before and 3 days after Gq activation in microglia. (C) Synaptic density after 3 days of treatment (normalized to before treatment) in slices treated with CNO or water. n = 28 (CNO), 21 (water) neurons. Unpaired t-test, ** P = 0.0095, F = 1.32 (D) Same data as in (C) but sex-separated. n = 11/17 (CNO female/male), 8/13 (water female/male) neurons. Sidak's multiple comparison test CNO – water Male, ** P = 0.002, Female, P = 0.98; Sidak's multiple comparison test Male-Female CNO, * P = 0.024, Water, P = 0.88. Shown are data from individual neurons, line and error bars show mean±SD.

4.2.2. Scavenging BDNF effectively prevents spine loss

BDNF plays a critical role in maintaining dendritic spine density and synaptic plasticity (Zagrebelsky et al., 2020). It influences spine morphology and density through its interaction with TrkB receptors, and any imbalance in this signaling pathway can result in structural instability and spine retraction (Kellner et al., 2014). Moreover, Cramer et al. (2022) demonstrated that after ischemia, microglia release BDNF, leading to the downregulation of glutamatergic and GABAergic synapses (Cramer et al., 2022). Based on these findings, we investigated whether BDNF signaling contributes to the observed spine loss following chronic Gq-DREADD activation in our study.

Results

To block BDNF signaling in our system, we applied chimeric TrkB-FC, a BDNF scavenger, during the CNO treatment. TrkB-FC binds to BDNF, preventing it from interacting with its native receptors, thereby blocking BDNF-mediated signaling. Slice cultures were incubated for three days in medium containing both CNO and TrkB-FC, or in control medium containing only TrkB-FC.

Quantitative analysis of synapse density revealed that scavenging BDNF prevented the spine loss driven by Gq-DREADD activation in microglia (Figure 9B). Further breakdown of synaptic density by sex revealed that the spine loss prevention by TrkB-FC was specific to male cultures. In female cultures, there was no significant differences in spine density between any of the treatment groups, indicating that BDNF scavenging did not alter synaptic density (Figure 9C, D) These results align with our previous findings, in which Gq-DREADD activation led to spine loss primarily in males, highlighting the differential impact of microglial Gq signaling between sexes.

To test if spine density levels were already different between the groups before treatment, we compared the number of spines per micrometer (spines/µm) across each group. Although there was a wide range of spine densities between the groups, there was no significant difference within the same sex (Figure 9A). This consistency in baseline spine density confirms that the observed effects on spine density are attributable to the experimental treatments rather than pre-existing differences. However, females exhibited a slightly lower spine density compared to males.

In summary, these finding reveal that chronic Gq-DREADD activation in microglia leads to significant spine loss in CA1 pyramidal neurons, specifically in male cultures. This effect is mediated through BDNF signaling, as scavenging BDNF effectively prevented spine loss. The sex-specific nature of these effects where females did not exhibit significant changes in spine density under the same conditions, highlights an important difference in how microglial activation affect synaptic remodeling between males and females.



Figure 9: Excitatory spine loss due to Gq activation in male microglia is rescued by blocking TrkB receptor activation. (A) Sex differences in spine density before treatment. n = 11/17 (CNO female/male), 8/13 (water female/male), 15/10 (TrkB FC + CNO female/male), 6/5 (TrkB FC + water female/male) neurons. Sidak's multiple comparison test for each treatment group has a p > 0.05. (B) Synaptic density after 3 days of treatment (normalized to before treatment) in slices treated with CNO or water together with the BDNF scavenger TrkB FC. n = 25 (TrkB FC + CNO), 11 (TrkB FC + water) neurons. Uncorrected Fisher's LSD, CNO TrkB FC vs. CNO, P = 0.2367, water TrkB FC vs. water, P = 0.5085, TrkB FC CNO vs. TrkB FC water, P = 0.6041, CNO vs. water, ** P = 0.0086 (C-D) Same data as in (B) but sex-separated. (C) Cultures from female mice. Uncorrected Fisher's LSD, p > 0.05 for each comparison. (D) Cultures from male mice. Uncorrected Fisher's LSD, CNO TrkB FC vs. CNO, ** P = 0.0071; CNO vs. water, ** P = 0.0022. Shown are data from individual neurons, line and error bars show mean \pm SD. Solid bars represent no TrkB FC conditions from Figure 2 C-D.

Results

4.3. EPSPs in acute slices after Gq-DREADD activation in vivo

The observed reduction in dendritic spine density, which is crucial for synaptic strength and plasticity, raised the question of whether these structural changes also have functional consequences at the level of synaptic transmission. Measuring field excitatory postsynaptic potentials (fEPSPs) allows us to assess the functional impact of the observed spine loss after Gq-DREADD activation in microglia on synaptic transmission and plasticity. fEPSPs are electrical signals recorded from a population of neurons in response to synaptic input, reflecting the collective excitatory activity of many synapses. By examining fEPSPs, we aim to determine if the structural changes we observed translate into deficit in synaptic plasticity and the ability to undergo long-term potentiation.

To investigate this, animals expressing Gq-DREADD in microglia were fed with wet food containing either CNO or water (as control) for three days. Following this treatment, we performed field recordings in the CA1 region of the hippocampus while stimulating Schaffer collaterals with a single theta-burst stimulation (TBS) in acute brain slices obtained from these pretreated animals (Figure 10A).

