The Role of P2Y12 in Non-Pathological Microglial Functions during Synaptic Plasticity

by

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Dedication

To my parents who taught me to never stop asking questions.

I haven’t.
Biographical Sketch

The author was born in Leominster, Massachusetts and grew up in Minnesota. He attended Lafayette College in Easton, Pennsylvania and graduated with a Bachelor of Science degree with Honors in Neuroscience. He began doctoral studies in Neuroscience at the University of Rochester School of Medicine & Dentistry, Rochester, New York, in 2010. He was awarded an F31 Predoctoral NRSA Fellowship in 2013 and pursued his research in glial biology and synaptic plasticity under the direction of Dr. Ania K. Majewska.

The following publications were the result of work conducted during doctoral study:


Abstract

Synaptic plasticity is critical for neurodevelopment and proper function of the adult nervous system. Studies show that microglia play critical roles in neurodevelopment, but mechanisms driving these roles are poorly understood. I explored purinergic signaling as a potential mediator between microglia and neurons during synaptic plasticity. Purinergic signaling has been implicated in microglial behavior, but studies focused on inflammatory roles. Non-inflamed microglia highly and selectively express the purinergic receptor, P2Y12, which functions in microglial chemotaxis. I posited that purinergic signaling contributes to the microglial motility underlying synapse surveillance and may be critical for microglial roles in synaptic refinement. My evidence suggests that P2Y12 disruption prevents ocular dominance shifts indicative of synaptic plasticity. P2Y12 disruption also decreases microglial process complexity, without affecting basal microglial process dynamics. In addition, microglial process dynamics appear to be regulated by arousal with increased surveillance during slow-wave sleep-like states. I find that noradrenergic signaling, contributing to arousal, is sufficient to suppress microglial process dynamics and inhibit microglial P2Y12-mediated roles in synaptic plasticity via microglial β2 adrenergic receptors. These data suggest microglia are active participants in cortical network remodeling in adolescent synaptic plasticity primarily during sleep states. These results not only describe novel neuro-immune interactions in the non-pathological brain, but provoke broader considerations of the importance of sleep in microglial roles during neurodevelopment and in neuropathology.
Contributors and Funding Sources

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CHAPTER I
Introduction

Overview of glial biology

Over the last several decades, a growing body of research has demonstrated that non-neuronal, glia cells play increasingly diverse and critical functions in normal and abnormal neurophysiology\textsuperscript{1,2}. Though once thought to be simply passive structural support cells for neurons, glia have been shown to influence and regulate important brain functions from structural alterations in neuronal networks during neurodevelopment and synaptic plasticity to modulating the excitability and tuning of neurons in adult physiological and pathophysiological conditions. Although neurons are still the principal units of information flow in the brain, it is now clear that they operate in concert with associated glia and that critical signaling between neurons and glia maintains an adaptive and functional nervous system. The importance of this relationship is evident in the wide range of neuropathologies associated with glial dysfunction\textsuperscript{3-5}. Although there is an increasing awareness and understanding of glial roles in the brain, many of the underlying mechanisms governing neuron-glial signaling and the functional importance of these interactions remain poorly understood.

The term “neuroglia” (Greek—‘glue’) was first used by Rudolf Virchow in 1858 to describe the non-neuronal cells interspersed throughout the brain parenchyma\textsuperscript{6}. Evident by their adapted name, the main purpose of glia was thought to be solely structural support, maintaining the intricate geometry of neuronal circuits and acting as connective material. Throughout the latter half of the 19\textsuperscript{th} century it became clear that neuroglia, or simply glia, consisted of heterogeneous cellular populations with diverse morphological
characteristics and distributions\textsuperscript{7}. However, the full appreciation for glial diversity was not fully realized until the widespread use of immunohistochemical techniques and transcriptional characterizations allowed for classification of distinct subtypes of glial populations. Experimental evidence then began to accumulate implicating for example, oligodendrocytes in axon myelination and astrocytes in metabolic coupling. Although these emerging functional roles for neuron-glia interactions began to gain appreciation among traditionally neuro-centric neuroscientists, glia were still considered to be passive support cells in the central nervous system whose dynamics were dictated by neuronal states and needs. The hierarchical consideration of important cellular targets in understanding behavior in healthy and diseased states has predominantly focused on the function and dysfunction of neurons. This bias towards studying neuronal properties was understandably influenced by technological limitations of the time and by the phylogenetic prevalence of neurons found across animal species. The core understanding that behavior was intimately dependent on the electrical excitability of neurons and that this excitation could be measured in the intact animal using electrophysiological methods meant that most research focused on electrical readouts in neurophysiology. This historical trajectory of neuroscientific research created a view of the nervous system with impoverished understanding of the roles of glia.

Today, the advent of light-based methods (such as genetically encoded calcium indicators, fluorescent glutamate sensors, two-photon microscopy etc.) for probing cellular function has led to an increasing range of tools to study neurons and glia\textsuperscript{8}. Experiments exploring glial functions with these techniques should not be done in a vacuum, but rather integrated into the extensive and ongoing research programs understanding neuronal contributions to behavior with more traditional techniques. In this
way, a more comprehensive, “systems” level of understanding can be achieved whereby
eurons and glia are considered integral components contributing to adaptive behavior
classic of the nervous system.

Overview of microglia

As mentioned previously, early histological observations describing variability in
glial morphology and distribution indicated that the collective term “glia” encompassed
heterogeneous cell populations with presumably diverse functions. Early studies
examining pathological tissue introduced the concept that some glial populations
acquired altered morphologies surrounding lesions in injured tissue. In 1878, Carl
Frommann described glia in the post-mortem brain and spinal cord from a patient with
multiple sclerosis as having enlarged somas and fewer processes that appeared
thicker. These observations were corroborated by Franz Nissl, Ludwig Merzbacher and
Alois Alzheimer. However, a more detailed description of these transforming glial cells
was not described until the early 20th century. Pio del Rio-Hortega, a student of Ramon y
Cajal, first comprehensively described these cells between 1919-1927 and termed them
“microgliocytes” or “microglia” based on their small size relative to other larger glial
populations termed “macroglia.” Although others had proposed a class of glia that
migrated into the brain in development and changed morphology in response to injury,
he was the first to unmistakably visualize microglia using a modified silver carbonate
stain. Simply using this technique on tissue from non-pathological and pathological
brain samples, he comprehensively classified microglia and proposed a series of
postulates regarding microglial origins, morphology, distribution, and behavior. He
postulated that amoeboid microglia migrate into the brain from the mesoderm during
early development and take up residence throughout the brain while adopting a ramified morphology throughout maturity. He proposed that in the adult brain, microglia occupy distinct territories, and that following pathological insult, transform their morphology into an amoeboid form with the capacity to proliferate, migrate to sites of injury and phagocytose damaged cellular material. Surprisingly, these visionary postulates have largely remained true to this day despite debates challenging them into the 21st century. For these contributions, del Rio-Hortega is generally regarded as the father of microglia.

**Microglial origins and unique profiles**

Much research characterizing microglia has been done since the work of del Rio-Hortega. Today, microglia are generally described as the immunosurveillant macrophages of the central nervous systems (CNS) with mesodermal origins and a monocytic lineage. This difference in lineage from other cell types constituting the brain parenchyma is reflected in the fact that microglia possess very different expression profiles and exhibit distinct behaviors underlying microglia-specific functions. To understand microglial roles in the brain, it has been necessary to investigate how they infiltrate the brain and how they behave after adopting a widespread residence within the parenchyma. Historical studies using the silver carbonate stain allowed for microglial visualization throughout the course of neurodevelopment. The proposal that microglia migrate into the brain during prenatal development and reside there throughout adulthood was first comprehensively described by William Robertson and Pio del Rio-Hortega and corroborated by John Kershman. More recent and sophisticated experiments using in vivo lineage tracing support the notion that microglia are derived from primitive macrophages born in the embryonic yolk sac and infiltrate the brain during
embryonic days 7-10 in mice. Consequently, it is generally accepted that microglia originate from myeloid-derived monocytes that take up residence in the early developing brain where they enact their functions throughout the lifetime of the animal.

Once microglia take up residence in the brain and mature, their proliferation and turnover occur at a relatively slow rate. It was unclear, however, whether resident microglial populations in adulthood were replenished by self-proliferation or if peripheral monocytes from the blood continually replenished the brain’s microglial population. Experiments using irradiation and bone marrow transplantation with GFP-tagged hematopoietic cells showed GFP-expressing microglia-like cells within the cerebellum, hippocampus, and striatum after transplantation, supporting the peripheral replenishment hypothesis. These results produced the theory that peripheral circulating mononuclear phagocytes regularly entered the brain, contributing to the microglial population. However, subsequent studies showed that monocyte extravasation to the brain was artificially induced by whole-body irradiation, which compromised the blood-brain barrier (BBB) and stimulated neuroinflammation. Experiments using head-shielded irradiation or parabiosis demonstrated that relatively few peripheral monocytes pass the BBB during normal adulthood. Thus, it is clear that environmental factors during development induce a population of microglia to express a phenotype distinct from peripheral macrophages that persists into adulthood and that this population proliferates independently from circulating monocytes. These data indicate that under non-pathological conditions, the blood-brain barrier amongst other protective roles, partitions circulating monocytes and microglia into two distinct immune cell populations that are independently maintained. In accordance with this dichotomy, microglia possess a number of distinctive attributes that creates further dissimilarity from
their monocyte relatives including a unique morphology and proteomic expression profile. These microglial attributes indicate that microglia are specially tailored to functional roles within the brain parenchyma, the extent of which are still being elucidated.

After taking up residence in the embryonic brain, microglia transform to their adult phenotype. During early development microglia have amoeboid morphologies, reminiscent of macrophage phagocytes, and they slowly mature to their distinctive ramified morphology characterized by intricately arborized processes. Though microglia retain similar gene expression markers of mononuclear phagocytes of their lineage (CD11b, F4/80, etc.) they also display striking differences\(^ {23,24}\). For example, microglia produce less superoxide dismutase\(^ {25,26}\) and acquire striking expression of a purinergic receptor, P2Y12, which is absent in most macrophages\(^ {27}\). In fact, recent studies have demonstrated that microglia possess an entirely distinct sensome generated by unique expression patterns\(^ {10,11}\). Not only do these patterns differ from ectodermal CNS cells (neurons, astrocytes, oligodendrocytes, etc.), but they diverge significantly from the most closely related macrophages of the same lineage found in other tissues. Indeed, expression profiles of microglia within the brain are also significantly different from immortalized microglial cell lines and microglia transplanted into primary cultures suggesting that extracellular signaling within the brain maintains microglial physiology. These data suggest that microglia may play specialized roles in the brain parenchyma distinct from macrophages residing in other tissues indicating critical cross-talk between microglia and other cells of the central nervous system. Evidence suggests that astrocytes mediate this maintenance of microglial morphology and transcriptional profile, potentially through purinergic signaling\(^ {28-30}\) and TGF-β cascades. The fact that blood
monocytes and peripheral macrophages also adopt an altered branched morphology when cultured on astrocytes reinforces the notion that microglia originate from a myelomonocytic lineage and that astrocytic signaling with cells of the monocyte lineage is sufficient to induce expression changes in immune cells\textsuperscript{28}. Thus, microglia roles in the CNS seem to be cultivated by the cellular environment in part by astrocytes, and this suggests that microglial functions within the brain are tailored and maintained by communication with other cell types.

**Microglial roles in immune responses**

Rooted in the discovery of microglia using pathological tissue, microglial research has focused predominantly on their responses to immune challenges. From these studies, it is clear that microglial signaling is incredibly multifaceted and three levels of signaling complexity must be acknowledged to appreciate gradations in microglial behavior. First, depending on the nature and severity of insult, a wide range of signaling combinations are sensed and responses produced by microglia. Second, microglial responses to extracellular cues are based on both intracellular states and extracellular receptor populations. Third, microglial response is a cumulative and dynamic outcome of competitive pathways that are both stimulus-driven and dose-sensitive\textsuperscript{1}. The heterogeneous population of receptors and sensors on microglia convey two important types of information: presence of damaging signaling or absence of continuous healthy signaling, the so called ‘on’ and ‘off’ signals\textsuperscript{31}. This allows microglia to respond to known pathogens (e.g. Toll-like receptors) as well as unknown pathogens (e.g. lack of CX\textsubscript{3}CR1-CX\textsubscript{3}CL1 or CD200-CD200R signaling)\textsuperscript{32,33}. These considerations not only underlie the
pillars of signal transduction found in every cell type, but also resonate particularly within the immune cell populations as fundamental mechanisms of host defense.

Despite these differences between microglia and tissue-specific macrophages, the classic characterization of microglia is that, like their closely-related macrophage cousins in the periphery, they also represent the first line of defense in the immune-privileged brain, predominantly serving immune defense roles. This characterization is rooted in the observation that microglia are able to respond to pathological insult with morphological and transcriptional transformation to an ‘activated’ state with characteristics both similar and dissimilar to macrophages. Microglia are able to present antigens via major histocompatibility complex II (MHC-II) expression and to secrete immunoactive compounds such as cytokines. However, microglia are less efficient in these processes than peripheral macrophages, leading them to be widely considered novice immune cells34. Interestingly, expression comparisons between microglia and peripheral immune cells indicate that during pronounced immune challenges, microglia express a wide range of chemoattractant cytokines (i.e. chemokines) that are known to attract other immune effectors from the periphery. This suggests that microglia possess a limited range of classical immune functions and likely serve as homeostatic gauges and limited responders that, when necessary, signal to the peripheral immune system, attracting more competent immune cells during severe pathophysiological insults where blood-brain barrier breakdown is deemed necessary.

Microglia have been shown to respond vigorously to two major classes of immunoreactive compounds of the innate immune system. Danger associated molecular patterns (DAMPS) indicate cell dysfunction and injury and include proteins such as
nucleotides$^{35,36}$, HMGB1$^{37}$, and S100$^{38}$. In contrast, pathogen associated molecular patterns (PAMPS) indicate foreign agents (e.g. viral RNA, bacterial membranes, flagella) and stimulate a host of Toll-like receptors (TLRs) and other pattern recognition receptors$^{39}$. Microglial responses to these molecules fall under roles in innate immunity. In addition, as mentioned above, microglia are capable of presenting antigens and engaging the adaptive immune system$^{40}$. In response to DAMPS and PAMPS, microglia release a myriad of cytokines, chemokines, neurotrophic factors, and cytotoxic factors$^{41}$. In turn, microglia sense these proteins in an autocrine and paracrine fashion, generating the characteristic amplification of immune defense. The characterization of microglial activation states has been based on the M1/M2 macrophage classification in which M1 states are classified as “pro-inflammatory” and M2 states as “anti-inflammatory.” Presumably, induction of the M1 activation by proinflammatory cytokines (e.g. IL-1β, IFN-γ, IL-6, TNF-α etc.) predominates early injury response and functions as pathogen elimination and host defense$^{17}$.

Conversely, induction of M2 activation by anti-inflammatory mediators (e.g. IL-4, TGF-β, IL-10 etc.), although stimulated concomitantly during M1, predominates late injury response and functions as damage containment and tissue repair$^{41,42}$. However, given the complexity and gradations of microglial activation, any rigid classification will not accurately reflect in vivo variation$^1$. This complexity has been reflected in the adoption of terms such as “M3” states and various other non-classical states or “alternatively activated” states. Though this gradient of responsiveness is thought to reflect a diverse repertoire of response mechanisms tailored to pathological diversity, these “alternatively activated” states may also indicate non-pathological responses in the healthy brain, a notion that will appear recurrently in this thesis.
Figure 1: Microglia respond to immune challenges with a change in morphology and increased phagocytosis. A. Microglia migrate to a cortical stab wound and adopt an amoeboid morphology (epifluorescent image, microglia genetically expressing GFP in fixed tissue). B. Low magnification view of panel A. Note the extensive migration and proliferation around the site of injury. C. Microglia in the healthy cortex with ramified morphology (confocal image, Iba-1 stained microglia in fixed tissue). D. Microglial decrease ramification following LPS administration (5 mg/kg, i.p., same preparation as C). E. Microglia form phagocytic cups (red arrows) following removal of skull and dura (two-photon image, genetic GFP labeling in vivo). F. Microglial lysis following phagocytosis of chlodronate-filled liposomes injected into cortex (epifluorescent image, genetic GFP labeling in fixed tissue). Scale bars = 50μm (A), 100μm (B), 40 μm (C,D), 30μm (E), and 75μm (F).
In addition to alterations in expression profiles, microglia demonstrate diverse changes in morphology during activation states (Figure 1). Classic studies describing microglial morphology in pathological tissue noted a pronounced shift in morphology associated with immune challenge. Similarly to their macrophage relatives, microglia near the site of injury adopt a striking amoeboid morphology devoid of primary process arborization that mirrors the appearance of phagocytic macrophages. This shift in morphology is most pronounced local to the site of injury with gradations in process shortening and thickening at intermediate distances (Figure 1A, B). This behavior reinforced the notion that ramified microglia in the healthy brain act as immune sentinels poised to sense injurious challenges and react by adopting a classic macrophage phenotype. These early studies in fixed tissue led to the historical description of microglia as maintaining “resting,” ramified phenotypes that were drastically altered following the sensing of pathological stimuli such as lipopolysaccharide (LPS), a component of bacterial membranes (Figure 1C,D).

These descriptions not only set the precedent for investigating microglial morphology as a proxy for function, but implanted the assumption that microglia in the absence of pathology were static sentinels, inert under normal neurophysiology. Based on these observations, the standard view of microglial function was that microglia detect abnormal changes in the local environment and generate appropriate responses to perturbations. To this end, microglia express a wide variety of receptors and sensors to monitor extracellular and intracellular homeostasis, as well as the appropriate machinery to produce immune reactions⁸. With their intimate spatial proximity to the brain parenchyma, they serve as the first line of detectors and effectors in brain pathogen defense. Results from standard methodologies for studying microglial activation (e.g.
LPS- stimulation) have misrepresented microglial states as being binarized, ‘all-or-none’ activations\textsuperscript{1}. In reality, microglial stimulation and response is a multi-dimensional gradient, dependent on a confluence of interactive extracellular signals and competitive intracellular pathways (Figure 2).

Regardless, microglial dysfunction has been linked to exacerbated damage in most neuropathologies including stroke, trauma, viral/bacterial infection, cancer, epilepsy, neurodegenerative disorders and neurodevelopmental disorders\textsuperscript{37-42}. Ultimately, despite extensive work on understanding microglial activation in pathology, relatively little is known about the roles and properties of quiescent microglia in the healthy brain.

**Figure 2:** Changes in microglial morphology during activation occur on a gradient. Schematic of representative changes in microglial morphology following an immune response. Microglia change from a resting ramified morphology (green) to a more activated phenotype with short, thick processes and less ramification (yellow). Targeted process extension and distal phagocytosis (orange). Amoeboid phagocyte devoid of primary processes (red). Resuming a relatively ramified morphology following resolved immune response.
Microglial roles in the non-pathological brain

The notion that microglia fulfill exclusively host-defense roles in the CNS has begun to be challenged by several lines of research in the last 15 years indicating that microglia may play roles in the non-pathological brain. Most work has centered on the role of microglia in early neurodevelopment. Microglia involvement during early neurodevelopment seems intuitively plausible given the link between extensive cell-death, axonal remodeling, and synaptic pruning that occur during this period and the ability for microglia to phagocytose dying cells and clear cellular debris (Figure 1E, F). It is possible that through evolution, microglia repurposed immune machinery to aid in non-pathological processes. Traditionally, studies of proteins associated with neuroimmunology have been described during pathology, but not necessarily in the healthy brain. It was assumed that basal levels of these factors existed in the normal brain as a non-functional background expression until neural insult provoked the rapid and presumably functional upregulation that is now a hallmark of neuroinflammation. However, given interesting dynamics in both spatial and temporal expression of these proteins in the developing and healthy, adult brain, studies began to uncover functional roles for classic immune molecules in normal neurophysiology. One might hypothesize that if immune-related proteins are involved in brain homeostasis, it is likely that they play anti-inflammatory roles in neuroinflammatory conditions. Surprisingly, a number of proinflammatory cytokines and effector proteins have been connected to normal brain development and physiology.

Microglial roles in early neurodevelopment
Early evidence of microglial involvement in non-pathological processes came through the discovery that immune molecules associated with microglial functions affected neuronal network remodeling. One such molecule is major histocompatibility class-I (MHC-I). MHC-I proteins are involved in antigen presentation during activation of the adaptive immune system. Whereas MHC class II proteins involve presentation of extracellular antigens and are expressed predominantly by immune cells, MHC-I proteins involve presentation of intracellular components following proteasome degradation and are expressed by most cells in the body. Neurons express MHC-I proteins, but surprisingly show particularly high levels of expression in areas undergoing synaptic plasticity such as the adult hippocampus and developing visual system. MHC-I has been shown to co-localize with a post-synaptic marker (PSD-95) and was found, serendipitously, to be involved in retinal ganglion cell axon guidance to the lateral geniculate nucleus. MHC-I deficient mice showed decreased ability to prune incorrect projections. In addition, studies have shown that MHC-I expression variance can lead to changes in quantity or size of synapsin-labeled puncta. Electrophysiological recordings demonstrated that mice deficient in MHC-I displayed increased miniature excitatory post-synaptic current (mEPSC) frequency with a corresponding increases in vesicular glutamate transporter (vGlut) staining. Therefore, from these studies, it is clear that MHC-I have predominantly presynaptic effects. Whether these indications of structural change represent a MHC-I role in synapse elimination or homeostatic tuning is still being explored. Given the classic function of MHC-I in tagging cells for destruction and phagocytosis, it seems likely that it facilitates a synaptic pruning role that is performed by resident microglia.
In a related line of work, evidence has suggested that DAP12 (an adaptor protein associated with receptors for MHC-I and exclusively expressed by microglia in the brain) is important in post-synaptic receptor modulation. In DAP12 loss-of-function mutants, decreased glutamate receptor subunit 1 and 2 (GluA1, GluR2) expression in PSD fractions was observed\(^{48}\). The AMPA/NMDA receptor ratio was also increased\(^{49}\) with corresponding increases in synaptic AMPA-mediated rectification currents\(^{48}\). Thus, although changes in MHC-I expression seem to affect pre-synaptic elements, proteins related to its receptors (DAP12), normally found in natural killer cells, seem to have post-synaptic effects, suggesting that perhaps they operate in concert to mediate both pre- and post-synaptic refinement. Of note, DAP12 loss-of-function microglia overexpressed proinflammatory proteins such as IL-6, IL-1β, and iNOS indicating a more activated expression phenotype. Further work is needed to resolve the neuronal signaling responses to MHC-I/receptor activation, as well as whether DAP12 function in microglia simply prevents uncontrolled inflammation signaling from impeding neuronal function. However, it seems possible that neuronal MHC-I presents self-antigens that microglia recognize during a plasticity period of particularly high parenchymal modification. Inhibiting the MHC-I receptor adaptor protein, DAP12, prevents microglial recognition of neurons, provoking an inflammatory response which affects both presynaptic and post-synaptic properties. Accordingly, neurons might reduce self-antigen presentation as a mechanism to induce microglial engulfment of inappropriate synapses.