In slices from the control group (without CNO administration), TBS induced stable long-term potentiation (LTP), with fEPSP amplitudes reaching approximately 300% of baseline levels 50-60 minutes post-induction (Figure 10B). In contrast, slices from animals treated with CNO exhibited significantly reduced LTP, with fEPSP amplitudes reaching only 150% of baseline levels during the same time window. Gq-DREADD activation in microglia impaired LTP in slices from both male and female mice (Figure 10D).

The input-output relationship, which measures synaptic strength in response to increasing stimulation intensities, showed no significant differences between the control and CNO-treated group (Figure 10C). This indicates that the observed reduction in LTP was likely due to specific changes in synaptic plasticity mechanisms rather than an overall reduction in synaptic efficacy.



Figure 10 Gq activation in microglia reduces excitatory drive and LTP in CA1. (A) GqDREADD+ mice were treated for 3 days with CNO or water before acute slice recordings were performed. fEPSPs were recorded in stratum radiatum in CA1 where LTP was induced by TBS. (B) Timeline of fEPSP responses (baseline normalized slope) to stimulation of CA3 axons in slices from mice treated with CNO or water. Data are shown as mean±SEM. Inset shows example fEPSP traces before and 1 hour after stimulation in either CNO or water treated conditions. n = 15 (CNO + TBS), 16 (CNO + ctrl), 21 (water + TBS), 8 (water + ctrl) recordings. (C) Input-output curves of fEPSP amplitude vs stimulation intensities. n = 15 (CNO + TBS), 21 (water + TBS) recordings. Unpaired t-test, P = 0.58, t = 0.56, df = 28. (D) Quantification of fEPSP slope (baseline normalized) 1 hour after LTP induction with TBS in CNO or water-treated conditions, sex separated. Ordinary two-way ANOVA Treatment, P = 0.01, F (1, 32) = 7.11, Sex, P = 0.91, F (1, 32) = 0.01, Treatment x Sex, P = 0.91, F (1, 32) = 0.01. Shown are data from individual recordings, box and error bars show mean±SD.

4.4. Behavioral experiments

Given that synaptic plasticity was affected after Gq-DREADD activation in microglia in acute hippocampal slices (see previous results), and considering the observed spine loss, we decided to test spatial learning and memory formation during Gq activation in microglia. Both functions are highly dependent on the hippocampus. To assess whether the microglial activation-induced changes in synaptic plasticity correlate with deficits in spatial memory, we used the Morris water maze. We compared animals expressing Gq-DREADD (GqDREADD+) to animals without Gq-DREADD (GqDREADD-). Both groups were fed with CNO for four days, during the training phase of the Morris water maze (Fig. 11A).

Previous studies have demonstrated that acute activation of Gq-DREADD in microglia results in the increase of pro-inflammatory cytokines mRNA expression levels, including TNF- α , IL-1 β , and IL-6 (Saika et al., 2021). This response resembles LPS-induced microglial activation, which is known to elicit sickness behavior in adult mice (Batista et al., 2019; Dantzer et al., 2008). Based on these findings, we hypothesized that Gq-DREADD activation would also lead to altered behavior in our experimental mice.

4.4.1. Open field exploration prior to Gq-DREADD activation

To rule out the possibility that the genotype itself (GqDREADD+ vs GqDREADD+) affected the behavior, we performed an Open Field experiment, to quantify general activity and exploratory behavior for both groups prior to CNO treatment. Both groups showed normal exploration behavior, and quantitative analysis of various parameters, including distance moved, time spent in the perimeter, and time spent in the center, revealed no significant differences between the two groups (Fig 11B-D). Specifically, both GqDREADD+ and GqDREADD- animals exhibited similar levels of locomotor activity, as indicated by comparable distance moved (Figure 11B), similar time spent in the perimeter (Figure 11C) and in the center of the arena (Figure 11D), suggesting that baseline anxiety and exploratory behavior were not affected by genotype.

4.4.2. Spatial learning and memory while Gq-DREADD activation in microglia

To assess spatial learning and memory, both groups of animals were fed CNO for four days, starting one day prior to the beginning of the training trials (TT). In total, all animals completed 11 TTs over the course of three days, in which mice had to learn the location of a hidden platform that was placed in the east quadrant of the maze. During these TTs, both groups performed similarly, with no significant differences in escape latency, distance to platform, or swim speed (Figure 11E-G). The escape latency (Figure 11E); distance to platform (Figure

11F), and swim speed (Figure 11G) all showed similar trajectories between GqDREADD+ and GqDREADD- groups, indicating that mice from both groups learned the location of the hidden platform at a similar rate, and there were no deficits in motor abilities or navigation capabilities due to the CNO treatment.

Differences emerged during the probe trials, which were conducted without the platform present, to assess how well the mice retained the information they had learned during training. During the first PT, GqDREADD+ animals showed a significantly greater preference for the east quadrant, where the platform had been located, compared to controls (Figure 11H, I). By the end of the three days of training, the differences between GqDREADD+ and GqDREADD- mice had disappeared, with both groups spending a similar amount of time in the target quadrant, indicating comparable spatial memory performance (Figure 11I). Another measure of water maze performance, the average distance to the platform position during the probe trials, was not different between both groups on any training day (Figure 11K), suggesting that the GqDREADD effect was relatively subtle.

To assess long-term memory retention, a remote memory probe trial was conducted seven days after the TT, without any additional training or CNO treatment in between. At this stage, GqDREADD+ mice exhibited impaired memory retention compared to controls. Specifically, GqDREADD+ mice spent significantly less time in the east quadrant and were farther from the former platform location compared to GqDREADD- mice (Figure 11H, I, K), suggesting that their spatial memory had degraded over time. By comparing the performance across all PTs within the same group, the control group (GqDREADD-) showed the expected learning curves, with significant improvement in time spent in the target quadrant and distance to platform over the three days of training and stable performance during the remote memory test (Figure 11J, L). In contrast, GqDREADD+ animals showed less improvement over the three days and a significantly impaired performance during the remote memory test, suggesting a deficit in remote memory retention. Male and female mice (open/solid triangles in Figure 11I and K) were equally affected by microglia Gq activation; we pooled the results to increase the statistical power.

Somewhat contrary to our expectations, these findings indicate that the Gq-DREADD activation in microglia does not induce sickness behavior, as evidenced by the similar performance of both groups in open field exploration and during the training trials. While spatial learning was largely intact, remote memory was impaired, in spite of the fact that Gq-DREADD was only activated during memory formation (training period), not during remote recall. The results Results

indicate that memories formed during microglia Gq-DREADD activation are less stable, which is consistent with the weaker LTP we found in the electrophysiological experiments.



Figure 11 description on next page.

Behavioral experiments

Figure 11: Gg activation in microglia affects remote memory in mice. (A) Experimental design: 3 weeks after treatment with tamoxifen, GqDREADD+ or GqDREADD- mice explored an Open Field (OF) arena: 1 day after the start of CNO treatment mice of both groups were trained to find a hidden platform in a Morris water-maze (TT: training trial); feeding with CNO was stopped after experimental Day 3, with probe trials happening on Day 2 (to asses STM: short-term memory), Day 3 (LTM: long-term memory, STM II: shortterm memory day 2) and Day 10 (Remote memory). (B-D) Distance moved, time spent in perimeter and time spent in center during open-field test. Sidak's multiple comparison test GqDREADD+ vs GqDREADDfor each time bin p > 0.05. Data shown as mean \pm SEM. (E-G) Escape latency, distance to platform and swim speed during the Morris water-maze training trials. Sidak's multiple comparison test GqDREADD+ vs GqDREADD- for each trial bin p > 0.05. Data shown as mean \pm SEM. (H) Heatmaps of average mouse location during the probe trials; guadrant East and platform location are marked. (I) Time spent in guadrant E during the probe trials of GgDREADD+ and GgDREADD- mice (male: empty triangles, female: solid triangles). Tukey's multiple comparison test (GgDREADD+ vs GgDREADD-): STM, *P = 0.0444; LTM, P = 0.2585; STM II, P = 0.7337; Remote, * P = 0.0298. Data shown as mean ± SD. (J) Time spent in quadrant E during the probe trials. Tukey's multiple comparison test (GqDREADD+: STM II vs Remote, *P = 0.0327. GqDREADD-: STM vs STM II, **P = 0.0036; STM vs Remote, *P = 0.0224; LTM vs STM II, ** P = 0.0028; LTM vs Remote, *P = 0.0181). Data shown as mean ± SD. (K) Average distance to platform during the probe trials of GqDREADD+ and GqDREADD- mice (male: empty triangles, female: solid triangles). Tukey's multiple comparison test (GqDREADD+ vs GqDREADD-): STM, P = 0.1711; LTM, P = 0.0718; STM II, P = 0.8490; Remote, *P = 0.0163. Data shown as mean \pm SD. (L) Distance to platform during probe trials. Tukey's multiple comparison test (GqDREADD+: LTM vs STM II, *P = 0.0375; STM II vs Remote, *P = 0.0205; GqDREADD-: STM vs STM II, **P = 0.0091; STM vs Remote, *P = 0.0201; LTM vs STM II, ****P < 0.0001; LTM vs Remote, ****P < 0.0001). Data shown as mean ± SD. TAM tamoxifen, OF open field, TT training trial, STM short-term memory, LTM long-term memory. n = 13 (GqDREADD+), 12 (GqDREADD-) animals.

5. Discussion

5.1. Gq-DREADD activation in microglia leads to a morphological and functional shift

In my study, I used the DREADD technology to investigate the effects of selective activation of Gq-signaling in microglia on hippocampal neurons, focusing on synaptic changes. First, I characterized the expression and function of the Gq-DREADD in microglia. I demonstrated that the Gq-DREADD is selectively expressed in microglia, and its chemogenetic activation results in an increase in intracellular calcium levels and a shift from a highly ramified state to a less complex morphology. These changes indicate that microglia shifted from their normal surveillance state, where they monitor the brain environment, to a more activated state, which is commonly associated with pro-inflammatory activity.