Another traditional immune factor associated with non-pathological roles in the brain is the cytokine tumor necrosis factor alpha (TNF-α). TNF-α has a well-documented neuroinflammatory role in brain pathology where it is upregulated and rapidly released, and upon binding to its receptor, induces NF-κB transcription and promotes caspase-
facilitated apoptosis. However, elegant experiments have demonstrated that basal levels of glial-secreted TNF-α plays a role in synaptic scaling during critical periods of neuronal network tuning \(^9\). Neurons plated on wild-type glia display characteristics of synaptic scaling, however, when plated on TNF-α-KO glia, this observation is absent. Furthermore, in hippocampal neuron cultures, exogenous TNF-α application induced AMPA receptor translocation to the membrane, consequently increasing the amplitude and frequency of mEPSCs \(^{50,51}\). Conversely, applying soluble TNF-α receptors (to remove TNF-α from the extracellular fluid) had the reversed effect of decreased AMPARs at the membrane and decreased amplitude and frequency of mEPSCs \(^{50,51}\). It was also shown that TNF-α application decreased inhibitory synapse strength \(^{50}\). Using the ocular dominance plasticity model and TNF-α KO mice, TNF-α was shown to be important for strengthening synapses from the open ipsilateral eye, but not weakening of the synapses from the deprived contralateral eye \(^{52}\). The authors proposed that weakening occurs first and TNF-α maintains ‘synaptic homeostasis’ with subsequent synaptic strengthening. Although microglial release of TNF-α has never been directly tested in the aforementioned experiments, microglia express TNF-α at much higher levels relative to any other glia within the parenchyma making them the likely source of this cytokine even under physiological conditions.

A more direct implication of microglia in early neuronal development has been described through studies of the action of the complement pathway in early retinogeniculate refinement. The classic complement pathway is a well-studied immune pathway that regulates phagocytosis during host-defense and neuroinflammation. It has been shown that C1q expression (a component of the complement pathway) in the developing lateral geniculate nucleus (LGN) reaches peak levels during activity-
dependent pruning stages, and co-localizes with pre- and post-synaptic markers (SV2 and PSD-95, respectively). Also, C1q KO and C3 KO mice displayed disrupted retinogeniculate synapse elimination with persistence of weak synapses that are normally removed during development. C3 is another component of the complement pathway that binds to its receptor CR3 on microglia and other immune cells to initiate phagocytosis, implicating microglia in a failure to prune weak synapses in the absence of C3/CR3 signaling. These results support the conclusion that complement facilitates the same role during development that it normally facilitates during inflammation, namely, tagging cellular material for phagocytosis and degradation. Further work demonstrated that C1q/C3 mediated synapse pruning was activity-dependent and occurred via microglial phagocytosis. Indeed, older work has suggested that soluble cleaved complement components act as microglial chemoattractants inducing cytoskeletal rearrangement. These experiments suggest that microglia engage a classic immune mechanism to support normal brain development through activity-dependent synaptic pruning. In light of the MHC-I results and the relationship between complement pathways and MHC-I during inflammation, it is probably that the two pathways interact. Additional studies will need to elucidate these possibilities.

Finally, substantial work has been done examining the role of CX3CL1 (fractalkine) during synaptic pruning. Fractalkine is a chemotactic cytokine that can exist in both a membrane-bound form and soluble form following proteolytic cleavage. Neurons express a high level of CX3CL1 during development and microglia are the only cells in the brain that express its only receptor, CX3CR1. Notably, CX3CR1 KO mice displayed decreased microglial phagocytosis of PSD95 labeled material and an increase in immature spines with corresponding electrophysiological decrease in
maturity\textsuperscript{56}. The results of these studies have been difficult to interpret as CX3CR1 KO animals purportedly have decreased microglial infiltration into the hippocampus during development and, in fact, the authors claimed that the deficit in pruning was not due to a deficit in fractalkine signaling, but rather a lower density of microglia. However, it has been documented that ligand binding to CX3CR1 in microglial promotes quiescence and is considered a basal signaling (‘off signaling’) pathway signifying normalcy unless disrupted\textsuperscript{17}. Thus, perhaps complement pathway molecules tag weak synapses for pruning and neuronal absence of CX3CL1 expression allows/promotes microglial phagocytosis of these spines. Indeed, other studies examining cognition in CX3CR1\textsuperscript{KO} mice revealed increases in hippocampal LTP with impaired environmental-enrichment effects\textsuperscript{58}, as well as impairment in Morris water maze and contextual fear conditioning tasks\textsuperscript{59}.

Though these studies have implicated microglia in early neurodevelopmental (P6-15), it has been unclear whether non-pathological microglial functions are relegated to this period of development or persist into adolescence and adulthood. It should be noted that microglia at these early developmental time points display significantly less ramified morphologies than in adulthood, suggesting that functions specific to early development are specific to this transient morphology. Therefore, the morphologies microglia adopt in later development and adulthood may underlie substantially different contributions to normal neurophysiology.
Microglia morphology and motility under non-pathological conditions

As was described by del Rio-Hortega, microglia in the adolescent and adult brain display strikingly different morphologies than during early development and under pathological conditions. Microglia in the healthy, mature brain have small somas ~15 μm in diameter with highly branched primary, secondary, and tertiary processes extending up to 50 μm away from the soma (Figure 3A). These processes establish microglial domains with limited overlap between neighboring microglia analogous to astrocytic domains (Figure 3B). The intricately branched morphology of microglial processes also puts them in close proximity with most elements of the brain parenchyma although perisynaptic regions are generally held by astrocytic processes (Figure 3B). As described earlier, this ramified morphology is maintained until stimuli that disrupt brain homeostasis induce a rapid change in microglial morphology. This reaction to insult has
made understanding microglial physiology under non-pathological conditions particularly challenging as cultured preparations of microglia display activated morphological phenotypes with more amoeboid-like appearances (Figure 4A, B).

Although cultured microglia can induce process outgrowth and mild ramification (indicating quiescence) after several hours of serum-free medium, it is clear that in addition to morphological discrepancies, *in vitro* genomic expression does not accurately reflect resting microglia *in vivo*. To date, *in vitro* studies investigating microglial properties require substantial parenchymal trauma to acquire culture or slice preparations. Inevitably this tissue perturbation activates microglia along multiple axes, a state shift that may never go back to physiological baselines (Figure 4A, B).

In order to study microglial functions in the healthy brain, immunohistochemistry on fixed tissue has been necessary to acquire snapshots of microglial expression and morphology under normal conditions. Historically, most work investigating microglial
dynamics has relied on *in vitro* or *in situ* preparations with the caveat that microglia are at best, moderately activated. Measuring microglial dynamics and signal transduction under healthy brain conditions was nearly impossible and largely unexplored until a striking set of experiments published in 2005 used genetic, fluorescent microglial labeling and two-photon microscopy to image microglial dynamics in live, anesthetized mice\textsuperscript{60,61}. These seminal studies opened up entirely new considerations for microglia under physiological conditions and led to a paradigm shift in understanding baseline microglial behavior.

Two important conclusions from these studies were immediately evident. First, whereas microglial processes were assumed to be relatively static until pathological insult, it became clear that instead these processes constantly survey the brain parenchyma at an astounding rate of ~1.5 \( \mu \text{m/min} \)\textsuperscript{60}. In addition, microglial processes could dynamically interact with astrocytes and neurons, and miniature phagocytotic cups could be seen engulfing and retracting parenchymal material. Second, following a small focal laser ablation, processes of neighboring microglia rapidly advanced towards the insult site, surrounding the injury and shielding it from the rest of the parenchyma within an hour after induction\textsuperscript{60}. This pronounced and rapid response was shown to be recapitulated with mechanical injury and with local release of adenosine triphosphate (ATP) from a micropipette. Though somal and process chemotaxis in response to nucleotides had been demonstrated in cultured microglia for several years, this *in vivo* evidence provoked new considerations for the role of purinergic signaling in microglia and early process responses on the minute timescale\textsuperscript{61}. Given the presumably high metabolic load of continuous and widespread surveillant process activity, two functional postulates for such motility were proposed. The microglial interaction with cortical
elements and phagocytosis of material suggested that microglia actively monitor and modulate the microenvironment domain surrounding each microglial soma. This ‘housekeeping’ role implied that microglia serve more chronic maintenance functions rather than simply reacting to large-scale insults. In addition to maintenance roles, the remarkable speed at which microglial processes responded to injury suggested that the pronounced motility also increases the efficiency of process surveillance mechanism for major injury.

Figure 5: Microglial processes are highly motile in the non-pathological brain and survey cellular structures at a high rate. A. Two-photon image of microglial motility in vivo. Images were taken every 5 minutes and six time points were overlaid in separate colors. White pixels represent stationary structures. Note the predominantly stationary soma and primary processes with highly motile secondary and tertiary processes. B. Two-photon still image of microglial processes extending towards nearby dendritic spines (microglia genetically labeled with GFP pseudocolored red, neurons with YFP pseudocolored green). Scale bars = 25μm (A), 10μm (B).

Following these discoveries, the general view of microglia as static immune cells did not change. The newly described motility in the healthy brain was paradoxically labeled “resting motility.” The general consensus was that microglial process motility in the uninjured brain represented a mechanism by which microglia increased the rate of homeostatic immune surveillance to produce a more rapid immune response to injury.
However, the high rate of interactions between microglia and other brain elements basally in the non-inflamed brain raised new questions regarding their potential functional significance.

**Microglial responses during dark-rearing and ocular dominance plasticity**

Although microglia in the non-pathological brain display pronounced surveillant motility, it was unclear whether microglial processes moved randomly through the parenchyma or if they displayed targeted movement to elements. Two hallmark studies demonstrated that indeed, microglial processes make frequent contacts with synapses in the healthy brain ($62,63$). These dynamics were observed in the live animal using two-photon microscopy and verified using electron microscopy. By using electron microscopy it became clear that occasionally microglial processes make physical contact with synapses directly in the synaptic cleft, a position normally held by astrocytic processes. The first set of experiments demonstrated that microglia contact dendritic spines in an activity dependent manner as enucleating an eye to deprive input to the cortex caused microglia to decrease their contacts with spines in the contralateral visual cortex. In addition, lowering the body temperature of the mouse or applying tetrodotoxin (TTX) to the retinas decreased neuronal activity in the visual cortex and decreased microglial-synapse interactions$^{63}$. Finally, cortical ischemia using photochemical occlusion of the middle cerebral artery caused microglial processes to prolong their interactions with presynaptic terminals in the somatosensory cortex. Following this prolonged contact, a subset of contacted terminals disappeared suggesting that microglial contacts in periods of ischemia may determine whether the terminal is removed. Though these challenges to brain homeostasis are relatively severe, they
underscore the ability of microglial processes to respond to alterations in cortical network homeostasis.

This work was corroborated by studies from our lab using similar methods\textsuperscript{62}. Images collected using electron microscopy demonstrated that synapses associated with a nearby microglial process were more likely to have increased extracellular space surrounding the synapse. This result was particularly intriguing because enzymes such as matrix metalloproteinases (MMPs) known to degrade components of the extracellular matrix (ECM) have been shown to be important for synaptic spine dynamics and synaptic plasticity. Additionally, microglia are one of the main producers of MMPs within the brain suggesting that degradation of ECM may be one mechanism by which

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{Microglia make dynamic putative contacts with dendritic spines on the order of minutes. Two-photon imaging of microglial processes (red) surveying nearby dendritic spines (green). Top panel- low magnification of dendritic branches. Bottom panel- high magnification time lapse series of dashed box in top panel. Note the targeted motility to several dendritic spines along the same length of dendrite. Scale bar = 15\textmu m. Labeling is the same as in Figure 5B.}
\end{figure}
microglia enact changes at the single synapse level. Using two-photon imaging to monitor microglial contacts with dendritic spines, it was shown that microglia preferentially contact small, immature-like spines and that this contact causes a temporary increase in spine size during the contact, perhaps due to enzymatic degradation of the surrounding ECM. However, it was also shown following two days, spines contacted by microglia on the first day were more likely to be eliminated. These results demonstrated that microglia may play directive roles in baseline transient dendritic spine activity. Interestingly, following a more physiological alteration in cortical activity using visual sensory deprivation to both eyes (i.e. dark adaptation), microglial processes displayed decreased motility and lost the preferential targeting of dendritic spines of different size. Spines that were contacted tended to shrink rather than grow as in under control conditions. On the ultrastructural EM level, following sensory deprivation microglial synaptic cleft interactions and inclusions increased, suggesting increased associations between microglia and synapses and increased microglial phagocytic activity. These results demonstrated for the first time that physiological alterations in sensory experience induced a change in neuron-microglial dynamics suggesting that microglia are able to respond to non-pathological stimuli in a dynamic and targeted manner. Furthermore, these data suggest that microglia may play proactive roles in the strengthening and elimination of synapses through signaling propagated in a contact dependent manner.

In order to determine whether microglia respond to periods of synaptic plasticity, our lab began studying microglial behavior during ocular dominance plasticity. Ocular dominance plasticity (ODP) is a well-studied model of cortical synaptic plasticity first described by Hubel and Wiesel in adolescent cats\textsuperscript{64}. Following monocular deprivation
(MD) of one eye, they found that the responsiveness of neurons in the contralateral binocular cortex shifted. Neurons that had biased responsiveness to input from the deprived eye began to respond more robustly to input from the open, ipsilateral eye. This shift of responsiveness from the closed eye to the open eye was termed an “ocular dominance shift.” Since the work of Hubel and Wiesel, ODP has been used as a cortical model of activity-dependent plasticity to better understand the general mechanisms of cortical plasticity, not only within the visual system, but across other sensory systems as well.

From these studies, several important characteristics of ODP became apparent. First, the receptiveness of the brain to undergo network remodeling in ODP is relatively confined to the visual critical period in adolescence⁶⁴. Critical periods are unique time windows in development where the brain has reached a mature state of gross connectivity but is particularly sensitive to sensory experience. Perturbations of sensory experience within this window produce a rapid shift in neuronal responsiveness. Since Hubel and Wiesel demonstrated ODP within the cat visual critical period, other lines of work have shown that critical periods exist for other sensory systems such as the auditory and somatosensory systems. Though the opening and closing of critical periods differs in time across sensory systems based on the timeline of cortical maturation for each system, it highlights the generalizability of mechanisms in ODP across other cortical regions. Second, shifts in neuronal responsiveness in the visual cortex occur in two distinct phases over the course of days⁶⁵. The first period of ODP is characterized by a loss of responsiveness to the closed, contralateral eye and occurs in the first 1-4 days following MD. The second period of ODP is characterized by an increased responsiveness to the open, ipsilateral eye and occurs following 4-7 days of MD⁶⁶.
Our lab began characterizing microglial behavior during ODP to test whether microglia play non-pathological roles in adolescent development during periods of experience-dependent cortical plasticity. Since microglial behavior has been predominantly studied during inflammatory “activation,” hallmarks of classical responsiveness were first explored. As discussed earlier, microglial morphology has been extensively used as a readout for microglial reactivity. During the mouse visual critical period, animals were deprived for varying lengths of time and microglial behavior measured in the binocular visual cortex. Surprisingly, microglia exhibited a variety of changes in their dynamics including altered morphology, motility, cellular contacts, and phagocytosis profiles. Importantly, these behaviors were not accompanied by classical markers of microglial activation, suggesting that microglial response to MD is not a traditional immune response. I will describe this work in greater detail in Chapter III. Importantly, this work clearly demonstrated that microglia react rapidly to ocular dominance plasticity with an alternative activation phenotype independent of classical immune responses. What signaling pathways and molecular mechanisms mediate microglial responses to ODP? Do microglia play active, critical roles in the plasticity process or do they simply respond passively to neuro-intrinsic mechanisms? These two questions formed the basis of my thesis.

In order to find molecules and signaling pathways that underlie the microglial behavior in ODP previously described by our lab, I created a list of criteria that a suitable candidate would need to match: 1) I reasoned that any candidate factor important for microglial signaling under non-pathological conditions should be expressed in the healthy brain; 2) The candidate factor should be regulated either directly or indirectly by neuronal activity; 3) The factor should have actions on microglia distinct from
inflammatory states; 4) The candidate factor should have a receptor or signaling partner expressed on microglia; 5) The factor should induce morphological or motility changes in microglia. I then performed an extensive literature search for potential candidates that matched these criteria. The best candidate molecule from my search that satisfied all these conditions was the purine, adenosine triphosphate (ATP). Here, I will summarize purinergic signaling broadly and detail why the purinergic receptor P2Y12 became my primary target.

**Purinergic Signaling**

Purines are a broad class of heterocyclic aromatic compounds consisting of a pyrimidine ring adjoined by an imidazole ring. Two of the most common purines, adenine and guanine form the nucleosides, adenosine and guanosine when bound to ribose. Phosphorylation of adenosine creates its derivatives, adenosine mono-, di-, and triphosphate (AMP, ADP, and ATP). In addition to being core components of DNA and RNA, purines are important mediators of cellular metabolism serving as a cellular energy substrate. Therefore purinergic signaling is evolutionarily conserved and widespread across all organisms. It not only serves as an intracellular metabolic gauge but also as intercellular communicator. Purinergic receptors (purinoceptors) fall into three families: adenosine receptors (A or P1), ionotropic purinergic receptors (P2X), and metabotropic purinergic receptors (P2Y). Much work since the 1970’s has been done on classifying and characterizing these receptors based on signal transduction mechanism, pharmacological kinetics, and genetic/molecular homology. Purinoceptors can be found in every cell type of every tissue, suggesting they are one of the most abundant receptor types in mammals. This includes the brain, where an extensive expression of
receptors from each family has been observed. In addition, purinergic signaling has been implicated in numerous nervous system processes such as growth and development as well as cytotoxicity and pathology. It is therefore critical to understand the localization and properties of these receptors to understand normal and abnormal functioning in the brain.

In the normal brain, purinergic neurotransmission was first demonstrated in sympathetic nerves\(^2\). Today it is clear that neurons and glia express purinoceptors, which facilitate functional crosstalk between these cells\(^70\). In development, it has been shown that glial release of ATP generates spontaneous neuronal activity in the cochlea, synchronizing network output\(^71\). In adulthood, evidence suggests that astrocytic networks can communicate through ATP-stimulated calcium waves\(^72\) and that neurons co-release ATP with glutamate\(^73\). In addition, activity-dependent decreases in extracellular calcium induces astrocytic release of ATP, potentially inhibiting neurotransmission through a feedback pathway\(^74\). This evidence suggests that purinergic signaling plays crucial roles in neurophysiology both in the pathological and non-pathological brain.

In the pathological brain, numerous studies have demonstrated that purinergic signaling plays a central role in neuroinflammation. Dysfunctional cells undergoing various forms of cell death have been shown to release ATP, which in turn stimulates production of inflammatory cytokines in nearby immune cells\(^75\). This indicates that high concentrations of ATP are released surrounding pathological insult. ATP is an ideal indicator of cell lysis because it is present at high intracellular concentrations and normally absent from the extracellular parenchyma. It can be rapidly released but quickly
degraded, and it can be detected by surveying microglia. Given their role as immunocompetent sentinels in the brain, microglia express heterogeneous populations of purinoceptors sensitive to extracellular adenine and uracil nucleotides. Presumably, the primary function of these receptors is to detect and respond to changes in brain homeostasis during neuropathological insults. However with the advent of new microglial roles, microglial purinergic signaling may also play a role in normal brain physiology. Here, I will summarize what is known about microglial purinoceptor expression and provide evidence for why purinergic signaling may play roles in non-pathological microglial functions.

**Purinergic receptors on microglia and known roles**

Efforts to identify microglial purinoceptor expression have historically produced mixed and inconsistent results. Two factors contribute to this ambiguity. First, microglial purinoceptor expression changes during development and during inflammation. Second, purinoceptor expression is different in primary cultures of microglial and microglial lines than freshly isolated microglia. Thus, statements about microglial purinoceptor expression must take into account the technique used as well as conditions sampled. Early reports indicated that microglia express nearly every type of purinoceptor, however given the potential confounds introduced by culturing microglia, these results were mostly artifacts of the cultured microglia. No single study has convincingly characterized complete purinoceptor expression in microglia, but comparisons across studies indicate several commonalities. Microglia seem to predominantly express A1, A3, P2X4, P2Y6, P2Y12, P2Y13, and GPR34 during normal physiological conditions. During inflammation, microglial upregulate A2a, P2X4, P2X7, and P2Y6, and
downregulate P2Y12 and P2Y13. In addition, during development it seems that microglia highly express P2X1, but lose this expression by adulthood\textsuperscript{79}.

Microglia typically express A1 and A3 receptors at rest and upregulate A2a during inflammation\textsuperscript{79}. All adenosine receptors are of the G-protein coupled receptor super family with A1 and A3 associating with G\textsubscript{i/o} alpha subunits and A2a associating with G\textsubscript{s}. A1 stimulation is thought to induce anti-inflammatory pathways, specifically modulating proliferation\textsuperscript{80}. Although generally not present in resting microglia, A2a receptor upregulation has been demonstrated in microglial cultures exposed to LPS or amyloid-beta. Remarkably, ATP stimulation following microglial activation induced chemorepulsion and A2a was shown to mediate process retraction. Although controversy exists on whether microglia express A3 or not, it has been demonstrated that blocking A3 or depleting extracellular adenosine by application of adenosine deaminase ADP induction of chemotaxis\textsuperscript{81}. It is still unclear how these mechanistic insights relate to interplay between A3 and P2Y12 signaling, or the relative contributions of each receptor.