Intracellular calcium signaling plays a critical role in regulating the ramified morphology of microglia. Under physiological conditions, microglia exhibit low basal levels of calcium activity, with localized, transient calcium signals associated with minor process adjustments during surveillance (Eichhoff et al., 2011; M. È. Tremblay et al., 2011). This low calcium activity is generally mediated by transient receptor potential (TRP) channels and purinergic receptors (P2Y12, P2X7 and P2Y6) and plays a role in modulating microglial motility and their ability to interact with synapses without inducing a full immune response (Kettenmann et al., 2011). In response to injuries, inflammation and neuronal distress, microglial intracellular calcium levels increase, driving various functional responses in microglia, such as process retraction, leading to morphological changes, from a highly ramified to a less ramified or amoeboid state and cytokine release (Hoffmann et al., 2003; Imai & Kohsaka, 2002; Mizoguchi & Monji, 2017; Umpierre et al., 2020). This less ramified shape is typically associated with a higher phagocytosis activity (Hanisch & Kettenmann, 2007). Notably, the activation of the Gq-linked GPCR P2Y6 has been shown to enhance microglial phagocytosis. Upon UDP binding, the P2Y6 receptor triggers intracellular calcium signaling and promotes cytoskeletal changes to enable microglia to efficiently clear apoptotic cells and debris during neuroinflammation (Koizumi et al., 2007; Neher et al., 2011).

In my study, microglial morphological changes occurred upon CNO activation of the Gqcoupled DREADD, which stimulates PLC, leading to the production of IP₃ and DAG. IP₃ binds to its receptor on the ER, causing the release of calcium from intracellular stores (Armbruster et al., 2007a), which in turn triggers the retraction of processes, the hallmark of a more proinflammatory state. These results are in line with previous research, demonstrating that activation of Gq-DREADD in microglia induces an increase in intracellular calcium signals and an upregulated expression of TNF- α , IL-1 β and IL-6 mRNA (Binning et al., 2020; Grace et al., 2018; Saika et al., 2021).

Together, these findings suggest that chemogenetic activation of Gq-DREADD in microglia effectively induces a transition into a pro-inflammatory phenotype, notably in the absence of traditional pathological stimuli such as injury or infection. Microglia Gq activation not only leads to increased calcium levels but also drives morphological changes, which might trigger the release of cytokines typically associated with inflammation (Binning et al., 2020; Hoffmann et al., 2003; Mizoguchi & Monji, 2017; Saika et al., 2021). By using the Gq-DREADD strategy, we can selectively study the effects of activated microglia on neuronal function, isolating the impact of microglial activation from other confounding factors.

5.2. Gq-DREADD activation in microglia induces structural and functional changes in neurons

The primary objective of my study was to explore the effects of Gq activation in microglia on neuronal function. I successfully demonstrated that chemogenetic activation of microglia results in both structural and functional alterations in neurons, as observed *in vitro* and *in vivo*. In hippocampal slice cultures, Gq-DREADD activation reduced the number of excitatory synapses on CA1 pyramidal neurons. Notably, this reduction was only evident in slice cultures derived from male pups, highlighting a sex-specific response to microglial activation. Synapse loss was contingent on intact BDNF signaling, raising questions about the relationship between neurotrophin secretion, microglia activation and spine density.

Additionally, I demonstrated that Gq-DREADD activation in microglia *in vivo* attenuates the ability of synapses to undergo plastic changes, resulting in an impaired LTP *ex vivo*. Similar input-output relationships in control and CNO group suggested that LTP impairment was not due to a decrease in baseline synaptic transmission, but rather linked to deficits in the mechanism of synaptic potentiation. Interestingly, Gq-DREADD activation during spatial training did not prevent spatial learning or short-term memory; instead, it specifically affected remote spatial memory. In line with the plasticity deficits measured *ex vivo*, the remote memory deficit was not sex-specific (Table 4).

The discrepancy in sex-specific effect observed between *in vitro* and *in vivo* may be explained by several mechanism, including differential microglial activation pathways, the developmental stage of microglia, and the varying role of BDNF in synaptic regulation. Additionally, the distinct experimental environments (*in vitro* vs. *in vivo*) may contribute to the variability in microglia-
neuronal interaction and their downstream effects on synaptic plasticity and memory (discussed in detail in chapter 5.4).

5.3. Cytokines and BDNF: modulators of synaptic density and synaptic plasticity

Previous research, as well as the one presented in this thesis, shows that Gq-DREADD activation leads to an increase in intracellular calcium and may trigger a release of proinflammatory cytokines such as IL-1 β , TNF α , and IL-6 (Binning et al., 2020; Hoffmann et al., 2003; Mizoguchi & Monji, 2017; Saika et al., 2021). Under normal physiological conditions, these cytokines are expressed at low levels and support memory and plasticity by modulating synaptic strength (Bourgognon & Cavanagh, 2020). For instance, low levels of IL-1ß support spatial learning and contextual memory, enhancing processes like LTP, which is crucial for memory consolidation (Depino et al., 2004; Goshen et al., 2008). Similarly, TNF-α facilitates synaptic scaling by regulating AMPA receptor trafficking, which maintains the balance between excitatory and inhibitory neurotransmission(Beattie et al., 2002; Stellwagen & Malenka, 2006). However, in the context of inflammation, elevated expression levels and prolonged exposure has been associated with synaptic weakening, disruption of LTP and ultimately result in increased spine loss (Cunningham et al., 2009; Kempuraj et al., 2017; Murray & Lynch, 1998; Pickering et al., 2005; Stellwagen & Malenka, 2006; Viviani et al., 2003). Specifically, microgliaderived TNFa and IL-1ß reduce AMPA receptor phosphorylation and modulate the subunit composition of both NMDA and AMPA receptors, leading to impaired excitatory synaptic transmission (Kleidonas et al., 2023; LEONOUDAKIS et al., 2004; Riazi et al., 2015). By modifying receptor subunit composition, these cytokines can alter the strength and plasticity of excitatory synapses, thereby inhibiting critical processes underlying memory and learning. Additionally, chronic elevation of IL-1^β impairs LTP and memory by activating MAPK pathways and interfering with NMDA receptor function (Hein et al., 2010; Hoshino et al., 2017, 2021; Vereker et al., 2000).