Microglia predominantly express three P2X receptors: P2X1, P2X4 and P2X7. All P2X receptors are ligand-gated ion channels, highly permeable to calcium. One study has identified P2X1 in the developing brain, but it is unclear if this expression carries into adulthood and no role has yet been ascribed to it in development. Interestingly, it has been shown to be upregulated following status epilepticus perhaps indicating a role in tissue regeneration\textsuperscript{82}. Much of the work on microglial P2X4 expression and upregulation comes from studies of or neuropathic pain in spinal allodynia\textsuperscript{83}. Importantly, mice lacking P2X4 did not develop tactile allodynia\textsuperscript{84}. In the presence of extracellular calcium, P2X4
stimulation opens a non-specific cation permeable channel, but in the absence of extracellular calcium, opens a transmembrane pore permeable to large molecules. The importance of this dual-function is still unclear\textsuperscript{85} although spreading calcium waves indicative of damage may be involved.

Similarly, P2X7 is upregulated during inflammation\textsuperscript{86}. It was shown that P2X7 was sufficient to activate microglia and results in proliferation and induction of inflammatory signaling cascades\textsuperscript{86}. Like other P2X receptors, P2X7 selectively binds to ATP, but unlike other P2X receptors, has a relatively low sensitivity (mM vs. μM range). At low concentrations of ATP stimulation, P2X7 permits non-selective cation flow, including calcium. Interestingly, upon continual ATP stimulation, P2X7 is able to form a transmembrane pore permeable to large molecules (up to 900 Da)\textsuperscript{86} in manner similar to P2X4. Thus, most roles attributed to P2X receptors in microglia seem to revolve around injury response.

Although one study indicated that microglia express P2Y1, P2Y2, and P2Y4, the experiments were done in N9 microglia lines and microglial cell culture and there was relatively little expression relative to actin\textsuperscript{87}. Thus, it is unclear if these expression patterns correlate to \textit{in vivo} expression. Recently, P2Y14, a UDP-glucose sensitive receptor has been detected in lymphocytes and neutrophils. Though these cells types share a related lineage, it is unclear if microglia express P2Y14 \textit{in vivo}\textsuperscript{88}. Similarly, although microglia seem to express P2Y13, the functional role it plays remains largely unexplored\textsuperscript{27}. Regardless, it has been shown that microglia robustly express P2Y6 and P2Y12.
P2Y6 is a G_3-protein coupled receptor with downstream activation of phospholipase C (PLC, which opens intracellular Ca^{2+} stores via InsP_3) and NFATc1 and NFATc2 through the phosphotase calcineurin. In a seminal paper, it was found that UDP-stimulated P2Y6 receptors on microglia produced actin reorganization that lead to phagocytotic cup formation. Furthermore, kainate induced seizures provoked microglia to upregulate P2Y6 and phagocytic activity in vivo. Additional experiments showed that P2Y6 stimulation also stimulated microglial production of chemokines CCL2 (monocyte-chemoattractive protein 1) and CCL3 (macrophage inflammatory protein one-alpha). These data lead to the theory that damaged cells release UTP, which is normally absent from the interstitial fluid. Subsequent hydrolysis to UDP and microglial stimulation may serve as an ‘eat-me’ signal with additional recruitment of peripheral monocytes. The fact that it is significantly expressed in resting microglia may serve a role in homeostatic function and perhaps phagocytosis of small parenchymal material, although this speculation has yet to be supported.

Overall, the purinergic receptors expressed by microglia seem to fall into two broad categories relating to injury detection/homeostasis and injury response. In resting microglia, microglial surveillance is mediated by P2Y12 and A3 facilitates process extension, while P2Y6 acts as a phagocytosis triggering detector. During injury response, A2a mediates process retraction while P2X4 and P2X7 mediate somal chemotaxis to injury sites and release of proinflammatory cytokines. Understanding the dynamic expression of these receptors will continue to elucidate microglial involvement in pathology and normal physiology.

**P2Y12 and current knowledge**
Within the last decade, the importance of purinergic signaling to microglial chemokinetics and chemotaxis has been elucidated through a variety of experimental techniques\textsuperscript{2}. Through these studies, P2Y12 has emerged as an important mediator of microglial motility. Although classically studied and characterized as an early effector for injury response, evidence suggests that P2Y12 may serve non-inflammatory functions.

The first evidence for P2Y12’s role in microglial chemotaxis came from studies using Boyden and Dunn chamber assays to measure microglial chemotaxis and motility in vitro\textsuperscript{90}. In the Boyden chamber assay, enhanced migration was observed with and without an ATP gradient indicating that it caused chemokinetic, rather than chemotactic effects. However, Dunn chamber results showed ATP-stimulated microglial chemotaxis towards the source. ADP was shown to have a stronger chemokinetic effect than ATP, implying that ATP stimulation was due to metabolite production via ectonucleotidases. Nonetheless a poorly hydrolyzable ATP analogue (ATPγS) had similar effects. Importantly, chemotactic responses were observed with a concentration range of 10-50 μM, within the extracellular physiological range of the non-pathological brain under certain conditions. No impairment in motility was observed when removing extracellular calcium or pretreating cultures with PPADS (P2X antagonist) ruling out the involvement of ionotropic purinergic receptors. Pretreating the cultures with A3P5PS (P2Y\textsubscript{1} antagonist) had no appreciable effect on membrane ruffling, whereas pertussis toxin (PTx) inhibited the ATP stimulatory effect, implicating G\textsubscript{i/o}-protein coupled receptor involvement.

At the time, P2Y12 (known as P2T\textsubscript{AC} for its mechanism of inhibiting adenylyl cyclase) had been studied primarily in blood platelets and an antagonist had been
developed, AR-C69931MX (i.e. cangrelor) as an anti-thrombotic treatment. Pretreating cultures with AR-C69931MX inhibited membrane ruffling and ATP-stimulated chemotaxis\(^3\). In addition immunohistochemistry and phalloidin staining showed Rac activation with actin reorganization. Thus, a mechanism was proposed whereby \(G_{i/o}\)-protein signaling influenced Rac activation via phosphoinositide 3-kinase gamma (PI3K\(\gamma\)). This influential study solidified \(G_{i/o}\)-coupled P2Y receptors as potential mediators of microglial motility.

Initially P2Y12 had been pharmacologically targeted in platelets, but it was not cloned until later the same year\(^9\). Eventually, it was shown to be expressed by blood platelets and microglia\(^5\), but it was unclear if other cell types expressed P2Y12. Given a common lineage and similar genomic profiles, macrophages were strong candidates, but surprisingly, peripheral splenic macrophages had little to no expression\(^{10,27}\). In addition, P2Y12 staining did not colocalize with NeuN or GFAP. This made therapeutic anti-thrombotic interventions targeting P2Y12 particularly intriguing given the relatively limited cell specificity. Despite advances in understanding P2Y12’s involvement in platelet dynamics, comparatively little research has explored its role in microglia. Though convincing \textit{in vitro} culture work had espoused P2Y12’s involvement in microglial motility, translating these results \textit{in vivo} was problematic given technical limitations. Indeed, even the dynamics of microglial injury response and purinergic signaling roles were largely unexplored in live animals. This uncertainty remained until a remarkable paper addressed these unknowns and firmly connected the role of ATP in microglial response to neural damage.
A month after microglial processes were shown to dynamically survey the healthy brain parenchyma using genetic fluorescent labeling and two-photon microscopy, another group of researchers used the same experimental setup to implicate purinergic signaling in microglial reactivity to injury. As discussed previously, the researchers replicated observations of rapid process extension towards insult by inducing a small focal laser ablation to the brain parenchyma⁶¹. Within a half hour, microglial processes had converged on and surrounded the damaged tissue, containing the site with minimal somal displacement. This response was reproduced using mechanical disruption via a glass electrode. To test whether purinergic signaling mediated this response, the researchers applied various concentrations of exogenous ATP prior to laser ablation. The microglial response was inhibited with ATP concentrations of 1 and 10 mM but not 100 μM, presumably indicating receptor saturation. This finding was corroborated with apyrase pretreatment, an enzyme that hydrolyzes ATP to AMP. In addition, applying exogenous ATP with a glass electrode produced a similar response to mechanical or laser ablation. From these results, it was concluded that ATP or ADP mediates fast microglial process response to injury in vivo.

In order to delineate the purinergic receptors responsible, the researchers pretreated the parenchyma with reactive blue 2 (RB2, predominantly P2Y2, 4 antagonist), PPADS (predominantly P2X antagonist), and suramin (broad P2 receptor antagonist) prior to laser ablation. They found that RB2 and PPADS inhibited the microglial response, whereas suramin did not. However, they claim that this evidence supports the involvement of P2Y receptors by claiming that suramin blocks P2X receptors and RB2 and PPADS blocks P2Y receptors. It is possible that antagonistic properties of these agents have since been refined, although understanding these
results may require further experiments (for summary of antagonists, see ref.\textsuperscript{15}). Regardless, involvement of purinergic signaling was compellingly shown. Furthermore, blocking connexin hemichannels using flufenamic acid or carbenoxolone reversibly inhibited the microglial response, linking astrocytic release of ATP (or other factors) to microglial process extension. In this model, the researchers concluded that ATP-stimulated astrocytic release of ATP likely induced microglial process extension towards the injury site\textsuperscript{14}. Combining these results with the \textit{in vitro} studies suggested that P2Y12 may mediate these described responses \textit{in vivo}.

Around the same time these results were published, P2Y12 was shown to be expressed in the cortex and spinal cord, with expression notably higher in cortex\textsuperscript{92}. In addition, microglial intracellular calcium transients showed the highest sensitivity to 2MeSADP (poorly hydrolyzable ADP analogue), implicating P2Y12/ 13. Importantly, relative to other purinergic agonists, P2Y12 has an exquisite sensitivity to ADP (or 2MeSADP), causing calcium transients in the nanomolar concentration range whereas other agonists had micromolar (e.g. UTP, UDP) or millimolar (e.g. ATP, BZATP) ranges. This distinguishing sensitivity could shed light on the functional role of P2Y12 in detecting even slight perturbations in the extracellular milieu and generating an appropriate dynamic response.

Further studies described mechanistic components of P2Y12 stimulation and subsequent membrane ruffling and chemotaxis. Beta one integrin (β1 integrin) was shown to be highly expressed in microglia, and antibody inhibition decreased chemotaxis in the Dunn chamber assay\textsuperscript{93}. However, increasing cAMP through forskolin inhibited translocation of β1 integrin to the microglial membrane. This inhibition was
abolished with co-application of a protein kinase A (PKA) inhibitor (KT-5720), establishing a temporary mechanism whereby P2Y12 stimulation decreases adenylyl cyclase, reducing cAMP levels and subsequently reducing activated PKA, leading to downstream translocation of β1 integrin to the membrane. In addition, induction of microglial chemotaxis also required P2Y12-stimulated PI3K activation and Akt phosphorylation, as blocking PI3K with wortmannin and LYS294002 inhibited Akt phosphorylation and chemotaxis94,95.

Remarkably, the same report showed diminished ATP-stimulated chemotaxis with shRNA knockdown of P2X4, indicating its involvement. ATP-induced membrane ruffling was only inhibited by the P2Y12 specific antagonist (AR-C69931MX) and not the P2X4 antagonists (e.g. PPADS), raising the interesting possibility of distinct somal motility and process motility mechanisms. Given the upregulation of P2X4 and downregulation of P2Y12 during pathological states, this may also be evidence for separate functional motilities as well. It is possible that P2Y12 mediated motility serves primarily early sensor and housekeeping functions, whereas somal displacement during long-term, larger-scale pathology is driven by P2X4 expression and activity over a longer time course.

Mitogen activated protein kinase (MAPK) has been linked to cell motility, but surprisingly inhibiting MAPK had no effect on ATP-stimulated microglial motility. It was demonstrated that microglial chemotaxis required initiation of an outward potassium current, a process independent of PI3K activity but dependent on P2Y receptor stimulation96. Also, following P2Y12 stimulation, downstream activation of phospholipase C (PLC) and release of intracellular Ca²⁺ stores via IP₃ are necessary for chemotaxis,
but this has also been debated\textsuperscript{21}. These results suggest that P2Y12 stimulation leads to a variety of downstream cellular pathways that contribute to neuroinflammatory responses, but that the effect of chemotaxis is not dependent on these pathways. Rather, chemotactic and inflammatory pathways proceed in parallel.

Consistent with this dissociation between downstream P2Y12 mediated signaling pathways, it has been shown that P2Y12 stimulation does not automatically induce TNF-\textgreek{a} efflux as seen in LPS activation\textsuperscript{97}. More importantly, it seems the environment in which P2Y12 stimulation occurs is important. Pretreating microglia cultures with TGF-\textbeta resulted in increased chemotaxis over controls whereas LPS pretreatment decreased chemotaxis. IFN-\textgamma pretreatment did not have an effect\textsuperscript{98}. Furthermore, TGF-\textbeta pretreatment increased P2Y12 expression while LPS pretreatment decreased expression. Given the known anti-inflammatory association with TGF-\textbeta, it seems that P2Y12 expression is tied to anti-inflammatory gene expression, fitting with its presumed homeostatic roles perhaps including facilitating synaptic plasticity.

In pathology, P2Y12 has been implicated in generation of neuropathic pain. Following peripheral nerve injury, it was shown that P2Y12 expression significantly increases on the ipsilateral spinal cord 3-10 days post-injury\textsuperscript{99}. Following status epilepticus, P2Y12 expression increased significantly 24 and 48 hours after seizures\textsuperscript{82}. It is strange then that P2Y12 is considered to be involved in early injury response especially since it can be downregulated as quickly as 8 hours following LPS activation\textsuperscript{7}. The evidence for P2Y12's anti-inflammatory association, described above, seems to suggest that microglial process motility is important during injury recovery and regeneration, rather than early injury response. In fact, antagonizing P2Y12 decreases
myelinated axon phagocytosis and subsequent tactile allodynia in spinal cord injury. These results seem to suggest that P2Y12 serves a more basic protective chemotactic role than pronounced injury response.

Despite all this evidence for P2Y12’s role in microglial chemotaxis, the pivotal connection between P2Y12 and microglial process motility in vivo came in a landmark paper from the Julius lab in 2006. The data from this paper also convincingly demonstrated that P2Y12 met all of the criteria for a candidate signaling molecule between neurons and microglia during ODP. Using P2Y12\(^{KO}\) mice and a custom P2Y12 antibody, P2Y12 was shown to localize exclusively to microglial membranes, both on the soma and distal processes (Figure 7).\(^7\) Not only was P2Y12 absent from astrocytes, but also absent from splenic macrophages. They also showed a strong positive relationship between P2Y12 expression and ramified morphology. Following administration of LPS, which as discussed before leads to a transformation of microglia from a ramified to amoeboid morphology, P2Y12 was found to be downregulated over the course of 24 hours. In fact, P2Y12 expression showed a strong linear correlation with the number of

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Figure 7: Ramified microglia in the non-pathological brain express high levels of P2Y12. Confocal image of cortical microglia with representative P2Y12 expression along processes and soma. Left panel - GFP genetically expressed at the CX3CR1 promoter. Middle panel – P2Y12 immunostaining. Right panel- merge. Note the enriched expression and localization of P2Y12 at distal ends of terminating processes. Scale bar = 10μm.
primary processes signifying ramification. The downregulation of P2Y12 was confirmed at both the protein and mRNA level. In culture, microglial membrane ruffling to ADP, ATP, and 2MeSADP was shown to be abolished in P2Y12\(^{-/-}\) microglia, with M-CSF (purinergic receptor independent) still able to induce ruffling. The chemotaxis of microglia along an ATP gradient in a Dunn chamber preparation also showed markedly decreased chemotaxis in P2Y12\(^{-/-}\) microglia. Finally, using the P2Y12\(^{-/-}\) mouse line crossed to the CX3CR1 GFP knock-in mouse line (to label microglia genetically), the lab was able to show that the receptor was necessary to for microglial process motility towards ATP or ADP both \textit{in situ} and \textit{in vivo}. Using two-photon microscopy in live, anesthetized mice, they showed that the strong characteristic microglial process responses to local application of ATP or laser ablation injury were completely abolished in P2Y12\(^{-/-}\)\(^{\text{Ko}}\) animals.

All these results indicated that purinergic signaling via P2Y12 met the criteria for a possible signaling candidate between microglia and neurons under non-pathological conditions. ATP, as an energy substrate, is present at high concentrations within every cell of the brain and its release has been tied to synaptic actions in the healthy nervous system. It seemed possible given the metabolic importance of ATP that neuronal activity (or inactivity) might regulate ATP release directly from neurons or indirectly from astrocytes. The wealth of literature described above indicated that microglial ramification in the healthy brain is highly correlated with P2Y12 expression, suggesting the P2Y12’s function is intimately tied to the behavior of ramified microglia. Although high extracellular concentrations of ATP act as DAMP signals indicating cell injury and compromised membrane integrity, small local release of ATP could signal under non-inflammatory conditions. With P2Y12’s high sensitivity, these transient events could
generate chemotactic crosstalk without triggering a pronounced immune response. Finally, given P2Y12’s high and selective expression in microglia within the brain, it was intuitive that P2Y12 would be important for microglia-specific roles and pharmacological and genetic manipulations would be easier to interpret. Therefore, I proposed a series of experiments to explore the role of P2Y12 behavior in microglia under non-pathological baseline states and during periods of synaptic plasticity. The results of these aims will be described in the following three chapters.

**Chapter II: P2Y12 in Physiological Microglial Behavior**

In order to test whether P2Y12 function was involved in microglia-neuron crosstalk during ODP, I needed to first establish the effect of P2Y12 disruption on baseline microglial behaviors. The experiments detailed in Chapter II reflect this baseline characterization. Included in this characterization is a validation of the pharmacological manipulations used to test P2Y12 function as well as description of the motility metrics used throughout these studies. Based on the observations of microglial behavior during ODP, I first needed to characterize basal microglial behavior following genetic or pharmacological disruption of P2Y12 in order to make conclusions about the effects of P2Y12 disruption during ODP.

**Chapter III: P2Y12 in Ocular Dominance Plasticity**

The ultimate goal of this thesis project was to determine whether P2Y12 mediates microglial behaviors during ODP and whether microglial responses to MD are critical components of the plasticity itself. The experiments described in Chapter III aim to resolve these goals. Specifically, I revisited the microglial dynamics during ODP observed in previous experiments but under conditions of P2Y12 disruption. Not only do
these experiments test the reproducibility of the previous observations in the lab (now as control comparisons to P2Y12 disruption) but also describe how P2Y12 functions in microglial dynamics in response to synaptic plasticity. In addition, by demonstrating abrogated synaptic plasticity under conditions of P2Y12 disruption, these experiments also demonstrate a novel role of microglia as integral components of adolescent cortical synaptic plasticity.

**Chapter IV: P2Y12, Noradrenergic Signaling, and Sleep/Wake States**

Following the data collected in the experiments described in Chapter II and III, I began to explore physiological states and signaling pathways that influenced P2Y12 function. In particular, I explored the ability of norepinephrine to inhibit purinergic-mediated chemotaxis in microglia via activity of β2 receptors. I tested the hypothesis that noradrenergic signaling in microglia acts as a negative regulator of purinergic signaling, and explored the role of this regulator in ODP. Finally, to understand how fluctuations in noradrenergic signaling might influence microglial behavior in physiological conditions, I began to investigate microglial dynamics between sleep and wakeful states. These experiments served to clarify a broader picture of the physiological constraints and regulation of microglial actions during periods of synaptic plasticity.
CHAPTER II

P2Y12 in Physiological Microglial Behavior

Introduction

In order to investigate the role of P2Y12 in mediating microglial behavior during ODP, I first needed to characterize the effects of P2Y12 on baseline behavior. In this manner, any alterations in microglial behavior in P2Y12 disrupted during ODP could be directly compared to the appropriate baseline P2Y12 disrupted state. Since the goal of exploring the role of P2Y12 in ODP was to compare whether the previously described microglial behaviors in ODP were affected, I chose to focus on those metrics. In summary of those results, following ODP microglia displayed increased process arborization as quantified using Sholl analysis. These morphological changes were accompanied by a decrease in microglial process motility and an increase in synaptic cleft interactions and membrane-bound intracellular inclusions. Given these results, I designed experiments to test whether genetic or pharmacological disruption of P2Y12 affected baseline morphology, motility, synaptic cleft interactions or prevalence of inclusions.

Previous characterization of P2Y12 blockade in microglia was predominantly done in \textit{in vitro} preparations using cell cultures of primary microglia lines or immortalized lines. In these studies, P2Y12 antagonism decreased microglial membrane ruffling and chemotaxis\textsuperscript{90}. Though these studies were important in characterizing the signaling cascades downstream of P2Y12 activation, the culture conditions and resulting activated phenotypes of microglia in these preparations made it difficult to determine how the
results generalized to the ramified behavior of microglia in the intact brain parenchyma. The most comprehensive study investigating P2Y12 function in the intact brain was done in 2006\textsuperscript{27}. Although the main results of that publication demonstrated abrogated microglial responses to ATP release or local laser ablation in the cortex of live P2Y12\textsuperscript{KO} mice, a baseline characterization of microglia was also performed. However, this data was presented in the supplemental information without example images and the experiments had several weaknesses.

The characterization included a survey of microglial density across the striatum, cortex, hippocampus, retina, and spinal cord. Although there were no significant differences in microglial density across all regions, only 2 animals per genotype were analyzed, and the large error bars indicated modest variability. In addition, the authors state that there were no obvious deficits in microglial morphology. Nevertheless, for morphological characterization, the authors used area occupied by processes originating from individual microglia as quantification, which may not capture nuances in morphological complexity. Furthermore, only 1 animal per genotype was examined using 12 sections per animal. P2Y12 KO’s displayed a decreased area occupied by microglial processes, though this did not reach significance (p=0.29). However, in order to perform statistical tests, the sample size must have been the 12 sections collected from each individual animal. Given that the standard error of the mean displayed large variance with n=12, and only microglia from the retina were analyzed, the conclusion that P2Y12\textsuperscript{KO} microglia have normal morphology was unconvincing.

In this study, the authors also claim that baseline microglial motility is unaffected by P2Y12 ablation. For quantification, the authors analyzed 8-16 processes per animal,
3 animals from each genotype, and measured the average process length change over a 10 minute time scale. Microglia from P2Y12KO mice had decreased baseline motility, however these results did not reach significance (p=0.16). In addition, the authors did not specify the brain region in which microglial motility was quantified and heterogeneity in microglia motility has not been conclusively disproven. Furthermore, the characterization of microglial baseline was only conducted in a constitutive genetic model leaving the possibility that genetic compensation might account for a lack of effect. Taken together, the statements that baseline microglial morphology and motility are unaltered by P2Y12 disruption were unconvincingly demonstrated. Therefore, I designed experiments to determine if a separate characterization using alternative quantification replicated these conclusions.

**Methods**

**Animals**

Experimental protocols were carried out in strict accordance with the University of Rochester Committee on Animal Resources (UCAR) and conformed to the National Institutes of Health Guidelines. Experiments were conducted on mice with a C57/Bl6 background during the visual critical period (P25-35). For fixed tissue experiments, C57/Bl6 controls were compared to P2Y12KO mice. For in vivo imaging CX3CR1GFP/+ controls were compared to CX3CR1GFP+/ P2Y12KO animals (kindly provided by Dr. Maiken Nedergaard).

**Histology**

Whole brains were collected following transcardial perfusion and overnight post-fixation with paraformaldehyde (4%). Tissue was cryoprotected and coronal sections were cut on a freezing microtome (Microm; Global Medical Instrumentation, Ramsey,
MN) at 50 μm thickness. Sections were processed free-floating at room temperature (RT). Sections were rinsed with phosphate buffered saline (PBS) and endogenous peroxidase activity and non-specific binding blocked using H₂O₂/methanol and bovine serum albumen, respectively. Sections were then incubated in a primary antibody solution (24 hours, 4°C, anti-Iba1, 1:1000, Wako #019-19741; anti-P2Y12, 1:1000, courtesy David Julius) followed by secondary antibody solution (4 h, RT, Alexa-Fluor 488, 594 or 647, 1:500, Invitrogen), mounted and coverslipped.

For examination of microglial ramification, areas contained entirely within binocular primary visual cortex were identified and imaged on a Zeiss LSM 510 confocal microscope (Carl Zeiss, Thornwood, NY). For each section, a z-stack encompassing the entire thickness of the section was collected with a z-step of 1μm at a magnification of 20x, 40x or 100x. Analysis was performed offline in ImageJ. Z-stacks were smoothed and compressed into a single z-projection. For analysis of ramification, microglia in 40x images whose entire process arbor was contained within the image were randomly and individually selected and cropped into a new image. Each image was thresholded to generate a binarized outline of the process arbor, filtered to remove artifacts, and analyzed with an automated Sholl analysis plugin (kindly provided by the Anirvan Ghosh lab, UCSD).

**Two-photon Microscopy**

A custom two-photon laser-scanning microscope was used for in vivo imaging (Ti:Sapphire, Mai-Tai, Spectraphysics; modified Fluoview confocal scan head, 20X lens, 0.95 N.A., Olympus). Excitation was achieved using 100 fs laser pulses (80MHz) at 920 nm. For baseline motility experiments a 580/180 (GFP) filter was used. For laser ablation experiments, a 565 dichroic and 580/180 (GFP), 578/105 (rhodamine) filters were used.
Mice were anesthetized with a mixture of fentanyl (0.05mg/kg, i.p.), midazolam (5.0mg/kg, i.p.) and dexmetatomadin (0.5mg/kg, i.p.). During surgery and imaging, body temperature was maintained at 37°C. Imaging was carried out using 4-5x digital zoom and 1 μm z-step size. Time lapse imaging used 4-5 minutes imaging intervals over 1-2 hours. Image analysis was carried out offline in ImageJ or in Matlab using custom algorithms.

**Microglial motility**

Microglial motility analysis was performed in ImageJ and Matlab using custom algorithms. Time-lapse imaging consisting of 40 μm deep z-stacks were collected every 4 minutes, 12 times, for a total of 48 minutes. Single image 15 μm Z-projections were created for each time point and lateral motion artifact was corrected. Z-projections were then thresholded and time points compared. RGB overlays were created for each pair of time points (0-4min, 4-8min etc.) such that red pixels (retraction) were present in the first time point, but absent in the second. Green pixels (extension) were present in the second, but not in the first, and yellow pixels (stable) were present in both time points. Motility index (representing change over 4 minutes) was then calculated for each RGB overlay as the sum of all extension and retraction pixels divided by yellow pixels and averaged across all RGB overlays. A custom MatLab program was used to compare pixels across multiple RGB overlays (e.g. 0-4min vs. 4-8min). Stability index was calculated as the proportion of extension pixels (green) in one RGB overlay that became stable (yellow) in the subsequent overlay divided by the total extension (green) pixels in the first overlay. Conversely, an instability index was calculated as the proportion of stable (yellow) pixels in one overlay that became retracted (red) in the subsequent overlay divided by the total stable (yellow) pixels in the first overlay. Finally, the stability
histogram compared the relative stability over multiple overlays of pixels that became stable at any time point in the imaging session (but omitting all pixels that were stable for the entire imaging session, generally somas or primary processes).

**Laser Ablation**

Laser ablations were produced by performing a point-scan for 3-4s at 780 nm using ~75 mW at the sample. Quantification of microglial response to laser ablation was measured as previously described by calculating the number of microglial processes entering from an outer radius (y, ~80 μm from ablation) into an inner radius (x, ~40 μm from ablation) as a function of time. Time-lapse z-projections were produced and thresholded to normalize background variability. The total number of pixels in x and y were measured across time (Rx(t); Ry(t)) and ablation response was calculated using the equation:

\[ R(t) = \frac{(Rx(t) - Rx(0))}{Ry(0)} \]

The vasculature was labeled using a retro-orbital injection of 4% tetramethylrhodamine dextran in saline to ensure that the blood brain barrier was not majorly compromised by laser injury.

**Electron Microscopy**

EM was performed as previously described. Briefly, primary visual cortex was prepared with 0.1% sodium borohydride (in 0.1M PBS), washed, and processed freely floating following a pre-embedding immunoperoxidase protocol. Sections were incubated with anti-Iba1 (48 h, RT, 1:1,000, Wako), goat anti-rabbit IgG conjugated to biotin (2 h, RT, Jackson Immunoresearch) and streptavidin-horseradish peroxidase (1 h, RT, Jackson Immunoresearch). Immunoreactivity was visualized with diaminobenzidine (0.5 mg/ml) and H₂O₂ (0.03%) (DAB Peroxidase Substrate Kit; Vector Laboratories). Sections
for EM were post-fixed (1% OsO4), dehydrated, treated with propylene oxide, impregnated in Durcupan (24 h, RT, EMS), mounted between ACLAR embedding films (EMS), and cured (48 h, 55°C). Binocular primary visual cortex (bV1) was excised and ultrathin sections (60-80 nm) were cut (Reichert Ultracut E) and images generated (Hitachi 7650 Transmission Electron Microscope, Gatan Erlangshen camera). Approximately 50-80 pictures were randomly taken at 30,000X in Layer 2 of bV1 (~10 μm from pial surface) corresponding to ~ 1,000 μm² of neuropil per animal. Cellular profiles were identified using a series of criteria previously defined in single-ultrathin sections. All subcellular profiles that were difficult to identify were classified as “unknown”. For quantification, measurements were normalized to the area of individual microglial processes to account for the fact that large processes have the ability to interact with more brain elements and contain more inclusions than smaller processes.

Electron Microscopy Element Classification

Dendritic Elements

Dendritic shafts cut longitudinally were recognized by their irregular contours, elongated mitochondria parallel to their central axis, frequent protuberances (spines, filopodia, small branches), and synaptic contacts with axon terminals (see below). When cut transversally, dendritic shafts were identified by their rounded morphology, frequent occurrence of mitochondria and microtubules, and distinguished from unmyelinated axons by their larger diameter. Dendritic spines cut longitudinally often protruded from dendritic shafts, displayed rounded morphologies and were generally free of mitochondria. They were characterized primarily by the presence of electron-dense accumulations (postsynaptic densities) at sites of synaptic contact with axon terminals.

Axon terminal
Axonal terminals were distinguished from other subcellular profiles based primarily on the presence of 40 nm diameter synaptic vesicles, with rounded to elongated morphologies, but also of synaptic contacts with dendritic shafts and spines. Axon terminals generally contained mitochondria.

**Astrocytes**

Protoplasmic astrocytes were recognized as electron-lucent structures seen to encase and wrap around other neuropil structures. As a result, astrocytes maintained irregular and angular shapes, distinguishing them from other neuronal profiles having a characteristic rounded shape. Perisynaptic astrocytic processes were defined by their direct contacts with axon terminals and/or dendritic spines.

**Microglia**

Microglial processes displayed irregular contours with obtuse angles, distinctive long stretches of endoplasmic reticulum visible through the DAB staining, electron-dense cytoplasm, numerous large vesicles, occasional multi-vesicular bodies, vacuoles or cellular inclusions, and distinctive surrounding extracellular space, distinguishing them from astrocytes, oligodendrocytes or NG2-positive glial cells.

**Quantitative Analysis**

Quantitative analysis of synaptic cleft interactions, element contacts, and inclusions were calculated by dividing the number of events per Iba-1 labeled process by the area of the process (μm²) and averaging across processes (50-88 per animal). For measurement of microglial process areas, individual microglial processes were traced with the freehand line tool in ImageJ. A Grubb's outlier test was used once to remove outliers (these were exceptionally small process whose normalization yielded a very large number).
Code Availability

Matlab code for motility analysis is available upon request from the author.

Statistics

Statistical comparisons were made between animal cohorts using Prism VI statistical analysis software (GraphPad, La Jolla, CA). All n-values represent individual animals, and are comparable to standard n values used in similar experiments in the literature. All values reported are the mean ± standard error of the mean (SEM). For all analyses, α=0.05. Two-tailed unpaired Student’s t-tests, and one-way or two-way ANOVAs with Holm-Sidak post-hoc comparisons were used to compare cohorts where appropriate.

Results

In order to determine whether P2Y12 facilitates process ramification in ODP, I verified that P2Y12 is expressed in the non-pathological mouse primary visual cortex using immunohistochemistry (Figure 8). P2Y12 expression was highly enriched throughout the visual cortex and co-localized extensively with cortical microglia expressing GFP (CX3CR1\textsuperscript{GFP+/}). P2Y12 localized on microglial membranes of both the soma and distal processes with particularly high enrichment on terminal pseudopodia (Figure 8B). Quantifying the proportion of GFP+ microglia expressing P2Y12 indicated that 100% of visual cortex microglia are immune-positive for P2Y12 reflecting the pronounced and widespread expression of P2Y12 in the non-pathological brain (Figure 8C). Importantly, P2Y12 expression could not be detected in P2Y12\textsuperscript{KO} mice (Figure 9).
Figure 8: Microglia ubiquitously express P2Y12 in binocular visual cortex. Confocal images of CX3CR1\textsuperscript{GFP/\textsuperscript{+}} microglia (green) in binocular visual cortex stained for P2Y12 (magenta). Images were taken using a 10x (A) and 40x (B) objective. Left panels show GFP representing microglia, middle panels show P2Y12 immunoreactivity, and right panels represent the merge. C. High levels of co-localization between GFP and P2Y12 immunoreactivity were observed in CX3CR1\textsuperscript{GFP/\textsuperscript{+}} mice. Left panels- images with circles denoting expression in individual microglia. Right panel- graphing showing quantification in three animals. Every GFP+ microglia contained P2Y12 immunoreactivity. V1b = binocular visual cortex, V2L = lateral area of secondary visual cortex, DHC = dorsal hippocampal commissure. Scale bars = 100\,\mu m (A), 50\,\mu m (B, C).
Pharmacological and genetic characterization of P2Y12 disruption

In order to test the effects of P2Y12 disruption on baseline microglial function in the non-pathological brain, I needed to generate and validate genetic and pharmacological models of P2Y12 disruption. For the genetic P2Y12 ablation model, I used P2Y12KO mice on a C57/Bl6 background (kindly provided by Dr. Maiken Nedergaard). To image microglia in vivo, I crossed P2Y12KO mouse lines with CX3CR1GFP/GFP animals to generate CX3CR1GFP/+/P2Y12KO mice that have one functional copy of fractalkine receptor and one copy of GFP, but are devoid of P2Y12 expression. In accordance with previous reports, I verified that microglia in these mice have a severely diminished process response to laser ablation (data not shown). In addition, P2Y12KO mice had symptomatically decreased platelet clotting as evidenced by decreased blood coagulation.

In order to pharmacologically block P2Y12 signaling, I used the anti-thrombotic drug clopidogrel (see Table 6 & Figure 34). Clopidogrel is a highly selective and irreversible P2Y12 antagonist developed as an anticoagulant that requires hepatic metabolism to produce an active metabolite. Although information regarding its ability to
cross the blood-brain barrier (BBB) is sparse, original radiolabeling studies indicated that detectable signal was observed in the brain, and the chemical structure of clopidogrel is predicted to be highly BBB permeable. In addition, previous studies have demonstrated that peripheral administration of clopidogrel is able to suppress neuropathic pain by suppressing P2Y12 activity in the spinal cord, suggesting that at sufficiently high doses, the drug crosses the BBB. I used a relatively large clopidogrel dose of 50 mg/kg administered via intraperitoneal injection and validated drug delivery to the brain by measuring P2Y12-dependent laser ablation responses (Figure 10). Though microglial processes converged on the laser ablation core following clopidogrel administration, the rate of process recruitment was significantly slower in comparison to saline injected animals, indicating partial blockade of P2Y12 function (Figure 10C). As a control, I tested laser ablation responses following ticagrelor administration (10 mg/kg, i.p.). Ticagrelor is a highly selective P2Y12 antagonist that is not predicted to cross the BBB. I found that microglia in ticagrelor dosed animals had robust process responses to laser ablation indistinguishable from saline treated animals indicating that it does not readily cross the BBB.
Baseline microglial morphology – Sholl and skeleton analyses

After validating the genetic and pharmacological models of P2Y12 disruption, I tested whether these manipulations affected baseline microglial ramification. I chose to use two different analyses in order to comprehensibly quantify microglial morphology. I used Sholl analysis to provide an overall measure of morphological complexity and was previously used in my lab to describe changes in microglia following ODP. Sholl analysis
consists of drawing concentric rings outward from the soma and measuring the number of intersections with each ring as a function of distance from the soma (Figure 11E). Microglia typically have a few intersections close to the soma that represent primary projections. At distances of ~10-30 μm from the soma, there is a distinctive spike in processes representing secondary and tertiary branches. Typically microglial processes do not extend farther than 50 μm from the soma under basal conditions. First, I compared cortical microglial morphology from P2Y12^{WT} and P2Y12^{KO} animals using Iba-1 immunohistochemistry to visualize microglial process arbors. Although general qualitative observation of microglial morphology did not indicate gross abnormalities in microglia from P2Y12^{KO} animals (Figure 11A), Sholl analysis demonstrated a significant decrease in process intersections ~10-30 μm from the soma, indicating a reduction in secondary and tertiary branching (Figure 11B). This difference was reflected by an overall significant decrease in total intersections as measured by comparison of Sholl curve integrals.
To determine whether pharmacological P2Y12 disruption affects microglial morphology, I dosed animals with saline or clopidogrel (50 mg/kg, i.p.) 4 hours prior to tissue fixation and collection. Again, using Iba-1 as a marker of microglial morphology, I
analyzed process ramification using Sholl analysis. As before, qualitative observation did not indicate gross morphological deficits in clopidogrel treated animals relative to saline treated animals (Figure 11C). However, Sholl analysis revealed a significant decrease in intersections ~10-25 μm from the soma, representing a deficit in secondary and tertiary branching similar to P2Y12KO animals (Figure 11D). Accordingly, as in P2Y12KO animals, clopidogrel treated animals also had a significant decrease in total intersections relative to saline controls, though the deficit was not as pronounced perhaps due to incomplete blockade of P2Y12 using clopidogrel.
Though Sholl analysis provides an overall measure of morphological complexity, it does not provide specific information regarding process features. In order to better understand the deficits revealed by Sholl analysis, I analyzed the same microglia using skeleton analysis. Instead of counting intersections as a function of distance from the soma, skeleton analysis systematically reduces pixel width of binary images to produce specific deficits in microglia from P2Y12KO or clopidogrel treated animals. A. Example of skeletonizing analysis work flow. Single microglia are isolated from surrounding microglia and filtered using a binary threshold. Skeletonizing the microglia produces a single pixel outline of microglial processes, which is analyze for number of branches, junction points, longest process length, and total area. B-C. Skeleton analysis on P2Y12KO microglia demonstrated that there is a significant decrease in longest branch (C) compared to P2Y12WT microglia with no change in total area, number of branches, or number of junctions (B-C). However, clopidogrel treated animals exhibit microglia that have a significant decrease in branch number and junction number (D), and a near-significant decrease in longest branch and total area (E) compared to microglia from saline treated animals.

Figure 12: Skeleton analysis describes specific deficits in microglia from P2Y12KO or clopidogrel treated animals. A. Example of skeletonizing analysis work flow. Single microglia are isolated from surrounding microglia and filtered using a binary threshold. Skeletonizing the microglia produces a single pixel outline of microglial processes, which is analyze for number of branches, junction points, longest process length, and total area. B-C. Skeleton analysis on P2Y12KO microglia demonstrated that there is a significant decrease in longest branch (C) compared to P2Y12WT microglia with no change in total area, number of branches, or number of junctions (B-C). However, clopidogrel treated animals exhibit microglia that have a significant decrease in branch number and junction number (D), and a near-significant decrease in longest branch and total area (E) compared to microglia from saline treated animals.
a frame that is 1 pixel wide. This “skeleton” can then be analyzed along a number of dimensions, including total skeleton area, branch number, junction number, and longest process (Figure 12A). In order to expedite analysis, I developed a custom MatLab program to generate averages of each of these measures across all microglia analyzed. Microglia from P2Y12\textsuperscript{KO} animals only had a significant reduction in the average longest process relative to P2Y12\textsuperscript{WT} controls with no significant difference in average total skeleton area, average branch number, and average junction number (Figure 12B, C). Surprisingly, microglia from clopidogrel treated animals had a significant decrease in nearly every metric relative to saline injected animals (Figure 12D, E).

Overall, both genetic and pharmacological disruption of P2Y12 function produced a modest but significant reduction in morphological complexity of microglial processes in the non-pathological brain, particularly at distances from the soma where substantial secondary and tertiary branching are most prevalent. It is unclear why morphological deficits seem more pronounced in the genetic model than the pharmacological model using Sholl analysis but the opposite using the skeleton analysis. Though the skeleton analysis provides more detailed information regarding morphological characteristics, its automated generation of the process “bones” can create artifacts. For example, if processes form a loop (occasionally after 3D z-projection), the algorithm will split the loop at its shortest segment and count it as two separate processes. Consequently, this artifact can produce inflated branch and junction numbers. Therefore, unfortunately the skeleton analysis did not provide conclusive explanations for the deficits in morphological complexity discovered using Sholl analysis. In the future, the skeleton analysis algorithm will need to be characterized more carefully to understand how it behaves with particular morphology differences and imaging artifacts.
P2Y12 disruption and baseline motility

In order to determine whether microglia with disrupted P2Y12 signaling had decreased surveillant motility, I used CX3CR1\textsuperscript{GFP+/+} P2Y12\textsuperscript{KO} mice and two-photon microscopy to image baseline microglial process dynamics \textit{in vivo}. I used a thin-skull preparation rather than a cranial window to minimize immune perturbation that might cause immune activation of cortical microglia. I collected 12 z-stacks of microglia from layer II/III of the cortex every 4 minutes for a total of 48 minutes. This allowed me to track motility changes short and long timespans.

Rather than limit the number of microglial processes analyzed by individually tracing branches, I used RGB overlays of binarized images and custom MATLAB algorithms to generate a number of motility measurements (Figure 13). Each time point sampled consisted of a 40 μm deep z-stack from which 15 μm z-projections were created to yield a 2-dimensional (2D) image of microglial processes. Z-projections coordinates within the original z-stack were adjusted across time to account for motion artifacts in the z-dimension. Following the creation of 2D z-projections across time, time points were concatenated into an image stack and aligned in the x and y dimensions. The entire stack was then thresholded to create a binary image of white microglia on a black background. Background noise was removed and microglia cropped to yield ~3-5 microglia to be analyzed.

Binary 2D time points were then saved individually. RGB timelapses were created by overlaying two time points (0-4 min, 4-8 min, etc.) with the first time point marked with red pixels (RGB: 255,0,0) and the second time point marked with green pixels (RGB: 0,255,0). Pixels present in the first time point but absent in the second were
represented as red pixels and were considered retraction pixels, while pixels present in the second time point but absent in the first represented green pixels and were considered extension pixels. Finally, pixels that were present in both the first and second time points were represented as yellow pixels (RGB: 255, 255, 0) and were considered stable pixels. Microglia analyzed in this manner had predominantly yellow somas and primary processes indicating relative stability across the entire imaging session. However, dynamic formation and elimination of pseudopodia at the distal ends of microglia processes yielded green and red puncta respectively. A simple motility index was defined as the sum of extension pixels (green) and retraction pixels (red) divided by the number of stable pixels (yellow) (Figure 13A, B). This index provided a simple change/no change metric over the course of 4 minutes and was averaged across 11 RGB time lapse overlays collected from the original 12 time points. By dividing by the number of stable pixels, the motility index was normalized to the number of microglia analyzed per field of view.
Figure 13: RGB overlays provide an automated pixel-based analysis to measure changes in microglial motility profiles. A. Schematic showing the analysis of time lapse in vivo images for microglial motility. Images are first thresholded to remove background (left panel). Consecutive images are pseudocolored in red or green and overlaid (middle panel). Yellow pixels in the overlay images are stable between the two time points, green pixels represent newly extended processes and red pixels indicate retraction (right panel). This process is repeated for all consecutive time points during the imaging session (1 hour). B. Equations for the calculation of four microglial motility metrics. C. An example of a stability histogram heat map. Red indicates highly stable pixels (left panel). Removing pixels that do not change throughout imaging removes somas and primary processes from analysis (right panel). D. Stability histogram heat maps for microglia using non-binarized analysis that compares changes in the brightness of pixels rather than absolute intensity. Changing the threshold needed for brightness ratios to register as extension or retraction alters the included pixels for the stability histogram analysis. Note that a threshold brightness ratio of 0.33 under-thresholds microglia while 0.66 over-thresholds. Scale bar = 20μm.
In order to capture more detailed information of microglial process dynamics and fates over multiple time lapses, I generated a stability and instability index. Pixel values across time lapses were loaded into a binary 3D matrix using MATLAB in which the x and y dimensions represented the pixel coordinates and the z dimension represented time lapses (11, 2D matrices per imaging session). Zeroes represented black background pixels and ones represented yellow or stable pixels. By comparing two time lapses using this matrix, I could determine the proportion of extension pixels (green) from one time lapse that became stable (yellow) in the subsequent time lapse divided by the total extension in the first time lapse. Total green pixel counts were already generated for each time lapse when calculating the motility index and extension pixels that became stable were determined as binary values in the 3D time lapse matrix that switched from zero to one. This measure I defined as the “stability index,” and it essentially represents the probability that a microglial pseudopodia extending over the course of one 4 minute interval, will be stable in the second 4 minute interval. In the same manner, I defined the “instability index” as the proportion of stable pixels (yellow) in one time lapse that became retracted (red) in the subsequent time lapse divided by the total number of stable pixels in the first time lapse.