This inflammatory environment may facilitate the reduction in spine density and contribute to impaired LTP and memory deficits, as sustained cytokine release disrupts the balance of excitatory and inhibitory neurotransmission. However, one key finding in my study is the prevention of spine loss through the use of the BDNF scavenger TrkB-FC, which highlights the crucial role of BDNF signaling in microglia-induced synaptic changes. This suggests that BDNF signaling is a critical downstream effector in the structural and functional changes induced by microglial activation. Furthermore, these results indicate that the mere activation of microglia

and the potential release of cytokines may not be sufficient to trigger the observed synaptic changes. Rather, it appears that BDNF signaling, in conjunction with microglial activity, is necessary to drive the synaptic remodeling processes that lead to spine loss and alterations in synaptic plasticity and memory.

BDNF is a well-known modulator of synaptic plasticity and plays a pivotal role in both the formation and elimination of synapses. It is expressed as a proprotein proBDNF and a mature form mBDNF. The proBDNF form binds to p75 receptor and promotes synapse weakening and spine retraction (Yang et al., 2009), whereas the mBDNF preferentially binds to the neurotrophin receptor tyrosine kinase B (TrkB) enhancing excitatory neurotransmission and exerting neuroprotective functions (Minichiello, 2009; Tanaka et al., 2008). This BDNF-TrkB signaling can also act in an autocrine fashion within a single dendritic spine. The release of BDNF and subsequent activation of the TrkB receptor in the same spine are shown to be essential for both structural and functional LTP. This highlights the importance of BDNF in modulating synaptic plasticity and memory at the level of individual synapses (Harward et al., 2016). However, dysregulation in BDNF-TrkB signaling, such as an abnormal increase or decrease in BDNF levels, can disrupt synaptic plasticity and impair LTP.

My results support those of a recent study that uncovered a similar mechanism after oxygenglucose deprivation in hippocampal slice culture. The authors show that microglia release BDNF, and that a subsequent reduction in both glutamatergic and GABAergic synapse density was mediated by elevated levels of proBDNF and mBDNF, respectively. The authors implied that proBDNF, acting on the p75^{NTR} receptor, activates the Rho/Rac1 pathway, which is involved in actin remodeling, leading to synapse collapse. Notably, spine loss could also be prevented by BDNF scavenging using the TrkB-FC chimera (Cramer et al., 2022).

While my results are consistent with this study, the exact cellular source of BDNF remains to be clarified. BDNF can be expressed and released by different cell types including neurons and astrocytes. In neurons, BDNF release is induced by neuronal activity, while astrocytes and microglia release BDNF in response to extracellular nucleotides, pro-inflammatory factors or neuropeptides (Arévalo & Deogracias, 2023; Coull et al., 2005; Prowse & Hayley, 2021; Trang et al., 2009; Ulmann et al., 2008; Vignoli et al., 2016). However, there is an ongoing controversy regarding whether microglia directly release BDNF or trigger its release from other cell types. Several *in vitro* studies demonstrated that microglia express BDNF after LPS stimulation (Prowse and Hayley, 2021) and in response to ATP through P2X4R signaling (Coull et al., 2005; Malcangio, 2017; Trang et al., 2009). Additionally, conditional knockout of microglial BDNF impairs spine formation in the motor cortex and blocks neuronal hyperactivity in the

somatosensory cortex (Huang et al., 2021; Parkhurst et al., 2013c). However, other research indicates that microglia do not express or only express a very low level of BDNF, which may not be sufficient to modulate neuronal function (Ayata et al., 2018; Bennett et al., 2016; Denk et al., 2016; Honey et al., 2022; Kang et al., 2018).

In the context of my study, the Gq-DREADD activation in microglia may initiate the release of pro-inflammatory cytokines, which stimulate BDNF release from microglia or from nearby cells. I propose that BDNF works synergistically with pro-inflammatory cytokines to promote retraction or elimination of spines in a microglial activity-dependent manner. This finding aligns with previous studies showing that spine density is correlated with LTP capacity (Bourne & Harris, 2008; Kasai et al., 2010).

However, the mechanism through which BDNF signaling mediates synapse elimination is still uncertain: Does BDNF promote synapse trogocytosis (Weinhard, Di Bartolomei, et al., 2018a), tag synapses via complement proteins for microglial pruning (Stevens et al., 2007), or destabilize synaptic connections, leading to their natural retraction. Future work should focus on answering such questions, as untangling the cellular and molecular mechanism is crucial for the understanding of the observed effect.

Other directions that would be worthwhile pursuing are that of directly assessing the spine density of CA1 pyramidal neurons following Gq-DREADD activation in microglia *in vivo*, as well as investigating the effects of BDNF signaling in the same condition. While I could show BDNF-dependent spine loss on organotypic slice cultures, it is unlikely that BDNF levels in slice culture are identical to the *in vivo* situation. *In vivo* imaging with single-synapse resolution would be a suitable approach to address these questions in the future.