The stability index and instability index allow for a comparison of microglial behavior over two time lapses, that is three time points. However, although microglial processes move at a remarkable rate on the order of minutes, I wanted to generate a measure that captured the fate of processes across the entire imaging session. If a microglial process extended in one time lapse, then how long did it stay stable across the entire imaging session (multiple time lapses)? To answer this question, I generated a stability histogram (Figure 13C). Again, using the binarized 3D matrix representing
stable pixels across time, I generated a second matrix that was a weighted version of the first, rewarding pixels that were stable across consecutive time lapses (in the z dimension). This weighting was achieved by specifying that for any given stable pixel value, if the pixel was stable in the previous time lapse, then the new stable pixel value was the previous value plus one \( V_{t=1-2} = V_{t=0-1} + 1 \). Therefore, when a pixel becomes stable for the first time lapse, it is represented as 1 \( (V_{t=0-1} = 1) \). If that pixel remains stable in the next time lapse then the value becomes the previous value plus 1 \( (V_{t=0-1} + 1 = 2) \). If that pixel remains stable again for the third time lapse then the value for that time lapse would be 3 \( (V_{t=1-2} + 1 = 3) \). As soon as a pixel retracts, its value automatically becomes 0 again and the weighting is reset. Consequently, the highest stability pixel values are those that stay consecutively stable for the longest number of time lapses.

Collapsing the z-dimension vector by summing values across time, yields a single number for each pixel coordinate pair reflecting this consecutive stability. For an imaging session that contains 12 time points, there would be a total of 11 time lapses and 10 time lapse comparisons. The highest stability value \( (V_{\text{high}}) \) possible can be generated using the following equation:

\[
V_{\text{high}} = \frac{n \times (n + 1)}{2}
\]

where \( n \) represents the number of time lapse comparisons (in the case of 10 comparisons, \( V_{\text{high}} = 55 \)). This value would be obtained for a pixel that was stable throughout the entire imaging session as would be the case for somas and primary processes that remain relatively stationary or yellow throughout the time lapse comparisons. Because I wanted to know about the stability of processes once they moved, I designed the program to make all pixels that were stable across all time lapses
0 removed as background so that only dynamic pixel stability was analyzed. The new highest value can be represented with the equation:

\[ V_{\text{high}} = \left( \frac{n \times (n + 1)}{2} \right) - n \]

which would represent a dynamic pixel that extended in the first time lapse and remained stable for the rest of the imaging session. For 10 time lapse comparisons, this value would be 45. The stability histogram measure creates a histogram from 0 to the highest value observed across all pixels and divides it into 10 equal bins. The lowest bin is discarded as background and the remaining 9 bins are divided into three larger bins representing low stability pixels (sum bins 1-3), medium stability pixels (sum bins 4-6), and high stability pixels (sum bins 7-9). The proportion is generated by taking the number of pixels in each larger bin divided by the sum of all the pixels from the remaining 9 bins. This allows for a measure of stability that does not depend on the number of time points analyzed or the highest stability theoretically possible, but rather provides a distribution measure of process stability relative to the max stability processes in a given image. This relative scale of the histogram provides greater tolerance across image quality variability.
I found that microglia in P2Y12KO animals had no significant difference in microglial motility index, stability index, instability index, or stability histogram (Figure 14A-F). These results suggest that across the time span of 1 hour imaging sessions, baseline microglial process dynamics are unaltered by loss of P2Y12 function. The lack
of effect in motility index suggests that across 4 minute time lapses, the rate of extension and retraction of processes occurs at the same rate between P2Y12\textsuperscript{KO} and P2Y12\textsuperscript{WT} animals (Figure 14B). Fluorescence photobleaching or process retraction due to microglial activation over the course of the imaging session could potentially produce an artificially high motility index. This would register as a higher net rate of retraction than extension. To verify that microglial activation or photobleaching were not confounding the motility index, I analyzed extension and retraction pixels normalized to stable pixels independently (Figure 14E). This analysis demonstrated that extensions and retractions occur approximately at the same rate (~50% of the motility index each) yielding no net difference between indices across genotypes. The absence of altered stability index suggests that when P2Y12\textsuperscript{KO} microglia extend a pseudopodia that the process remains stable in the next 4 minutes to the same degree that P2Y12\textsuperscript{WT} microglia exhibit (Figure 14C). Accordingly, the likelihood at any given time point that a process will retract in the next 4 minutes is unaffected by P2Y12 deficiency as evidenced by the comparable instability index between genotypes (Figure 14D). Finally, the stability histogram results indicate that across the entire imaging session, microglial processes from P2Y12\textsuperscript{KO} animals are equally likely to stay stable for the same duration of time as microglial processes from P2Y12\textsuperscript{WT} animals (Figure 14F).

Motion artifact from animal respiration or headplate fixation can produce artificial motion as even slight shifts in pixel location across time will register as change in these equations. However, this fact does not mask an effect between the genotypes as the image quality and motion artifact was comparable between animal cohorts. Furthermore, automated alignment of stacks across time and pixel smoothing coupled with binary thresholding reduces small changes to a negligible background. It is possible that due to
the fact that the motility index, stability index, and instability index measure changes within a 4-8 minute window in this analysis, my results miss potential differences between the genotypes on longer timespans. However, consistent differences over longer periods of time should have been detected with the stability histogram. The fact that this measure yielded no difference between genotypes suggest that microglial processes from P2Y12$^{\text{KO}}$ animals behave similarly to P2Y12$^{\text{WT}}$ animals on both short term changes and long term behavior.

These results corroborate the lack of difference in baseline motility between genotypes observed by the Julius lab by using multiple novel measures, an increased sample size, and a larger sampling window. Other labs have measured baseline microglial motility in P2Y12 deficient mice since these experiments were performed and their results have also replicated these findings. The absence of alterations in microglial motility does not however contradict the decrease in microglial ramification observed previously. The analyses used to measure changes in microglial process motility do not provide a measure of morphology and it is wholly possible that motility in P2Y12$^{\text{KO}}$ animals occurs at the same rate as P2Y12$^{\text{WT}}$ animals despite a less ramified morphology. Additionally, the CX3CR1$^{\text{GFP+}}$/ P2Y12$^{\text{KO}}$ animals used in this experiment only allow for visualization of microglial processes, which does not allow for investigation of interactions between microglial processes and other elements of the brain parenchyma. In order to probe whether microglial interactions with other cells in the brain are altered by P2Y12 deficiency, I turned to electron microscopy to answer this question.

**Baseline inclusions and cleft interactions**
The previous work done in the Majewska lab demonstrated that on the ultrastructural level, microglial proximity to specific elements in the brain is changed during ODP. Specifically, microglia contained membrane-bound inclusions and were found at synaptic clefts more frequently. While the motility experiments demonstrated that P2Y12<sup>KO</sup> animals display normal baseline microglial dynamics, the morphology data suggested that microglial signaling and association with parenchymal elements might be affected with P2Y12 deficiency. I performed an experiment to determine if P2Y12<sup>KO</sup> microglia exhibited similar frequency in ultrastructural characteristics as P2Y12<sup>WT</sup> microglia during ODP. Although I will discuss P2Y12 function and microglial behavior in the context of ODP in Chapter III, the design of the experiment provided information regarding the ultrastructural characteristics of microglia under baseline conditions, which I will describe here.
Electron microscopy was used in conjunction with Iba-1 immunoperoxidase staining to help identify microglial processes at the ultrastructural level. Imaging was performed in layer II/III of the binocular mouse visual cortex on tissue collected from P2Y12KO and P2Y12WT animals (Figure 15A). Cellular identities and structures were identified as described in the methods with “The Fine Structure of the Nervous System”.

**Figure 15: P2Y12 disruption does not alter baseline ultrastructural behaviors.**

A. Representative electron micrograph of Iba-1 immunoreactive microglial processes in binocular visual cortex (a = perisynaptic astrocytic process, s = dendritic spine, t = axon terminal, d = dendrite, m = microglial process, * = extracellular space, arrow = cleft contact, arrowhead = inclusion). B. No statistically significant difference was observed in the number of microglial contacts with dendritic spines, axon terminals, astrocytes, or synaptic clefts in P2Y12WT microglia vs. P2Y12KO microglia. In addition there was no difference between the frequency of intracellular inclusions in or the amount of extracellular space around microglia from P2Y12WT mice compared to microglia from P2Y12KO mice (n=5, data presented here is the same baseline data as from Figure 23; statistics were run with the entire data set and can be found in Figure 23; post-hocs for these comparisons were non-significant, p>0.05). Scale bar = 200nm.

Electron microscopy was used in conjunction with Iba-1 immunoperoxidase staining to help identify microglial processes at the ultrastructural level. Imaging was performed in layer II/III of the binocular mouse visual cortex on tissue collected from P2Y12KO and P2Y12WT animals (Figure 15A). Cellular identities and structures were identified as described in the methods with “The Fine Structure of the Nervous System”. 

![Electron microscopy micrograph](image1)

![Graphs](image2)
System's serving as a reference guide. Images corresponding to ~1000 μm² of tissue were gathered at 30,000x magnification (~40-60 images per animal), and were chosen if they contained at least one distinguishable microglial process. Microglial processes were given a unique identifier and their process area, inclusion number, extracellular space, and contact profile recorded. Elements contacted were categorized into six groups: dendritic spines, axon terminals, dendrite shafts, astrocyte processes, synaptic clefts, and unknown. Each contacted element was divided by the area of the microglial process to normalize across small differences in number of microglia sampled in each animal and to control for the intuitive fact that larger microglial processes are more likely to contact parenchymal structures by chance due to a larger surface area (see Methods for additional details).

I found that in non-deprived P2Y12KO and P2Y12WT animals, microglia contacted dendritic spines and axon terminals with the highest frequency (~20-30% each of all contacts). Astrocyte processes and dendritic shafts were contacted less frequently (~10-15% each) and synaptic clefts rarely (~1-4%). A large proportion of contact identities were unable to be determined (~30%). There was no difference in the relative frequency of contacts with any of the elements between P2Y12KO and P2Y12WT animals (Figure 15B). Importantly, this result was especially evident with the frequency of microglial processes found at the synaptic cleft, which was consistently low across genotypes (Figure 15C). Likewise, there was no change in baseline microglial inclusions with microglia from both genotypes averaging less than 1 inclusion per process. Lastly, there was no difference in the frequency or area of extracellular space surrounding microglial processes (Figure 15D).
These results indicate that microglia lacking P2Y12 do not display discernable differences from wildtype controls at the ultrastructural level. The absence of baseline effects in parenchymal cell contacts corroborates the normal motility phenotype observed previously and does not provide explanation for the modest but significant decrease in P2Y12KO microglial ramification. Extracellular space surrounding microglial processes is thought to relate to microglial basal process movement because enzymatic degradation of the ECM is presumably necessary for microglial motility through the densely packed parenchyma. In this view, the lack of difference in extracellular space between genotypes is consistent with the comparable motility between genotypes and suggests that microglial mechanisms to navigate around cellular compartments are intact. If signaling pathways mediate basal microglial-membrane interactions with the various elements analyzed here, then P2Y12 deficiency does not appear to disrupt these functions.

**Conclusion – summary of baseline microglial behavior following P2Y12 disruption**

The overarching goal of the experiments detailed here in Chapter II was to establish a baseline profile of microglial morphology, motility, and cellular contacts in P2Y12 deficient animals. Microglia in these animals largely appear phenotypically normal with no gross abnormalities. These results are somewhat surprising given the intense and ubiquitous expression of P2Y12 across nearly every microglial membrane. However, the lack of obvious baseline phenotype validates studies from other laboratories that have reported relatively normal microglial appearance under basal conditions. Taken together, the evidence supports the notion that P2Y12 is not necessary for maintaining basic microglial maintenance and dynamics.
Observations from P2Y12 immunostaining replicate data from previous reports that P2Y12 is highly enriched on ramified microglia in the non-pathological brain. Though I did not counterstain tissue with classical markers of other cell types such as astrocytes, the 100% colocalization of P2Y12 with GFP+ microglia suggests that P2Y12 may serve as an important marker for mature, non-pathological microglia. In fact, previous studies have suggested that P2Y12 expression distinguishes microglia from invading peripheral macrophages. Given that infiltrating macrophages can adopt similar morphologies and overlapping expression patterns with microglia, being able to distinguish the relative contributions of each population by using P2Y12 as a delineating factor may be critically important in future studies of neuropathology where macrophage infiltration has been documented.

The laser ablation experiments demonstrated that clopidogrel’s active metabolite is able to cross the BBB at high enough concentrations to slow P2Y12 recruitment to ablation cores validating its use as a pharmacological model. Though the response was not abolished as seen in P2Y12-deficient mice, it was nonetheless significantly slower than saline. A very recent study using laser ablation response has demonstrated that clopidogrel does not affect the laser ablation response unless the BBB is compromised by focal injury. These results contradict the results I present here. Several explanations may account for this discrepancy. Although I used 4% rhodamine to label vasculature in order to avoid compromising large blood-vessels with the ablation, I may have inadvertently caused damage to smaller vessels that are harder to visualize and thereby compromising the BBB. However, results described in Chapter III discussing the effect of clopidogrel on ODP suggest that clopidogrel crossing the BBB is not solely an artifact of BBB breakdown induced by laser ablation. Another explanation is that the other study
used a smaller dose of clopidogrel than I used in my studies (20 mg/kg vs. 50 mg/kg). The rationale given behind the lower 20 mg/kg was that it more closely mimics doses used by humans as an anti-thrombotic. However, as demonstrated by the other study, increasing the dose of clopidogrel from 20 mg/kg to 40 mg/kg resulted in a nearly 6-fold increase in bleeding time suggesting that higher doses of clopidogrel may have non-linear effects.

The results obtained from the laser ablation experiments not only served as validation of the genetic and pharmacological models used in these experiments, but also reinforce the critical role P2Y12 plays in these microglial responses. Laser ablations, though local in spatial damage, represent a traumatic perturbation of tissue homeostasis. As the immune sentinels of the brain, it seems probable that microglia would make use of a large and diverse repertoire of damage response mechanisms to respond rapidly to these injuries. It is surprising that knocking out P2Y12, one receptor, could have such a pronounced effect on microglial process accumulation at the ablation core. Though P2Y12-mediated chemotaxis in response to laser ablation likely represents the earliest signaling response in a large cascade of parallel injury response pathways, the degree to which the rapid process recruitment is slowed by pharmacological inhibition or abolished by the genetic knockout suggests P2Y12 is necessary for rapid responses in brain pathology. This indicates that despite constitutive absence of P2Y12 across development in the P2Y12KO animals, genetic compensation is either absent or insufficient to fill the role of this receptor. Microglia express P2Y13 transcripts and this receptor has been shown to share a high degree of homology with P2Y12 as evidenced by its similarly high sensitivity to ADP specifically and its traditional coupling to Gαi/o signaling. However, translation and surface expression of P2Y13 is absent in P2Y12KO
animals suggesting that this likely candidate has unknown functional roles. Ultimately, the results described here reinforce previous reports to suggest P2Y12 is solely responsible for rapid microglial process responses to purines under non-pathological conditions.

The deficit in microglial ramification is an interesting and atypical result from these experiments. While the decrease in morphological complexity was modest, it was replicated in both the pharmacological and genetic models of P2Y12 disruption. The previous studies that demonstrated correlation between a ramified morphology and P2Y12 expression advocate a relationship between P2Y12 function and microglial function under non-pathological conditions. However, the conclusions from that study and the morphology results here clarify that this correlation is not a causal relationship as P2Y12 signaling disruption does not produce an “activated,” amoeboid microglial morphology. Therefore, what significance does a small reduction in ramification indicate? The ultrastructural analysis of microglial contacts does not provide an explanation as no irregularities in cellular contacts were observed. It is possible that minor purinergic targeting events similar in rapidity but smaller in magnitude to laser ablation responses occur under normal physiological conditions. These events could increase surveillance to a particular area of the brain without an observable preference for cellular contact. However, no data presented here supports that speculation and it is unclear how P2Y12 contributes to a typical, fully ramified microglia morphology.

The motility data presented here corroborates previous work demonstrating that baseline microglial dynamics are overtly normal in P2Y12 deficient animals. Nevertheless, other studies have suggested that baseline microglial dynamics are
dependent on purinergic signaling as the application of apyrase, an enzyme that rapidly degrades ATP, disrupts basal microglial motility. The lack of P2Y13 protein in microglia suggests that signaling is not through P2Y12 or P2Y13. As of yet, though several research groups have attempted to find the mechanism behind surveillant microglial motility, candidate receptors have not been supported. One alternative explanation that has not been explored to my knowledge is that ATP does not signal through a receptor but rather serves as a kinase substrate to facilitate dynamic extracellular signaling pathways necessary for process motility, such as cell adhesion or permissive actin reorganization. This hypothesis has neither been supported nor refuted, and future experiments may shed light on this possibility.

Finally, given that P2Y12 expression is downregulated following microglia activation in response to LPS, it would seem plausible that absence of P2Y12 might engage a DAMP signaling cascade creating a proinflammatory phenotype. The data presented here suggests this proposition is not the case. First, as discussed above, microglial morphology is still reminiscent of quiescent microglial phenotypes. Furthermore, there is no difference in cellular inclusions on the ultrastructural level suggesting that microglia are neither increasing the phagocytosis or lysosomal degradation of material under basal conditions in the absence of P2Y12. Unfortunately, the analysis performed on the electron microscopy data here does not allow for the conclusive identity of the intracellular inclusions and it is possible that P2Y12 deficient microglia have a proinflammatory expression that is not detectable by the methods used in this study. To more extensively characterize P2Y12KO microglia in the future, a more comprehensive proteomics approach should be considered to investigate subtle changes in microglial expression. Overall, P2Y12 disruption largely does not affect
baseline microglial behavior, except in a small, perplexing reduction in morphological complexity. These results imply that any alterations in microglial behavior during ODP in the context of P2Y12 depletion are not a consequence of an altered basal microglial state.
CHAPTER III

P2Y12 in Ocular Dominance Plasticity

Introduction

Ocular dominance plasticity (ODP) is a well-studied model of cortical synaptic plasticity first described by Hubel and Wiesel in kittens. Following monocular deprivation (MD) of one eye, they found that the responsiveness of neurons in the contralateral binocular cortex shifted. Neurons that had biased responsiveness to input from the deprived eye began to respond more robustly to input from the open, ipsilateral eye. This shift of responsiveness from the closed eye to the open eye was termed an "ocular dominance shift." Since the work of Hubel and Wiesel, ODP has been used as a cortical model of activity-dependent plasticity to better understand the general mechanisms of cortical plasticity, not only within the visual system, but across other sensory systems as well.

From these studies, several important characteristics of ODP became apparent. First, the receptiveness of the brain to undergo network remodeling in ODP is relatively confined to the visual critical period in early development. Critical periods are unique time windows in development where the brain has reached a state of gross connectivity but is particularly sensitive to sensory experience. Perturbations of sensory experience within this window produce a rapid shift in neuronal responsiveness. Since Hubel and Wiesel demonstrated ODP within the cat visual critical period, other lines of work have shown that critical periods exist for other sensory systems such as the auditory and
somatosensory systems. Though the opening and closing of critical periods differs in time across sensory systems based on the timeline of cortical maturation for each modality, this highlights the generalizability of mechanisms in ODP across other cortical regions. Second, shifts in neuronal responsiveness in the visual cortex occur in two distinct phases over the course of days. The first period of ODP is characterized by a loss of responsiveness to the closed, contralateral eye and second period of ODP is characterized by an increased responsiveness to the open, ipsilateral eye.

Vision in mice is relatively underdeveloped in comparison to other vertebrates such as cats and humans as mice prefer to live in more spatially confined locations and rely heavily on whisker somatosensation to receive sensory information from the external environment. Despite poor visual acuity and a more lateral eye orientation, mice exemplify many of the tenets of visual cortical organization displayed by more visual mammals. Though they lack the distinct organization of ocular dominance columns present in mammals that utilize binocular vision more regularly, mice do have both monocular and binocular regions of primary visual cortex, the latter which displays ODP in response to monocular deprivation. As in cats, ocular dominance plasticity in mice occurs most robustly during a visual critical period, which peaks in adolescent mice (~P28). The genetic tractability and easy maintenance of mice has made them attractive candidates for probing the molecular mechanisms governing ODP and the opening/closing of visual critical periods. The ease of imaging intact mouse brains using two-photon microscopy has also made them attractive models for studying cellular structural dynamics during periods of synaptic remodeling. These reasons prompted our laboratory to use ODP in mice as a model to explore glial contributions to cortical synaptic plasticity.
Our lab began characterizing microglial behavior during ODP to test whether microglia play non-pathological roles in adolescent development during periods of experience-dependent cortical plasticity. Since microglial behavior has been predominantly studied during inflammatory “activation,” hallmarks of classical responsiveness were first explored. As discussed earlier, microglial morphology has been extensively used as a readout for microglial reactivity. During the mouse visual critical period, animals were deprived for 12 hours, 1, 2, 4, and 7 days (12hMD, 1dMD, 2dMD, 4dMD, 7dMD) or left non-deprived (ND) and their brains were fixed for immunohistochemistry. Using antibodies against the microglia specific protein ionized calcium binding adaptor protein 1 (Iba-1), microglia in the binocular visual cortex were imaged using confocal microscopy and their morphologies quantified using Sholl analysis (as described in Chapter II).
Figure 16: Microglia display rapid changes in morphology following MD. A. Images showing Iba-1 immunoreactive microglia in fixed sections of binocular visual cortex at different times following MD (ND = non-deprived; MD = monocular deprivation). B. Sholl analysis of microglia at different time points following MD. Note the hyper-ramification occurring following 12 hours of contralateral eye closure. (n=3-4, two-way ANOVA, p<0.0001, see Appendix for post-hoc comparisons). C. Images showing microglia in binocular visual cortex both contralateral and ipsilateral to the deprived eye, as well as primary somatosensory cortex contralateral to the deprived eye within the same animals in ND and 12hMD conditions (separate animal cohort from that shown in A-B, E-F). d. Hyper-ramification is restricted to the contralateral binocular visual cortex. (n=4-6, two-way ANOVA; p<0.0001 F(1,150) = 379.9; see Appendix for post-hoc comparisons). Scale bars = 50 µm. Soma size (E, one-way ANOVA; p>0.05) and somatic Iba-1 expression (F, Kruskal-Wallis one-way ANOVA, p>0.05) were not different at any time point after deprivation. RLL and CEL performed this experiment.
Sholl analysis of microglial morphology in response to monocular deprivation revealed a robust increase in hyper-ramification after as little as 12 hours of MD (Figure 16 A, B). This hyper-ramification was particularly pronounced around 20-30 μm away from the soma where microglial branching is greatest (see Appendix for post-hoc summaries). Interestingly, this increase in process complexity remained elevated at 1 day and 2 days of MD eventually decreasing at 4 days of MD and returning to ND levels by 7 days of MD. This hyper-ramification was limited to the binocular visual cortex contralateral to the closed eye (where plasticity is most evident). Hyper-ramification was not observed in the ipsilateral binocular visual cortex or the somatosensory cortex (Figure 16C, D). Following MD, there was no significant change in somal area or Iba-1 intensity, microglial density (Figure 16 E, F) or MHC-II and CD45 expression, which are implicated in immune responses (Figure 17A, B). Finally, there was no change in the density of microglia in contralateral visual cortex, ipsilateral visual cortex, or contralateral somatosensory cortex (Figure 18A, B).
Figure 17: Microglia do not show upregulation of classical immune markers following MD. A-B. Images showing immunoreactivity for MHCII (A) and CD45 (B) in fixed sections of binocular visual cortex at different times following MD. A negative control was included where the primary antibody was omitted. No expression of either marker was noted in ND or MD conditions. Lipopolysaccharide (LPS) injection (5 mg/kg, i.p.) robustly upregulated the expression of both markers. Scale bar = 100µm (first panel), 500µm (last panel). CEL performed this experiment.

Figure 18: Microglial density is unchanged by MD. A. Image of Iba-1 immunoreactive microglia in the contralateral binocular visual cortex after 12hr MD. B. Microglial density was comparable between contralateral binocular visual cortex, ipsilateral binocular visual cortex and primary somatosensory cortex of the same animals. Densities were unchanged by 12 hours of MD in all three regions (n=4-5 per group, One-way ANOVA, p>0.05). Scale bar = 100µm. CEL performed this experiment.
Taken together, these data suggest changes in microglial process arborization occurred independently of other hallmarks of microglial activation and represent an alternative activation from the classic immune response. Furthermore, tracing analysis of microglial processes revealed that microglial motility decreases significantly after 2 and 4 days of MD compared to ND controls (Figure 19C, D). Therefore, microglia display hyper-ramification and decreased process motility in response to MD without proliferation, alteration in somal size or intensity, and upregulation of classical immune signaling molecules.
To gain insight into microglial interactions with synapses after MD, electron microscopy was used to analyze microglia-synapse interactions in the binocular visual cortex at the ultrastructural level. Iba-1 positive microglial processes were characterized by their irregular contours and surrounding extracellular space. They were found to frequently make contact with synaptic elements such as dendritic spines, axon terminals and perisynaptic astrocytic processes (Figure 20A, B). Occasionally, microglial

Figure 19: Microglial motility, but not density, changes after MD. A. Example images showing microglia in binocular visual cortex chronically imaged in vivo. Microglial cell bodies were marked in images taken at 0, 2, and 4 days to compare microglial density over time in non-deprived (ND) and deprived (MD) mice. Scale bar = 100 μm. B. Quantification of microglial density as a ratio between the density observed after 2 or 4 days (D2, D4 respectively) and the density observed at day 0 (D0) in the same animal. No significant change in microglial density was observed (n=4-8 per group, Student's t-tests, p>0.05). C. Images showing time lapse imaging of microglial motility in the binocular visual cortex. Insets show traced portions of the microglial arbor. Notice the retraction (arrow) and extension (arrowhead) of processes on the time scale of minutes. Scale bar = 25 μm. D. Quantification of microglial motility on Days 0, 2 and 4 in the same animals (n=5 per group). Microglia were less motile in contralateral binocular visual cortex following 2 and 4 days of MD (two-way ANOVA, p<0.001, Holm-Sidak post-hoc, **p<0.01). Scale bars = 100μm (A), 25μm (B). RLL performed this experiment.
processes were found adjacent to synaptic clefts. To quantitatively compare these interactions between conditions, and control for the fact that large processes have the ability to interact with more brain elements and contain more inclusions, quantified observations were normalized to the size of the microglial process analyzed. While the analysis showed a trend towards an increase in the number of microglial contacts with dendritic spines and axon terminals during the early response to deprivation (1-4 days MD), these differences were not statistically significant (Figure 20B; one-way ANOVA; \( p > 0.05 \)). The number of microglial contacts with synaptic clefts, however, was significantly increased by MD (Figure 20C, one-way ANOVA, \( p < 0.05 \)) and remained elevated throughout the deprivation period (up to 7 days). Microglial processes also contained inclusions such as large vesicles, vacuoles, and endosomes containing cellular elements which may indicate active phagocytosis (Figure 20A). The number of processes with such inclusions was significantly elevated following deprivation (Figure 20D, one-way ANOVA, \( p < 0.05 \)) with the highest number of inclusions observed at 2 days MD, supporting an early role for microglia in ODP during the pruning of connections from the deprived eye.
To determine whether inclusions contain synaptic elements, immunohistochemistry was performed for GluA1, the main subunit of the cortical glutamate receptor, in the binocular visual cortex of ND and 4D MD CX3CR1$^{GFP/+}$ mice (Figure 21A). GluA1 surface expression has been shown to decrease during the early phase of ODP when synapses from the deprived eye are eliminated and increase when non-deprived eye responses strengthen$^{102}$. Three-dimensional confocal analysis showed that while most of the immunoreactivity for GluA1 was limited to puncta in the neuropil, some puncta were contained within microglial cell bodies and processes (Figure 21B). A
quantification demonstrated a significant increase in GluA1 puncta within the microglial cytoplasm after deprivation (Figure 21C; Student's t-test, p<0.0001), demonstrating active engulfment of synaptic material by microglia during periods of ODP.

In summary, following MD, microglia rapidly hyper-ramified selectively in the binocular visual cortex and remained hyper-ramified until 4 days of MD. This was accompanied by a decrease in process motility follow 2 and 4 days of MD, and occurred in the absence of somal characteristics, proliferation, or upregulation of classic immune mediators. On the ultrastructural level, microglial processes display increased frequency of membrane-bound inclusions and synaptic cleft interactions without a change in frequency of contacts with other parenchymal elements. Finally, following 4 days of MD,
microglia had increased co-localization of GluA1 positive puncta suggesting an increase in synaptic engulfment. This work demonstrated that microglia react rapidly during ocular dominance plasticity with an alternative activation phenotype independent of classical immune responses.

My results characterizing the role of P2Y12 in contributing to microglial baseline behavior demonstrated that other than a small decrease in ramification, microglia appear relatively unaffected by P2Y12 disruption by displaying robust baseline motility and typical cellular contact frequencies observed in normal animals (see Chapter II Results). Therefore, I investigated whether P2Y12 disruption affected the microglial changes observed during ODP previously in the lab, and if this disruption affected ODP itself.

**Methods**

**Animals**

Experimental protocols were carried out in strict accordance with the University of Rochester Committee on Animal Resources (UCAR) and conformed to the National Institutes of Health Guidelines. Experiments were conducted on mice with a C57/Bl6 background during the visual critical period (P25-35). All experiments and analyses were carried out blind to genotype and manipulation. Sex distribution is available in Supplementary Table 3.

*Monocular deprivation*

Animals were separated into non-deprivation (ND) or monocular deprivation (MD) cohorts on P28±1. MD animals were anesthetized (isoflurane, 5% induction, 3% maintenance) and right eyelids resected and sutured together. Eyes were not reopened
except for intrinsic signal imaging experiments. All analysis was carried out contralateral to the deprived eye (i.e. left hemisphere) unless stated otherwise.

**Histology**

Whole brains were collected following transcardial perfusion and overnight post-fixation with paraformaldehyde (4%). Tissue was cryoprotected and coronal sections were cut on a freezing microtome (Microm; Global Medical Instrumentation, Ramsey, MN) at 50 μm thickness. Sections were processed free-floating at room temperature (RT). Briefly, sections were rinsed and endogenous peroxidase activity and non-specific binding blocked. Sections were then incubated in a primary antibody solution (24 hours, 4°C, anti-Iba1, 1:1000, Wako #019-19741; anti-MHC-II, 1:7500 BD Pharmingen #556999; anti-CD45, 1:1000, Serotec #MCA1031G; anti-GluA1, 1:500, EMD Millipore #PC246) followed by secondary antibody solution (4 h, RT, Alexa-Fluor 488, 594 or 647, 1:500, Invitrogen), mounted and coverslipped.

For examination of microglial ramification and density, areas contained entirely within binocular primary visual or primary somatosensory cortex were identified and imaged on a Zeiss LSM 510 confocal microscope (Carl Zeiss, Thornwood, NY). For each section, a z-stack encompassing the entire thickness of the section was collected with a z-step of 1μm at a magnification of 20 and 40x. Analysis was performed offline in ImageJ. Z-stacks were smoothed and compressed into a single z-projection. For analysis of ramification, microglia in 40x images whose entire process arbor was contained within the image were individually selected and cropped into a new image. Each image was thresholded to generate a binarized outline of the process arbor, despeckled to remove imaging noise artifacts, and analyzed with an automated Sholl analysis plugin (kindly provided by the Anirvan Ghosh lab, UCSD). For analysis of
microglial activation, sections stained with either MHC-II, CD45 or Iba-1 were imaged on BX51 Olympus scope (Olympus, Tokyo, Japan) mounted with a Spot Pursuit RT color digital camera (Diagnostic Instruments, Sterling Heights, MI) at the same exposure settings. For quantitative analysis of microglial soma size and Iba-1 expression, microglial somas were outlined manually in ImageJ. The area and background subtracted mean intensity was measured and averaged for all microglia in a single animal. Microglial density was quantified on 20x confocal images by manually counting the number of Iba1 positive microglial cell bodies present within a measured area.

For quantification of co-localization of AMPA receptor subunits within microglia, a two-channel z-stack encompassing a 10µm section of tissue was collected with a z-step of 0.5µm at a magnification of 100x using confocal microscopy. Analysis was carried out offline in ImageJ. For each channel, z-stacks were smoothed and thresholded. For accurate, objective thresholding, the triangle method of auto-thresholding was selected. The number of GluA1 puncta localized within microglial cell bodies and processes was determined by multiplying thresholded, binarized z-stacks in each channel and quantifying the number of particles larger than 1 pixel. Co-localization was determined as the number of internalized puncta divided by the total area occupied by microglia.

Two-photon Microscopy

A custom two-photon laser-scanning microscope was used for in vivo imaging (Ti:Sapphire, Mai-Tai, Spectraphysics; modified Fluoview confocal scan head, 20X lens, 0.95 N.A., Olympus). Excitation was achieved using 100 fs laser pulses (80MHz) at 920 nm. For motility experiments a 580/180 (GFP) filter was used. Mice were anesthetized with a mixture of fentanyl (0.05mg/kg, i.p.), midazolam (5.0mg/kg, i.p.) and
dexmetatomadon (0.5mg/kg, i.p.). During surgery and imaging, body temperature was maintained at 37°C. Imaging was carried out using 1-10x digital zoom and 0.5-1 μm z-step. Time lapse imaging used 5 minute imaging intervals over 1-2 hours. Image analysis was carried out offline in ImageJ or in Matlab using custom algorithms.

**Microglial motility**

Microglial motility after MD was measured by individually tracing and measuring the length of the same individual microglial processes at 5 minute intervals over a one hour period. For each animal, analysis was performed on a single microglial process, defined as a process whose primary and secondary processes were clearly visible in their entirety in all twelve z-stacks over the hour period. The sections encompassing this process were identified for each of the twelve z-stacks and compressed into a single image z-projection. Beginning with the z-stack representing the 0 minute time point, each individual process was traced, measured, and numbered. This process was repeated for each z-stack in the hour period. Process motility was then calculated using the following formula: |(length of processX @timeB – length of processX @timeA)/5 minutes|.

Microglial motility indices were also calculated in ImageJ and Matlab using custom algorithms. Time-lapse imaging consisting of 40 μm deep z-stacks were collected every 5 minutes, 12 times, for a total of 60 minutes. Single image 15 μm Z-projections were created for each time point and lateral motion artifact was corrected. Z-projections were then thresholded and time points compared. RGB overlays were created for each pair of time points (0-4min, 4-8min etc.) such that red pixels (retraction) were present in the first time point, but absent in the second. Green pixels (extension) were present in the second, but not in the first, and yellow pixels (stable) were present in
both time points. Motility index (representing change over 4 minutes) was then calculated for each RGB overlay as the sum of all extension and retraction pixels divided by yellow pixels and averaged across all RGB overlays. A custom MatLab program was used to compare pixels across multiple RGB overlays (e.g. 0-5min vs. 5-10min). Stability index was calculated as the proportion of extension pixels (green) in one RGB overlay that became stable (yellow) in the subsequent overlay divided by the total extension (green) pixels in the first overlay. Conversely, an instability index was calculated as the proportion of stable (yellow) pixels in one overlay that became retracted (red) in the subsequent overlay divided by the total stable (yellow) pixels in the first overlay. Finally, the stability histogram compared the relative stability over multiple overlays of pixels that became stable at any time point in the imaging session (but omitting all pixels that were stable for the entire imaging session, generally somas or primary processes, see Chapter II for more details).

**Electron Microscopy**

EM was performed as previously described (Riad et al., 2000, Tremblay et al., 2010b). Briefly, primary visual cortex was prepared with 0.1% sodium borohydride (in 0.1M PBS), washed, and processed freely floating following a pre-embedding immunoperoxidase protocol. Sections were incubated with anti-Iba1 (48 h, RT, 1:1,000, Wako), goat anti-rabbit IgG conjugated to biotin (2 h, RT, Jackson Immunoresearch) and streptavidin-horseradish peroxidase (1 h, RT, Jackson Immunoresearch). Immunoreactivity was visualized with diaminobenzidine (0.5 mg/ml) and H₂O₂ (0.03%) (DAB Peroxidase Substrate Kit; Vector Laboratories).

Sections for EM were post-fixed (1% OsO₄), dehydrated, treated with propylene oxide, impregnated in Durcupan (24 h, RT, EMS), mounted between ACLAR embedding
films (EMS), and cured (48 h, 55°C). Binocular primary visual cortex (bV1) was excised and ultrathin sections (60-80 nm) were cut (Reichert Ultracut E) and images generated (Hitachi 7650 Transmission Electron Microscope, Gatan Erlangshen camera). Approximately 50-80 pictures were randomly taken at 30,000X in Layer 2 of bV1 (~10 μm from pial surface) corresponding to ~ 1,000 μm² of neuropil per animal. Cellular profiles were identified using a series of criteria previously defined in single-ultrathin sections. All subcellular profiles that were difficult to identify were classified as “unknown”. For quantification, measurements were normalized to the area of individual microglial processes to account for the fact that large processes have the ability to interact with more brain elements and contain more inclusions than smaller processes.

**Electron Microscopy Element Classification**

Elements were classified as described previously (see Chapter II Methods).

**Intrinsic Optical Signal Imaging**

P2Y12WT animals were injected with clopidogrel (50 mg/kg, i.p., Mylan S.A.S), ticagrelor (10 mg/kg, i.p. kindly supplied by Lundbeck Research USA, Inc.) or saline daily for 4 days beginning the day of deprivation. Following 4 days of ND or MD, animals were re-anesthetized with isoflurane and chloroprocaine (2 mg/kg), and sutured eyes reopened. The skull over contralateral visual cortex was exposed, cleared, covered with agarose (0.5%), and sealed with a coverslip. Animal anesthesia was maintained with isoflurane (0.75%) throughout imaging.

A custom-made intrinsic optical signal (iOS) imaging set-up was used to record activity in visual cortex during presentation of a visual stimulus (DALSA 2M30 CCD). The cortex was illuminated with 550nm light to identify vasculature and 700nm light for intrinsic optical signal collection. Images of left visual cortex were collected continuously...
while either the ipsilateral or contralateral eye was stimulated by white horizontal square-wave bar gratings on a black background moving upwards (90°) or downwards (270°) at a frequency of 8°/s for 6 minutes (30 cm from eyes). Visually evoked responses were collected for each eye individually. The normalized amplitude of the fast Fourier transform component of the intrinsic signal was averaged for each eye from responses to both stimulus directions and compared between eyes offline using MatLab to determine ocular dominance (OD). An ocular dominance index (ODI) was computed using the following equation: ODI = (average contralateral response – average ipsilateral response) / (average contralateral response + average ipsilateral response).

Code Availability

Matlab code for motility analysis is available upon request from the author.

Statistics

Statistical comparisons were made between animal cohorts using Prism VI statistical analysis software (GraphPad, La Jolla, CA). All n-values represent individual animals, and are comparable to standard n values used in similar experiments in the literature. All values reported are the mean ± standard error of the mean (SEM). For all analyses, α=0.05. Two-tailed unpaired Student’s t-tests, and one-way or two-way ANOVAs with Holm-Sidak post-hoc comparisons were used to compare cohorts where appropriate.

Results

**P2Y12 contributes to changes in microglial morphology during ODP**

Given the slight decreases in microglial ramification observed basally in P2Y12 disrupted microglia, I tested whether P2Y12 was necessary for the microglial hyper-
ramification observed during ODP. I analyzed 10 cohorts of mice (n=6 per cohort) comparing varying lengths of MD (ND, 12hMD, 2dMD, 4dMD and 7dMD) in both P2Y12\(^{WT}\) and P2Y12\(^{KO}\) animals. Immunohistochemical staining for Iba-1 was used to visualize microglia in layer II/III of binocular visual cortex contralateral to the deprived eye, and Sholl analysis was used to quantify microglial morphology (Figure 22 A).

![Figure 22: P2Y12 disruption reduces hyper-ramification during MD. A. Representative confocal images showing microglia in contralateral binocular visual cortex of P2Y12\(^{WT}\) and P2Y12\(^{KO}\) mice before and after MD of different durations. Scale bar = 25 \(\mu m\). B. Sholl analysis confirms that microglia in P2Y12\(^{WT}\) animals hyper-ramify following 12hMD (two-way ANOVA; p<0.0001). C. Ramification also occurred after 12 hours of MD in P2Y12\(^{KO}\) mice (two-way ANOVA, p<0.0001), although to a smaller extent than in P2Y12\(^{WT}\) mice. See Appendix for post-hoc comparisons. D. ND and 12hMD data from B, C to emphasize difference. CEL helped collect this data.](image-url)
In P2Y12<sup>WT</sup> animals, microglia displayed hyper-ramified morphologies within 12 hours following MD relative to ND controls, replicating the previous observations in our lab (see Appendix for post-hoc summaries). This hyper-ramification returned to baseline by 2 days of MD and remained near baseline at 4 and 7 days of MD (Figure 22B). In P2Y12<sup>KO</sup> animals, microglia also demonstrated increased hyper-ramification following 12 hours of MD (Figure 22C). In a similar manner to P2Y12<sup>WT</sup> animals, P2Y12<sup>KO</sup> microglial hyper-ramification returned to baseline by 2, 4 and 7 days of MD. Comparing the Sholl curves between genotypes at each deprivation time point revealed that ND P2Y12<sup>KO</sup> microglia had a decreased baseline process ramification relative to ND P2Y12<sup>WT</sup> controls replicating my previous results. Interestingly, P2Y12<sup>KO</sup> microglial hyper-ramification at 12 hours MD was also significantly lower than P2Y12<sup>WT</sup> microglial hyper-ramification suggesting that P2Y12 disruption inhibits the full hyper-ramification present in wildtype animals (Figure 22D). There was no difference between genotypes at 2 days MD, however, at 4 and 7 days, there was again a significant decrease in microglial ramification in P2Y12<sup>KO</sup> microglia relative to P2Y12<sup>WT</sup> controls. From these results it appears that P2Y12-deficiency does not block hyper-ramification in response to monocular deprivation but significantly attenuates it.