The observed LTP and remote memory impairment could be driven by mechanisms independent of spine loss or BDNF, potentially involving the direct effects of pro-inflammatory cytokines on synaptic receptor function, such as alterations in AMPA and NMDA receptor activity (Stellwagen & Malenka, 2006; Viviani et al., 2003). Therefore, it remains possible that LTP and memory impairments result from cytokine-mediated synaptic dysfunction, BDNF-dependent spine loss, or a combination of both.

5.4. Sex-specific effects in organotypic slice culture

One intriguing finding presented in this thesis is the sex-specific nature of the observed spine loss. Only neurons in male-derived hippocampal slice cultures exhibited significant spine loss following chronic microglial activation, whereas female neurons appeared to be resistant. This difference is consistent with previous research showing that microglial activity differs between male and female mice (Bobotis et al., 2023). Male microglia exhibit a higher expression of genes associated with immune response, chemotaxis and inflammatory pathways, while female microglia display increased expression of neuroprotective genes and genes related to cell-to-cell communication and cytoskeletal organization (Guneykaya et al., 2018; Villa et al., 2018). This suggests that male microglia are more prone to adopting a reactive inflammatory phenotype. Additionally, Vegeto et al., (2001) showed that estrogen prevents LPS induced inflammatory response in microglia. This protective effect of the sex hormone might also explain the preservation of synapses and the lack of spine loss in the female-derived cultures. Nevertheless, in our *ex vivo* measurements of synaptic plasticity and *in vivo* water maze experiments, both sexes were equally affected (Table 4.).

Table 4:	Overview	of sex-spe	cific effects

	Synapse density	LTP	Remote memory
male	**↓	\downarrow	\downarrow
female	ns	\rightarrow	\downarrow

This discrepancy in sex-specific effects could be due to several factors. A primary explanation could be differences in developmental stage. Slice cultures were derived from pups at postnatal days 4-6 (P4-6) and incubated for 3 weeks (21 DIV) prior to experiments. In contrast, *in vivo* activation of microglia for LTP and water maze experiments was performed in 12-20 weeks old animals. Sex differences in microglia, particularly their effects on hippocampal spine density and morphology, are most prominent during the early postnatal period and nearly disappear by P40 (Weinhard, Neniskyte, et al., 2018). Thus, both microglia and neurons in our slice culture experiments were not fully mature, consistent with the strong sex differences we observed. Unfortunately, it was not possible to culture mouse brain slices for much longer than 4 weeks: Imaging older cultures revealed a large fraction of microglia with retracted processes, suggesting an inflammation-like state. Our findings underscore the importance of considering both the age and the sex of the microglia when investigating microglia-neuron interactions.

5.5. Chemogenetic activation of microglia does not alter exploratory behavior

My study demonstrates that chemogenetic activation of microglia does not lead to sickness behavior, as evidenced by the absence of any significant alterations in escape latency, distance to the platform, or swim speed during the training trials in the Morris Water Maze. This finding Discussion

is particularly surprising given that Gq activation in microglia would likely elevate proinflammatory cytokine levels (Binning et al., 2020; Grace et al., 2018; Saika et al., 2021). In classical models of systemic inflammation, such as LPS or Poly I:C administration, proinflammatory cytokines induce robust sickness behaviors characterized by reduced locomotion, anorexia, social withdrawal, and lethargy (Cunningham et al., 2007, 2009; Dantzer et al., 2008). Additionally, these models lead to significant cognitive impairments, particularly in tasks related to memory and learning, as inflammation-driven cytokine activity disrupts hippocampal function (Qin et al., 2007). These behaviors are adaptive responses that help conserve energy during infection but can severely hinder studies that rely on normal motor and cognitive performance. Using models like LPS or Poly I:C for cognitive research is problematic, as sickness behavior profoundly affects behavioral readouts of cognitive performance (Cunningham et al., 2007; Raison et al., 2006).

The lack of sickness behavior in the present model highlights that specific activation of microglia via Gq-DREADD differs fundamentally from systemic LPS-induced activation. While LPS induces a systemic inflammatory response with widespread cytokine release, chemogenetic activation affects only the brain, which is apparently not sufficient to produce the sickness behavior. The absence of peripheral immune activation, which is a key driver of the LPS-induced sickness response, could explain the behavioral differences. The absence of sickness behavior is crucial as it enables the investigation of microglial activation effects on learning and memory without the confounding influences of behavioral and motor deficits. It has also important implications for animal welfare, as it allows studying the consequences of microglia activation *in vivo* with no apparent effect on the wellbeing of the experimental animals.

5.6. Gq-DREADD activation in microglia impairs remote memory, but not spatial learning

My study uncovered an interesting dissociation between LTP impairment and the behavioral outcomes related to spatial learning and memory. Despite the impaired LTP following Gq-DREADD activation in microglia, no deficit was observed in the spatial learning task during the training phase of the Morris water maze. However, a significant deficit was found in remote memory, particularly when spatial memory was assessed after seven days without further training. This suggests that the transition to long-term memory or the retrieval of remote memory is impaired after Gq-DREADD activation in microglia.