**P2Y12 disruption prevents decreases in microglial motility following MD**

Though my previous data suggested no difference in basal microglial motility in P2Y12<sup>KO</sup> mice, I tested whether changes in microglial motility during ODP were affected by P2Y12 deficiency. Previous observations in the laboratory indicated that microglial motility is significantly lower at 2 and 4 days of MD in CX3CR1<sup>GFP/+</sup> animals. I repeated this experiment using CX3CR1<sup>GFP/+P2Y12<sup>KO</sup></sup> and imaged microglial motility using two-
photon microscopy in ND and 4 day MD animal cohorts. I imaged microglial motility in binocular visual cortex over the course of 1 hour and quantified motility by creating RGB overlays and generating a motility index (described in Chapter II and in the methods section of this chapter). Following 4 days of MD, P2Y12\textsuperscript{KO} microglial motility was unchanged compared to ND controls, suggesting that microglia in P2Y12\textsuperscript{KO} animals do not respond to ODP with a decrease in process motility (Figure 23A, C). Manual microglial tracing and process velocity analysis was previously used to demonstrate a decrease in motility during ODP in P2Y12\textsuperscript{WT} animals rather than the motility index. To verify that the absence of microglial motility changes in P2Y12\textsuperscript{KO} animals was not due to a difference in analysis from the original experiment, I repeated the analysis using identical manual tracing (Figure 23A, B). This analysis replicated the motility index results and demonstrated that in P2Y12\textsuperscript{KO} animals, microglial motility remains unchanged follow 4 days MD.
P2Y12 signaling mediates microglial targeting to the synaptic cleft and increased phagocytosis following MD

Based on the microglial morphology and lack of motility decrease in P2Y12KO animals during ODP, I next tested whether the changes in microglial behavior observed at the ultrastructural in P2Y12WT animals during ODP were affected by P2Y12 deficiency. I chose to compare genotypes at 4dMD when wildtype animals show the greatest increases in synaptic cleft interactions and intracellular inclusions to ND baseline controls. Cortices were collected, stained with Iba-1, and imaged using electron microscopy as described in the Methods. In brief, Iba-1 labeled microglial processes and their surrounding cellular contacts were imaged in layer II/III of the primary binocular visual cortex contralateral to eye deprivation. Analysis of extracellular space surrounding

Figure 23: Microglial motility in P2Y12KO mice is not altered by MD. A. Two-photon time lapse images showing microglia in vivo in P2Y12KO mice. Traced processes in the boxed area are shown in insets in the upper right-hand corner of each panel. B-C. Quantification of traced processes (B) and motility index (C) showed no change in motility after deprivation in P2Y12KO mice (Student's t-test, p>0.05). Scale bar = 25μm.
microglial processes revealed no significant difference between MD conditions or genotypes (data not shown). Likewise, the frequency of microglial contacts with dendritic spines, axon terminals, and astrocytic processes was not different following 4 days of MD in P2Y12<sup>WT</sup> animals or P2Y12<sup>KO</sup> animals, and no differences were observed between genotypes (Figure 24A, B).

![Figure 24: P2Y12 is necessary for increased synaptic cleft interactions and inclusions following MD. A. Electron micrographs of Iba-1 immunoreactive microglial processes (a = astrocytic process, s = dendritic spine, t = terminal, d = dendrite, m = microglial process, * = extracellular space, arrow = cleft contact, arrowhead = inclusion) B. Quantification of microglial contacts with dendritic spines, axon terminals and astrocytes, yielded no significant difference in contact frequency contacted between P2Y12<sup>KO</sup> microglia and P2Y12<sup>WT</sup> nor across ND and 4dMD animals. C. However, following 4dMD, P2Y12<sup>WT</sup> microglia exhibited a significant increase in synaptic cleft interactions while P2Y12<sup>KO</sup> microglia remained at baseline following 4dMD (n=5, two-way ANOVA, Holm-Sidak post-hoc, p<0.05). D. Likewise, the number of microglial inclusions was also increased in P2Y12<sup>WT</sup> animals following deprivation but not in P2Y12<sup>KO</sup> animals (n=5, two-way ANOVA, Holm-Sidak post-hoc, p<0.001). Scale * = p<0.05, ** = p<0.01, *** = P<0.001. Scale bars = 200nm. GOS and EAK collected this data. GOS analyzed it.](image)

However, analysis of synaptic cleft contacts by microglial processes revealed a significant increase in contact frequency in P2Y12<sup>WT</sup> animals following 4dMD (Figure 24C). Importantly, this outcome replicated the previous data from our laboratory. Remarkably, an increased frequency of cleft contacts was absent in P2Y12<sup>KO</sup> animals.
following 4dMD with microglia contacting synaptic clefts at a frequency comparable to ND animals (Figure 24C). Similarly, analysis of intracellular, membrane-bound inclusions in microglial processes confirmed a significant increase in frequency of inclusions following 4dMD in P2Y12$^{WT}$ microglia relative to ND controls, reproducing previous data (Figure 24D). In P2Y12$^{KO}$ animals, this increase in inclusion frequency following 4 days of MD was absent. These data indicate that changes in microglial associations with parenchymal elements during ODP that were observed previously in our laboratory are dependent on P2Y12 signaling since P2Y12 deficiency abolishes these responses.

**P2Y12$^{KO}$ microglia do not exhibit increased GluA1 internalization following MD**

As described before, following 4dMD, microglia contain increased intracellular GluA1 staining suggesting augmented phagocytosis of material containing cortical glutamate receptors. To determine whether P2Y12 is necessary for this phagocytic behavior, intracellular GluA1 staining was analyzed in ND and 4dMD, P2Y12$^{KO}$ mice (Figure 25). Although GluA1-positive puncta were observed within GFP+ microglia, no increase in puncta co-localization was observed following 4dMD (Figure 25C). This result suggests that P2Y12 is necessary for the increased GluA1 phagocytosis observed in P2Y12$^{WT}$ animals.
P2Y12 is critical for ocular dominance plasticity

To summarize the results thus far, in P2Y12WT animals, MD elicits an increase in morphological process complexity, a decrease in process motility, an increase in synaptic cleft contacts, an increase in intracellular inclusions, and an increase in GluA1 phagocytosis. In P2Y12KO animals, following MD, microglia showed an attenuated increase in morphological process complexity, no change in process motility, unchanged synaptic cleft contacts, no change in intracellular inclusion frequency, and no increase in GluA1 phagocytosis. Overall, these experimental effects support the role of P2Y12 as being a critical component facilitating microglial responses to ODP. But, in the absence of these behaviors, is ODP functionally affected? Is P2Y12 necessary for the extensive
cortical remodeling taking place during ODP? Do microglia play an active role in cortical synaptic plasticity or do they simply play a passive glial role in response to changes in neuronal dynamics? To answer these questions, I investigated whether P2Y12 disruption has a functional outcome on shifts in neuronal responsiveness that effectively define ODP.

I used intrinsic optical signal (iOS) imaging to quantify responses to contralateral and ipsilateral eye input in binocular visual cortex and used the relative amplitudes of the hemodynamic response to calculate an ocular dominance index (ODI) defined as:

\[
ODI = \frac{\text{Response}_{\text{contra}} - \text{Response}_{\text{ipsi}}}{\text{Response}_{\text{contra}} + \text{Response}_{\text{ipsi}}}
\]

With normal visual experience, the mouse binocular visual cortex will display stronger cortical hemodynamic responses to stimulation of the contralateral eye than stimulation of the ipsilateral eye. This preferential activity to stimulation of the contralateral eye will yield a positive ODI value and represents the normal contralateral bias of the mouse visual cortex. If ODP occurs unhindered, then monocular deprivation of the contralateral eye should decrease the hemodynamic response to stimulation of that eye reducing the contralateral bias and producing an ODI closer to zero. This shift occurs within the first few days of MD and represents the weakening of contralateral input to the binocular visual cortex. Following 4-7 days MD, the hemodynamic response will begin to show a stronger response to stimulation of the ipsilateral eye relative to the contralateral eye, producing a negative ODI value. This ipsilateral shift represents the strengthening of inputs from the ipsilateral eye to the binocular visual cortex.
To test the role of P2Y12 in ODP, I compared ocular dominance shifts in P2Y12<sup>WT</sup> animals treated with saline versus P2Y12<sup>WT</sup> animals treated with clopidogrel, versus P2Y12<sup>WT</sup> animals treated with ticagrelor, or P2Y12<sup>KO</sup> animals. As expected, ND P2Y12<sup>WT</sup> animals treated with saline had a strong contralateral bias (Figure 26B, C). Likewise, ND clopidogrel-treated, ticagrelor-treated, and P2Y12<sup>KO</sup> animals also had comparable contralateral biases, suggesting that P2Y12 disruption does not cause

Figure 26: P2Y12 is necessary for ocular dominance plasticity. A. Schematic showing the intrinsic optical signal imaging apparatus used (left). Cortical responses to visual stimuli (a moving vertical bar presented on a monitor 30 cm in front of the mouse) were recorded as changes in the reflectivity of 700 nm light. This allowed the collection of phase maps (top right) which indicate retinotopy, and amplitude maps (bottom right) which indicate the strength of the cortical response. Amplitude maps obtained from stimulation of each eye independently were used to compute the ocular dominance index. B. Representative amplitude maps obtained from contra and ipsilateral eye stimulation in different conditions. C. Quantification of ocular dominance index shows robust shifts in saline- and ticagrelor-treated mice after MD, but no shift is observed in clopidogrel-injected and P2Y12 KO animals (n=5-7 per group, two-way ANOVA, Holm-Sidak post-hoc, * = p<0.05, ** = p<0.01, *** = p<0.001).
abnormal network organization early in development. Following 4dMD, animals injected with saline daily demonstrated an ocular dominance shift as evidenced by exhibiting similar hemodynamic responses to stimulation of the contralateral and ipsilateral eye, and thereby validating the ODP model. However, an ocular dominance shift did not occur in animals treated daily with clopidogrel (50 mg/kg, i.p.) or P2Y12KO animals, which retained robust contralateral biases following 4dMD (Figure 26B, C). Analyzing maximum single eye responses indicated that 4dMD predominantly results in the depression of deprived eye responses and no change in ipsilateral eye responses, consistent with ODP time courses described in the literature (Figure 27). Disrupted P2Y12 signaling appears to block this weakening of the contralateral response.

In order to test whether ODP is simply delayed by defective P2Y12 signaling, I imaged P2Y12KO animals after extending the deprivation period to 7 days. Remarkably,
these mice retained a strong contralateral bias indicating that ODP was still abrogated. However, P2Y12\textsuperscript{WT} animals treated daily with ticagrelor (10 mg/kg i.p.) displayed robust ocular dominance shifts following 4dMD suggesting that peripheral P2Y12 blockade has no effect on ODP and that the deficits in ODP found in clopidogrel treated and P2Y12\textsuperscript{KO} animals are due to blockade of P2Y12 in the CNS. Given the microglia-selective expression of P2Y12 in the brain parenchyma, overall these data suggest that homeostatic microglia are necessary for ODP, and that P2Y12 signaling plays a critical role in this process.

**Conclusion – summary of ODP phases and P2Y12 results**

The overarching goal of the experiments presented in Chapter III was to determine if P2Y12 is necessary for changes previously observed in microglial behavior during ODP, and to test whether P2Y12 itself is necessary for ODP. Although microglia in P2Y12\textsuperscript{KO} animals still exhibited an increase in process ramification following 12hMD, this response was significantly diminished when compared to P2Y12\textsuperscript{WT} animals. Microglia from P2Y12\textsuperscript{KO} animals also demonstrated no change in process motility, synaptic cleft contact frequency, intracellular inclusion frequency, and GluA1 phagocytosis, demonstrating that genetic ablation of P2Y12 is sufficient to disrupt all the previously observed changes in microglial behavior during ODP. Furthermore, ocular dominance shifts were disrupted using both pharmacological and genetic models of P2Y12 disruption implicating microglia and P2Y12 in ocular dominance plasticity for the first time.
Microglia respond to ODP at a remarkably rapid rate displaying hyper-ramification within 12 hours following MD. This response occurs before neurons undergo plastic changes suggesting that microglia are actively recruited early in plasticity and not simply responding to plastic changes that have already occurred. This initial hyper-ramification returns to baseline within the first phase of ODP, and may represent an initial homeostatic response to largescale neuronal changes in network activity following eye deprivation. Importantly, though diminished, $P2Y12^{KO}$ microglia display a hyper-ramification following 12hMD. These results indicate that hyper-ramification in $P2Y12^{KO}$ microglia occurs despite ODP being disrupted in these animals. These results suggest that another, yet unknown, mechanism contributes to this hyper-ramification, and that it is $P2Y12$-independent. These results also do not determine whether the hyper-
ramification is upstream of P2Y12-dependent functions or whether hyper-ramification is necessary for microglial contributions to ODP. Future studies will need to explore these possibilities.

These results demonstrate that P2Y12 is critical for most of the microglial behaviors in ODP described previously. The fact that P2Y12\textsuperscript{Ko} microglia did not exhibit increased synaptic cleft contacts following 4dMD may represent a deficit in synaptic cleft targeting mediated by purines and could perhaps contribute to the decreased hyper-ramification discussed above. The results discussed here do not establish a causal relationship between increased synaptic cleft interactions and increased intracellular inclusions/GluA1 phagocytosis in ODP; both behaviors are absent in P2Y12\textsuperscript{Ko} animals. Given that phagocytic processes require an elaborate set of cell-cell interactions, it is possible that decreased synaptic cleft contacts prevents the necessary cell-contact signaling to engage phagocytic machinery. This seems particularly plausible because unlike P2Y6, which has known roles in promoting phagocytic behavior, little evidence has linked P2Y12 stimulation directly to phagocytosis. It is also not clear if synaptic cleft interactions are targeted to a specific set of synapses, such as those undergoing depression. It is possible that purinergic signaling via P2Y12 simply causes microglial processes to move to the synaptic clefts with a higher unbiased frequency so that further local signaling can take place. However, if this were the case, I would expect a higher frequency of cleft interactions following MD. Despite the significant rise in synaptic cleft interactions following MD, these contacts still occur at a relatively sparse frequency. It seems more likely and efficient for purinergic signaling to facilitate microglial process targeting to specific synaptic populations. Unfortunately, the data presented here does
not allow for the identification of distinct synapse populations, such as contralateral vs.
ipsilateral inputs. Further work will need to be done in order to refine these observations.

The lack of decreased microglial motility in P2Y12\textsuperscript{KO} animals following MD is
surprising based on P2Y12’s known roles in promoting microglial process chemotaxis.
One possible explanation for these observations is that targeted interactions with cellular
components, perhaps with the synaptic cleft contacts described above, arrest microglial
processes for longer periods of time than under basal motility conditions. It is possible
that targeted processes engage increased adhesion with the ECM or cellular
membranes, decreasing basal motility. If the targeting is dependent on P2Y12 function,
then a decrease in synaptic contacts may suggest a deficit in process adhesion and
result in unchanged basal motility. Though much work has been done in culture to
describe downstream effects of P2Y12 signaling in integrin-mediated adhesion, it is
unclear if these same principles apply within the complex compartments of the densely
packed parenchyma. Future experiments will be needed to address these possibilities.

Finally, several lines of evidence from these studies suggest that microglia are
involved in the early phase of ODP. Microglial phagocytosis and internalization of GluA1
receptors suggest actions to decrease synaptic strength, which would correspond to
weakening of the contralateral inputs that occurs in the first phase of ODP. In fact, it has
been shown previously that GluA1 surface expression specifically is known to be
decreased\textsuperscript{102}. This data may indicate that microglia are engaged in active synaptic
pruning. Also, independently analyzing the maximum hemodynamic response for each
eye revealed that animals with P2Y12 disruption do not have a functional decrease in
the maximum contralateral response following 4dMD, whereas saline controls had
decreased contralateral responses comparable to ipsilateral responses. This would suggest that P2Y12 signaling facilitates the contralateral decrease in the first phase of ODP. The fact that P2Y12\textsuperscript{KO} animals at 7dMD still do not have a decrease in contralateral responsiveness, nor an increase in ipsilateral responsiveness, may indicate that inhibiting the first phase of ODP with deficient P2Y12 signaling prevents the second phase of ODP to proceed. However, microglial interactions with synaptic clefts in P2Y12\textsuperscript{WT} animals remain high for up to 7 days of MD, which leaves open the possibility that microglia contribute to the strengthening of non-deprived eye synapses. Unfortunately, I did not analyze synaptic cleft contacts in P2Y12\textsuperscript{KO} animals at 7dMD, so it is unknown whether P2Y12 disruption only affects behaviors observed in the first phase of ODP.

Overall, the data presented here suggest that not only are microglia critical components of experience-dependent synaptic plasticity in adolescent development, but that purinergic signaling through P2Y12 is necessary for this roles.
CHAPTER IV

P2Y12, Noradrenergic Signaling, and Sleep/Wake States

Introduction

The data presented in Chapter III demonstrated a novel, critical role for microglia in ocular dominance plasticity that depended on P2Y12 function. This result prompted a series of new questions regarding the source of purines that activate P2Y12 signaling during plasticity, the significance of microglial process targeting in synaptic remodeling, and the permissive physiological states that would allow microglia to participate in plastic processes. To answer these questions I searched for candidate mediators and physiological factors that would affect P2Y12 function and microglial process dynamics during synaptic plasticity.

Purinergic signaling is highly regulated under baseline physiological states. As discussed in Chapter I, high extracellular concentrations of ATP trigger pronounced inflammatory responses as they indicate compromised cell-membranes and cell death. To prevent ATP-induced immune reactions in the absence of pathology, extracellular ectonucleotidases hydrolyze purines at a very rapid rate, maintaining low extracellular ATP concentrations. While there is heterogeneity in the regulation of extracellular purinergic signaling across brain regions due to differential expression of many classes of endonucleotidases, ATP concentrations in the cortex are typically low. However, studies describing extracellular ATP concentrations across brain regions under different physiological states indicate that extracellular concentrations in the cortex are highest during periods of slow-wave sleep, suggesting that purinergic signaling may be
particularly active during this period\textsuperscript{106}. Coincidentally, structural remodeling of the cortex has been shown to occur at increased rates during slow-wave sleep and disrupting slow-wave sleep following MD has been shown to disrupt ocular dominance shifts\textsuperscript{107,108}.

This evidence suggests that there may be a connection between higher extracellular levels of ATP in slow-wave sleep and the fact that slow-wave sleep is necessary for structural synaptic remodeling and ODP. It seemed possible that P2Y12 function in microglia was influenced by sleep/wake states. Intuitively, it also seemed reasonable that microglial interactions with synaptic cleft compartments would be relegated to physiological states when neurons are not actively processing behaviorally-relevant sensory information. Also, in our previous deprivation studies, the shortest time point analyzed (12hMD) would have provided enough time for mice to engage in multiple slow-wave sleep periods suggesting that even the earliest microglial behaviors may depend on periods of slow-wave sleep. These concurrences prompted me to explore microglial dynamics between sleep and wakeful states.

However, there is a surprising paucity of previous research on changes in microglial dynamics between sleep and wake states. This is in part due to the fact that the majority of research done on microglial dynamics is performed in culture or slice preparations where natural sleep/wake transitions are understandably absent. Interestingly, most research investigating microglial process dynamics in vivo including the original papers that discovered basal microglial motility were done in anesthetized animals that display cortical neuronal activity reminiscent of slow-wave sleep. Based on these initial characterizations, most studies are conducted on the premise that surveillant microglial motility occurs continuously across physiological states, but to my knowledge,
characterization of basal microglial dynamics in wakeful states has not been published and only a few researchers study microglial behavior in awake-behaving animals. In order to determine if slow-wave sleep is necessary for permitting P2Y12 signaling in microglia-mediated synaptic remodeling, I first needed to determine if slow-wave sleep affects microglial dynamics under normal conditions. At the end of this chapter, I will summarize the experiments I conducted to explore these hypotheses.

If microglial dynamics are altered by physiological sleep/wake states, then what factors mediate these changes? Several different studies provided insight into this question. First, a landmark study demonstrated that there is an increase in extracellular space during periods of slow-wave sleep or the sleep-like states of anesthesia. Given the fact that the brain parenchyma is a highly dense network of convoluted cellular structures, an increase in extracellular space by itself might support increased microglial motility by reducing the mechanical resistance impeding microglial process extension. However, in the same paper, noradrenergic signaling was implicated in mediating the decreases in extracellular space observed during wakefulness. Studies have shown that wakefulness is induced and maintained in part by diffuse release of norepinephrine from locus coeruleus efferents across the brain, including the cortex. Although norepinephrine acts on many different cell types and has been implicated in a broad range of behavioral effects such as attention, a corollary function seems to be decreasing extracellular space during wakefulness. In order to explore the effects of noradrenergic-mediated decreases in extracellular space on microglial dynamics during wakefulness, I considered what direct effects norepinephrine might have on microglial physiology.
Although research on noradrenergic signaling in microglia has been performed in a diverse variety of experimental preparations and pathophysiological models, the emerging view is that norepinephrine exerts predominantly anti-inflammatory effects on immune cells, including microglia. For example, microglial stimulation with norepinephrine can decrease their production of cytokines in response to pathological stimuli\textsuperscript{110}. Unfortunately, little research had been done investigating the effects of norepinephrine on microglial motility under physiological conditions. However, one particularly compelling line of research not only demonstrated that microglia are particularly enriched with β2-adrenergic receptors under physiological conditions, but that microglial stimulation with norepinephrine is sufficient to inhibit P2Y12-mediated process extension to sources of ATP in 3D culture gels\textsuperscript{111}. Moreover, the expression of β2-adrenergic receptors in microglia was markedly decreased in response to the proinflammatory molecule, lipopolysaccharide (LPS), suggesting that the function of β2 adrenergic signaling in microglia was intimately related to physiological conditions. Transcriptome information from the database in another study confirmed that β2-adrenergic receptors are nearly exclusively expressed by microglia in the non-pathological brain. If norepinephrine release not only decreased extracellular space but also actively inhibited P2Y12-mediated microglial process extension via β2-stimulation, then it might restrict purinergic signaling in microglia to periods of slow-wave sleep critical for ODP. I therefore set out to determine if β2-stimulation \textit{in vivo} decreased basal microglial motility and whether chronic β2-stimulation during MD was sufficient to prevent the P2Y12-dependent role of microglia in ODP.

\textbf{Methods}
Animals

Experimental protocols were carried out in strict accordance with the University of Rochester Committee on Animal Resources (UCAR) and conformed to the National Institutes of Health Guidelines. Experiments were conducted on mice with a C57/Bl6 background during the visual critical period (P25-35) for ODP experiments or on CX3CR1GFP/+ during early adulthood (~P60) for the two-photon terbutaline, sleep/wake, circadian, and stress motility experiments. Whenever possible, sex distribution was balanced across animal cohorts.

Monocular Deprivation

Animals were separated into non-deprivation (ND) or monocular deprivation (MD) cohorts on P28±1. MD animals were anesthetized (isoflurane, 5% induction, 3% maintenance) and right eyelids resected and sutured together. Eyes were not reopened except for intrinsic signal imaging experiments. All analysis was carried out contralateral to the deprived eye (i.e. left hemisphere) unless stated otherwise.