Spatial learning involves rapid formation of short-term memories, which can support performance across multiple trials. This short-term memory does not necessarily rely on LTP, but on temporary synaptic plasticity changes in synaptic transmission in the hippocampus that allow for initial learning (D'Hooge & De Deyn, 2001; Morris, 1984). However, for the transition into long-term memory, a more robust synaptic plasticity such as LTP is required in order to consolidate and stabilize memory(Bliss & Collingridge, 1993; Morris, 1984). A recent study demonstrated that microglia activation using clodronate during the early phase of LTP inhibits the maintenance of late LTP, indicating that microglia are involved in memory-related processes at a specific time window (Raghuraman et al., 2019), which aligns with our results. Nevertheless, the long-term memory (LTM) probe trials were performed almost 18 h after the last training and both groups behaved the same, suggesting that the LTP persisted for this phase.

This discrepancy might also arise from the differential timing of Gq-DREADD activation during the water maze experiment in comparison to the acute slice recordings. In the MWM experiments, Gq-DREADD was activated during the training phase, whereas LTP was measured after three days of Gq-DREADD activation. Hence, we might not have seen any difference in LTM because the Gq-DREADD activation was too acute to actually induce structural and functional changes in the neurons as observed in the other experiments. Further experiments are needed in order to investigate if spatial learning is still intact when Gq-DREADD activation in microglia occurs before the training phase.

In summary, *in vivo* Gq-DREADD activation in microglia leads to LTP impairment, which may result from cytokine-mediated disruption of synaptic function or through BDNF-dependent spine density loss and contribute to remote memory deficits. The intact spatial learning suggests that initial encoding and short-term memory formation are less dependent on the sustained synaptic modifications required for long-term memory consolidation. Future studies should aim to dissect the specific molecular pathways by which Gq-activated microglia impair LTP and how this affects memory at different stages, particularly focusing on the role of cytokines and BDNF in synaptic plasticity and memory retrieval.

5.7. Open questions and alternative mechanism beyond cytokines and BDNF

While the involvement of pro-inflammatory cytokines and BDNF signaling provides a compelling explanation for the observed LTP impairment and synaptic changes, it is essential to recognize that other potential mechanisms may also contribute to these outcomes. In this

study, we focused primarily on the effects of Gq-DREADD activation in microglia on neuronal function, such as LTP and synaptic density. However, we have not yet explored how microglia themselves respond to chronic Gq activation beyond their morphological changes, or how astrocytes, which closely interact with microglia, may play a role in these processes. This lack of direct assessment of microglial and astrocytic responses presents a significant limitation in our current understanding.

One key observation in my study is the morphological shift in microglia following Gq-DREADD activation, characterized by a less ramified state. Microglial morphology is closely tied to their functional state, and these changes could influence motility, surveillance, and receptor expression. The shift in microglial morphology may impact microglial-synapse interactions, potentially affecting synaptic stability and plasticity. Recent studies have shown that the P2Y12 and CX3CR1 receptors present on microglia are critical for synapse surveillance, and that inflammation downregulates these receptors, reducing microglial interactions with synapses (Cardona et al., 2006; Haynes et al., 2006). The chronic Gq-DREADD activation may have led to reduced P2Y12 or CX3CR1 signaling, impairing microglial-synapse interactions and contributing to LTP deficits. Both the P2Y12 receptor and direct microglia-synapse contacts have been shown to be necessary for synaptic plasticity (Pfeiffer et al., 2016; Sipe et al., 2016).

Another potential mechanism that could explain LTP impairment involves the release of prostaglandin E2 (PGE2) by activated microglia and astrocytes. Pro-inflammatory cytokines, such as interleukin-1 β and tumor necrosis factor- α , stimulate the expression of cyclooxygenase-2 (COX-2) and microsomal PGE2 synthase-1 in microglia, leading to increased production of PGE2 during inflammatory responses (Ikeda-Matsuo et al., 2005; Johansson et al., 2013). PGE2 can negatively regulate synaptic strength through its interaction with EP2 and EP3 receptor subtypes, which modulate signaling pathways such as cAMP and CREB activity, influencing NMDA receptor function and contributing to LTP deficits (Ahmad et al., 2006; Akaneya & Tsumoto, 2006). Microglia and astrocytes play critical roles in neuroinflammation by producing PGE2, which amplifies the inflammatory milieu and alters synaptic environments (de Oliveira et al., 2008; D. Zhang et al., 2009). These combined effects of prostaglandins, pro-inflammatory cytokines, and microglial dysfunction are likely to contribute to synaptic impairments observed and further studies are needed to dissect these interactions.

These examples clearly illustrate that many questions remain unanswered, and further studies are required to fully understand the complex mechanisms underlying microglia-mediated synaptic changes and LTP impairment following Gq-DREADD activation. While the role of pro-

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inflammatory cytokines and BDNF signaling provides important insights, it is evident that additional mechanisms, including microglial motility, receptor expression, and astrocyte involvement, may play crucial roles in shaping the observed outcomes.

Future experiments should focus on analyzing the molecular and functional states of microglia during chronic activation. Techniques such as RNA sequencing could help identify changes in microglial gene expression, particularly those involved in inflammation, motility, and synapse interaction, such as P2Y12 and CX3CR1.

The potential role of astrocytes in modulating synaptic changes and LTP impairment cannot be excluded and needs to be investigated in-depth. Astrocytes are key regulators of synaptic homeostasis and are known to interact closely with microglia, particularly during neuroinflammation (Matejuk & Ransohoff, 2020). It is essential to explore whether astrocyte activation contributes to the observed effects, including the release of BDNF. Understanding how microglia and astrocytes communicate and coordinate their roles during chronic activation will be critical for gaining a comprehensive picture of the underlying cellular and molecular processes.

6. Conclusion

Despite the mentioned limitations and open questions, my study has demonstrated that Gq-DREADD activation in microglia serves as an effective and versatile model for investigating the interaction between microglia and neurons. By specifically activating the Gq signaling pathway in microglia, this model allows for precise manipulation of microglial activity and provides valuable insights into how chronic microglial activation affects synaptic structure and plasticity. The observed effects on synaptic spine loss, LTP impairment, memory deficits, and the involvement of BDNF signaling highlight the critical role that microglia play in modulating neuronal function. The microglial Gq-DREADD model offers a powerful tool for further unraveling the communication between microglia, neurons, and other glial cells, ultimately contributing to a deeper understanding of neuroinflammation, synaptic regulation, and neurological diseases. Conclusion

7. Literature

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Appendix

List of Abbreviations

7TM	Seven transmembrane alpha-helices
ACSF	Artificial Cerebrospinal Fluid
ADP	Adenosine Diphosphate
AMPA	α -Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ATP	Adenosine Triphosphate
AUC	Area Under the Curve
BBB	Blood-Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
Ca ²⁺	Calcium Ion
CA	cornu ammonis
CaMKII	Calcium/Calmodulin-Dependent Protein Kinase II
cAMP	Cyclic adenosine monophosphate
CCL2	Chemokine (C-C motif) Ligand 2
CD39	Cluster of differentiation 39
CD73	Cluster of differentiation 73
CNO	Clozapine-N-Oxide
CNS	Central Nervous System
Cre-ER	Cre Estrogen Receptor
CR3	Complement Receptor 3
CX3CR1	C-X3-C Motif Chemokine Receptor 1
DAG	Diacylglycerol
DAMP	Damage-Associated Molecular Pattern
DG	Dentate Gyrus
DIV	Days In Vitro
DREADD	Designer Receptor Exclusively Activated by Designer Drug
ECM	Extracellular Matrix
ER	Endoplasmic Reticulum
fEPSP	Field Excitatory Postsynaptic Potential
FOV	Field of View
GABA	Gamma-Aminobutyric Acid

GDP	Guanosine diphosphate	
GFP	Green Fluorescent Protein	
GPCR	G-Protein-Coupled Receptor	
GRK	G-Protein-Coupled Receptor Kinase	
GTP	Guanosine triphosphate	
hM3Dq	Human M3 muscarinic receptor	
hM4Di	Human M4muscarinic receptor	
IFN	Interferon	
IL	Interleukin	
IL-1β	Interleukin-1 Beta	
IL-6	Interleukin-6	
IL-10	Interleukin-10	
IL-33	Interleukin-33	
IP ₃	Inositol 1,4,5-Trisphosphate	
LPS	Lipopolysaccharide	
LTD	Long-Term Depression	
LTM	Long-Term Memory	
LTP	Long-Term Potentiation	
МАРК	Mitogen-Activated Protein Kinase	
Mg ²⁺	Magnesium Ion	
mBDNF	Mature Brain-Derived Neurotrophic Factor	
mGluR	Metabotropic Glutamate Receptor	
MWM	Morris Water Maze	
NA	Numerical Aperture	
Na⁺	Sodium Ion	
NF-κB	Nuclear Factor Kappa B	
NMDA	N-Methyl-D-Aspartate	
NMDAR	NMDA Receptor	
NO	Nitric Oxide	
PAMP	Pathogen-Associated Molecular Pattern	
PBS	Phosphate-Buffered Saline	
PCR	Polymerase Chain Reaction	
PFA	Paraformaldehyde	

Appendix

PKC	Protein Kinase C
PLC	Phospholipase C
PLCβ	Phospholipase C Beta
PMT	Photomultiplier Tube
PSD95	Postsynaptic Density Protein 95
PT	Probe Trial
p75NTR	p75 Neurotrophin Receptor
ROI	Region of Interest
ROS	Reactive Oxygen Species
RT	Room Temperature
SEM	Standard Error of the Mean
STM	Short-Term Memory
STP	Short-Term Plasticity
TBS	Theta-Burst Stimulation
TGF-β	Transforming growth factor beta
TLR	Toll-Like Receptor
TNF-α	Tumor Necrosis Factor Alpha
TrkB	Tropomyosin Receptor Kinase B
TrkB-FC	Tropomyosin Receptor Kinase B Fc Fusion Protein
TREM2	Triggering Receptor Expressed on Myeloid Cells 2
TRP	Transient Receptor Potential
ТТ	Training Trial
UDP	Uridine Diphosphate

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Declaration of oath

EIDESSTATTLICHE VERSICHERUNG

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne unzulässige fremde Hilfe verfasst habe. Alle Stellen, die wörtlich oder sinngemäß aus Veröffentlichungen oder anderen Quellen entnommen wurden, sind als solche kenntlich gemacht. Die Arbeit wurde in gleicher oder ähnlicher Form keiner anderen Prüfungsbehörde vorgelegt.

Hamburg, den 12.12.2024

Marie-Luise Brehme