Two-photon Microscopy

A custom two-photon laser-scanning microscope was used for in vivo imaging (Ti:Sapphire, Mai-Tai, Spectraphysics; modified Fluoview confocal scan head, 20X lens, 0.95 N.A., Olympus). Excitation was achieved using 100 fs laser pulses (80MHz) at 920 nm. For all motility experiments a 580/180 (GFP) filter was used. Mice were anesthetized with a mixture of fentanyl (0.05mg/kg, i.p.), midazolam (5.0mg/kg, i.p.) and dexmetatomadine (0.5mg/kg, i.p.). During surgery and imaging, body temperature was maintained at 37°C. Imaging was carried out using 4x digital zoom and 1 μm z-step. Image analysis was carried out offline in ImageJ or in Matlab using custom algorithms.

Terbutaline Motility Imaging
For terbutaline motility experiments, following a craniotomy with the dura intact, microglia were imaged with artificial cerebral spinal fluid (ACSF) only as the immersion media over the craniotomy. 30 μm z-stacks were collected every 2 minutes for 30 minutes prior to drug application and 60 minutes following drug application. Following the 30 minute baseline, ACSF-only immersion media was replaced with either ACSF-saline or ACSF-terbutaline (1mM) and microglia imaged for the remaining 60 minutes. 15μm z-projections were created and microglia aligned and overlaid every 2 minutes as discussed in Chapter II. Motility index was calculated without binarizing images to increase the sensitivity of changes in fluorescent brightness during process movement (as shown in Figure 13D). Grayscale images were overlaid to create ratios of greens, yellows, and reds. A 0.5 ratio was chosen for the pixel brightness threshold (pixel intensities ±50% were counted as extension or retraction). Motility indices calculated for each time lapse were normalized to the first time lapse of the imaging session to quantify relative changes in motility following saline or terbutaline administration. Overlays were also generated over 30 minutes by using t=0 and t=30 minutes as overlaid time points. Extending pseudopodia were qualitatively identified as green bulbous terminal endings, indicating formation of pseudopodia whereas red indicated retraction.

**Intrinsic Optical Signal Imaging**

On day 0, C57/Bl6 animals were monocularly deprived (MD) or left non-deprived (ND) and injected with saline (equiv. vol., i.p.), nadolol (10 mg/kg, i.p. Sigma) or clenbuterol/nadolol (5 mg/kg clenbuterol, 10 mg/kg nadolol, i.p.) twice a day (at zeitgeber time 6 and 18) for 4 days beginning the day of deprivation. On day 4 of ND or MD, animals were re-anesthetized with isoflurane and chloroproxithene (2 mg/kg), and
sutured eyes reopened. Intrinsic optical signal imaging was then performed as described previously (see Chapter III Methods).

**Anesthetized/Awake Motility Imaging**

For the anesthetized/awake experiments, following surgery, mice were either maintained on anesthesia or given a reversal agent to stimulate wakefulness. Imaging did not begin on awake animals until a response was observed by tactile stimulation of the hind limb and/or voluntary movement was obvious. 40μm deep z-stacks were collected every 4 minutes, 12 times, for a total of 48 minutes. Motility index, stability index, instability index, and the stability histogram were then computed in the same way detailed previously (see Chapter II Methods).

**Electrophysiology**

In a subset of animals, local field potential records were collected before and after anesthesia reversal by implanting tungsten electrodes (12Ω, MΩ, #577200, A&M Systems) in layer V of the primary motor cortex. Electrodes were mounted in headstages (CV-7b, Axon Instruments) and a reference connected to the skull surface. Cortical activity was amplified, digitized (MultiClamp700B, Digidata1322A, Axon Instruments) and recorded (pClamp9, Axon Instruments). Electrophysiological recordings were collected continuously over the course of 2 hours, 1 hour before anesthesia reversal agent, and 1 hour after. Recordings were analyzed offline (ClampFit, Axon Instruments). Local field potential recordings were processed by applying a low-frequency band-pass filter (low pass 100Hz, high pass 0.1Hz). A power analysis was performed and frequency bands were normalized to the maximum power and combined into 0.25Hz spectral bins.

**Circadian Motility**
For circadian rhythm experiments, animals were maintained on a normal 12:12 light/dark cycle with lights on at 6:00AM (ZT0) and off at 6PM (ZT12) and imaged at ZT6 and ZT18. Time lapse z-stacks of 40 μm were collected every 5 minutes, 12x, for a total of 60 minutes. Images were prepared for analysis and a motility index, stability index, instability index, and stability histogram were calculated as described before.

**Stress Motility**

For stress experiments, animals were placed in a restraint tube and placed in bright light conditions for 2 hours each day at zeitgeber 0 (ZT0) 3 days prior to imaging. Control animals were left undisturbed in their home cages. Stressed immediate cohorts were imaged immediately after tube restraint on the 3rd day. Stressed delayed cohorts were imaged 4 hours after the final tube restraint on the 3rd day.

**Code Availability**

Matlab code for motility analysis is available upon request from the author.

**Statistics**

Statistical comparisons were made between animal cohorts using Prism VI statistical analysis software (GraphPad, La Jolla, CA). All n-values represent individual animals, and are comparable to standard n values used in similar experiments in the literature. All values reported are the mean ± standard error of the mean (SEM). For all analyses, α=0.05. Two-tailed unpaired Student’s t-tests, and one-way or two-way ANOVAs with Holm-Sidak post-hoc comparisons were used to compare cohorts where appropriate.

**Results**

**Noradrenergic effects on microglial motility**
Although previous work had demonstrated a noradrenergic-dependent inhibition of microglial process extension in response to ATP, the experiments were carried out using isolated microglia in 3D gel cultures. In order to determine if β2-stimulation was sufficient to suppress microglial dynamics in a more physiological context, I tested whether β2-agonists affected basal microglial motility in vivo using two-photon microscopy.

In preliminary studies, I dosed CX3CR1^{GFP/+} animals with terbutaline, a β2-specific agonist that crosses the BBB, using a peripheral injection (10 mg/kg, i.p.). Though this allowed me to use a thin-skull preparation, less-intrusive than a craniotomy for microglial imaging, I found that stimulation of peripheral β2-receptors had sympathomimetic effects including increased respiration and heart rate. The initial qualitative results indicated that peripheral administration of terbutaline caused a retraction of microglial processes in the cortex. However, motion artifact from the increased respiration rate made motility index quantification difficult.

In order to avoid the confounds of peripheral sympathomimetic feedback on the CNS and increased motion artifact from increased respiration, I used a cranial window preparation and bath application of terbutaline (1mM in ACSF). The use of a cranial window introduced a new confound—microglial activation in response to removing the skull. Therefore, I attempted to minimize the influence of these confounds by applying terbutaline in the immersion media above the dura rather than through a pipette inserted into the cortex, by imaging microglia deeper in the cortex (~50-75 μm from the pial surface), and by collecting baseline motility prior to drug-application to monitor signs of microglial activation. In addition, I normalized motility to the start of baseline imaging so
that changes in microglial motility were relative to before and after terbutaline administration.

Following one hour after application of terbutaline (1mM, n=4) microglial motility was decreased by 25.3±3.9% relative to baseline, whereas saline application (n=3) had no effect (2±2.4% increase). Motility index in terbutaline animals was significantly decreased from saline controls 10 minutes following drug application and remained significantly decreased for the remainder of the imaging (Figure 29A, B). Importantly, there was no difference in baseline microglial motility prior to drug application. Qualitative analysis of terbutaline application revealed a striking retraction of secondary and tertiary microglial branching and a decrease in bulbous terminal endings on microglial processes, suggesting that β2-stimulation inhibits the formation and stability of microglial pseudopodia (Figure 29A). This effect was paralleled by an apparent increase in thin filopodia formation along primary processes, indicating that signaling underlying the initiation of microglial motility remained intact. These results demonstrated that microglial stimulation with terbutaline is sufficient to decrease baseline surveillant motility.
Noradrenergic effects on ocular dominance plasticity

Based on results that β2-agonists can impede microglial dynamics in vivo, I explored the effects of chronic β2-stimulation on ODP. If critical microglial roles in ODP are dependent on P2Y12-mediated process dynamics, then inhibition of P2Y12-
dependent motility by chronic β2-stimulation following MD may disrupt ODP. I tested this hypothesis by chronically administering β2-agonists throughout 4 days of MD (4dMD) and using iOS to functionally assess ocular dominance shifts. Terbutaline has a relatively short half-life (~3 hours). In order to minimize the number of injections needed to maintain chronic stimulation of β2 receptors over 4 days, I used another β2-agonist, clenbuterol. Clenbuterol is a BBB-permeable β2 selective agonist with a long half-life (~25 hours). To avoid the sympathomimetic effects of peripheral β2 stimulation, I co-administered nadolol with clenbuterol. Nadolol is a non-selective β receptor antagonist that is BBB-impermeable. Using this paradigm, ODP was assessed in animals that were dosed with saline, clenbuterol/nadolol, and nadolol only twice a day across 4 days following MD.
ND controls in each animal cohort had normal ODI values following 4 days of drug administration, indicating that chronic peripheral β2 blockade or central β2 stimulation does not affect the normal baseline contralateral bias (Figure 30A, B). Following 4dMD, saline dosed animals demonstrated a significant ocular dominance shift with contralateral responses similar to ipsilateral responses, validating the ODP...
model. Nadolol only dosed animals also demonstrated a significant ocular dominance shift following 4dMD, suggesting that peripheral β2-blockade does not impede ODP. However, following 4dMD, clenbuterol/nadolol dosed animals failed to exhibit an ocular dominance shift, indicating that clenbuterol prevents ODP. These results suggest that stimulation of β2 receptors in the CNS is sufficient to impair cortical synaptic plasticity.

The effect of wakefulness on microglial motility

The previous results demonstrating that β2-stimulation is sufficient to decrease microglial motility and disrupt ODP raises the interesting possibility that norepinephrine release in the cortex during wakeful states may suppress microglial dynamics and prevent microglia from carrying out their functions that aid in synaptic remodeling. However, it is possible that these data represent noradrenergic stimulation that is not equivalent to cortical concentrations of norepinephrine during periods of wakefulness and that the deficits in β2-stimulated microglia do not reflect differences in microglial dynamics between sleep/wake states. In order to determine if microglial dynamics change under more physiological conditions, I began to characterize microglial motility in different states and compare anesthetized, slow-wave sleep-like states, and wakeful conditions.

As mentioned previously, the majority of studies investigating microglial motility in vivo have been done in anesthetized animals that exhibit cortical network activity similar to slow-wave sleep states. In order to determine whether microglia in awake animals display altered process motility, mice were trained to be restrained during awake two-photon imaging. Microglial motility was compared between two cohorts of animals. The anesthetized cohort was maintained under anesthesia following thin-skull preparation for
the duration of imaging. The awake cohort of animals was given a reversal agent to
induce wakefulness following thin-skull preparation and was imaged following response
to tactile stimulation and voluntary movement. Microglia in awake animals exhibited
remarkably attenuated baseline motility in comparison to animals maintained under
anesthesia (Figure 31A, B). This was quantitatively validated by a significant decrease in
motility index, instability index, and shift in stability histogram. The stability index was not
significantly different, indicating that when processes did move, the same proportion
stayed stable in the subsequent time lapse. Awake animals displayed an approximately
50% reduction in motility index relative to anesthetized animals, indicating that wakeful
states dramatically suppress microglial process surveillance (Figure 31B). In the stability
histogram, awake animals demonstrated a significantly decreased proportion of low
stability pixels and a corresponding significant increase in high stability pixels (Figure
31E). This suggests that in awake animals, when microglial processes move, they
remain stable for longer periods of time relative to anesthetized animals that are more
likely to retract the process. Taken together, these results suggest that periods of
wakefulness may strongly attenuate basal microglial dynamics, limiting their capacity to
survey the parenchyma and interact with other cellular entities.

To make sure that differences in microglial motility in awake states was not due
to a difference in image quality or photobleaching, the total number of pixels from both
cohorts were normalized to the start of imaging and tracked across the entire imaging
session. Although unavoidable photobleaching was observed (~20% loss in pixels), no
relative difference in photobleaching was observed between awake and anesthetized
cohorts (data not shown).
In order to determine that anesthetized and awake animals had cortical network activity characteristic of slow-wave sleep and wakeful states, I implanted local field potential (LFP) recording electrodes in a subset of animals and recorded LFPs before and after an anesthesia reversal agent was administered (Figure 31F, G). I found that anesthetized animals displayed slow-wave oscillations in network activity with dominant power in the delta wave frequencies (0.1-4 Hz) thereby confirming previous observations that anesthesia induces a slow-wave sleep-like state. Following reversal agent administration, animals demonstrated a significant decrease in delta-wave power with a concomitant increase in theta, alpha, and beta wave frequencies corresponding to more asynchronous network activity that is characteristic of wakeful states. These results suggested that based on LFP recordings, cortical activity in anesthetized animals is reminiscent of slow-wave sleep and that anesthesia reversal is able to induce cortical activity similar to wakeful states.
However, anesthetized states are known to be significantly different from natural slow-wave sleep. In order to determine if microglial motility and LFP recordings during anesthesia are similar to natural sleep states, I collected data from awake animals that
fell asleep naturally during imaging. LFP traces indicated that natural sleep displayed similar characteristics of anesthetized animals. Interestingly, microglial motility appeared to be increased in sleep states relative to animals imaged during awake states, but not as pronounced as the increased motility in animals under anesthesia. This suggests that although anesthesia and natural sleep both facilitate increased microglial motility relative to wakeful states, there may be differences in magnitude with anesthesia causing a more pronounced effect. Unfortunately, these experiments suffer from a low sample size due to the difficulty of animals falling asleep naturally under the scope and future work will need to be done to make more definitive conclusions.

**Figure 32: Circadian rhythm does not affect microglial motility.** Animal cohorts were imaged at either 12PM or 12AM to determine if circadian cycles influence basal microglial motility rates. **A.** Example RGB overlays of microglial from each cohort. **B-D.** Motility, stability, and instability indices were found to not be significantly different between animals imaged at 12PM and 12AM, though there was a trend for increased motility at 12AM relative to 12PM (n=5-7 per group, Students t-test, p>0.05). **E.** Likewise, no significant shift in the distribution of process stability was found in the stability histogram between animals imaged at 12PM and 12AM (n=5-7 per group, two-way ANOVA, p>0.05).
Circadian rhythm plays an important role in governing the frequency and duration of sleep states across animal species and has been suggested to influence microglial morphology. In order to verify that differences in microglial motility under anesthetized and awake conditions were not due to changes in circadian rhythm, microglial motility in anesthetized animals was analyzed at 12PM and 12AM. No significant differences were found in motility, stability, or instability indices between these times, nor was there a shift in the stability histogram suggesting that circadian effects do not influence microglial motility in anesthetized animals (Figure 32).

Body restraint represents a significant source of stress for mice. Although animals were trained for three days prior to imaging to habituate them to restraint during imaging, it is possible that restraint stress suppresses microglial motility and accounts for the differences observed in microglial motility between anesthetized and awake states. To rule out this possibility, animals were restrained for 2 hours a day, for 3 days leading up to imaging. All animals were imaged under anesthesia and non-stressed animals were compared to animals imaged immediately after the last restraint period or 4 hours later. There was no significant difference in microglial motility between non-stressed animals, stressed animals imaged immediately following the stressor, and stressed animals imaged after a 4 hour delay (Figure 33 A, B). These results demonstrate that microglial motility under anesthesia is not affected by prior stress.
Conclusion – summary of noradrenergic effects, sleep/wake effects

The goal of the experiments detailed here in Chapter IV was to understand how sleep/wake states and noradrenergic signaling influence P2Y12-mediated microglial roles in ODP. I demonstrated that acute β2-stimulation is sufficient to inhibit baseline surveillant microglial motility in vivo. Additionally, chronic β2-stimulation is sufficient to inhibit ODP following 4 days of MD, which raises the interesting possibility that these two results are linked via β2 suppression of P2Y12-dependent roles in ODP. I demonstrated that microglia have decreased basal motility in awake mice compared to anesthetized mice, and that LFP recordings under anesthesia display similar properties to periods of...
slow-wave sleep. Furthermore, I established that differences in motility between sleep-like and wakeful states are not artifacts from circadian rhythm changes or restraint-induced stress. These results provide further incentive to investigate if microglia predominantly exert their effects in the non-pathological brain during periods of slow-wave sleep, and whether these effects are curtailed by physiological release of norepinephrine during periods of wakefulness.

Though terbutaline decreased microglial motility, this effect seemed to be a result of decreased microglial pseudopodia formation. In fact, following terbutaline administration, there appeared to be a significant increase in small filopodia formed along primary branches. These results suggest that pro-chemokinetic signaling in microglia, whether initiated by extracellular or intracellular sources, is still intact and microglia are attempting to extend processes. As it has been shown in a variety of chemotactic events, cell-adhesion to the extracellular matrix or the surfaces of other cells is critical for efficient chemotaxis. It is possible that β2-signaling prevents microglial process extension by simply destabilizing extracellular pseudopodial adhesion. This possibility may explain the impaired ODP observed with clenbuterol following 4dMD. As discussed previously in Chapter III, microglial processes hyper-ramify following MD with an increase in synaptic cleft contacts and decreased motility. Though not directly proven, these behaviors suggest that microglia may use functional pseudopodia to produce targeted chemotaxis towards synaptic clefts and then remain in those compartments for extended periods of time via pseudopodial adhesion. If this were the case, then even with intact P2Y12-signaling, microglial processes would be unable to reach synaptic compartments and might fail to facilitate weakening of the contralateral inputs to binocular visual cortex. Although these speculations are possible, future experiments will
be needed to determine how β2-stimulation specifically affects pseudopodia stabilization and whether this stabilization is required for functional microglial roles in ODP.

Though chronic stimulation with clenbuterol was able to prevent ocular dominance shifts following 4d of MD, it is possible that this effect is due to β2-stimulation on non-microglial cells. Although transcriptome analysis reveals that β2 is heavily enriched on microglia relative to any other cells in the CNS, many different cells in the periphery express β2 receptors. In order to minimize this potential effect, nadolol was co-administered with clenbuterol to prevent confounding peripheral contributions such as hemodynamic responses to β-receptor stimulation on blood vessels. Although the efficacy of nadolol antagonism was confirmed by the suppression of sympathomimetic behaviors, such as increased respiration, induced by clenbuterol alone, it is possible that the effects of clenbuterol in ODP are still due to peripheral β2-stimulation. These possibilities have not been ruled out in the work presented here. In order to more convincingly demonstrate that ODP impairment by clenbuterol is due to its action specifically on microglia, a CX3CR1^CreERT^/+ mouse line crossed with a β2^floxed^ mouse line could be used to allow for the selective removal of β2 receptors from the microglial lineages. If the effect of ODP impairment by clenbuterol administration is lost in these animals, this would suggest that microglial β2 expression is necessary for the impairment of ODP. This proposed experiment and future work will be needed to conclusively link the motility deficits in microglia with the disrupted ODP.

Though the characterization of microglial motility in sleep/wake states presented here provides preliminary evidence for decreased microglial dynamics during wakeful states, additional experiments will be needed to firmly establish this phenomenon.
Although anesthesia emulates many properties of slow-wave sleep such as a lack of consciousness and synchronous, oscillatory cortical activity, it is not physiologically identical. For example, mice sleep for short periods of time (~5 minutes) with increased frequency of these periods occurring early in the light cycle of their housing conditions. Therefore, microglial changes in motility over the time course of an hour under anesthesia do not accurately reflect physiological slow-wave sleep. Conversely, the effects of anesthesia do not dissipate immediately following administration of a reversal agent. Though mice respond to tactile stimulation, they may not be “fully awake” - that is attentive and behaving normally. It is possible that the frequency of slow-wave sleep episodes is more important for determining microglial motility than the duration of any one episode. In future experiments, mice will need to be imaged while awake and behaving and compared to periods of increased slow-wave sleep frequency. While preliminary, the results presented here indicate that changes in microglial physiology between sleep and wakeful states merits further exploration.
CHAPTER V

Discussion

Overall, the results presented in this thesis have demonstrated a novel role for microglia in ocular dominance plasticity and uncovered a mechanistic component, P2Y12, that is critical in this role. The results in Chapter II laid the foundation for describing the behavior during ODP investigated during Chapter III by suggesting that P2Y12 is not necessary for maintaining the bulk of baseline microglial behaviors including ramified morphology, surveillant motility, and basal cellular contact frequency. However, from the results in Chapter III, it is clear that microglial responses to MD are dependent on intact P2Y12 function as disrupting P2Y12 signaling attenuated microglia process hyper-ramification and abolished the decreased motility, increased synaptic cleft contacts, increased inclusion frequency, and increased GluA1 internalization that normally occur in microglia following MD. Ultimately through these alterations in behavior, or some yet unknown consequence of P2Y12 disruption, ocular dominance shifts are inhibited and functional ODP fails to occur. The results in Chapter IV provide evidence for new avenues of exploration investigating microglial roles in the non-pathological brain, including the role of noradrenergic signaling and physiological changes in sleep and wakeful states. Here, I will focus on briefly discussing some of the outstanding questions regarding microglia in the non-pathological brain and speculate how the results demonstrated here fit in with other research lines.

Microglia in the developing brain have significantly different morphologies and expression profiles from microglia in the mature brain. Although work has demonstrated
that microglia actively prune projections early in development\textsuperscript{54}, the question remains: how do mature microglia with ramified morphologies participate in neurophysiological brain function? Recent studies have shown that without mature microglial release of trophic factors, learning-dependent spine formation and subsequent behavioral improvement are significantly decreased\textsuperscript{112} suggesting that microglia play critical roles well past development and into adulthood. However, other studies have shown that cognition and motor performance are not affected by pharmacological elimination of mature microglia\textsuperscript{22}. Though it seems unlikely that removing an entire population of cells in the brain has no effect on behavior, it raises the question of what are microglia really required for under normal neurophysiological conditions in adulthood? In early development it makes sense that efficient removal of aberrant connections by microglia would be evolutionarily advantageous, but how would this translate to microglial roles in the adult brain? Here we show that altering signaling through a single receptor in ramified microglia, either genetically or pharmacologically can severely inhibit neuronal plasticity.

These results can be viewed in two different ways. On the one hand, the behaviors we observe in microglia and the critical roles they play in ODP may be evidence that on a smaller scale than monocular deprivation, microglia are constantly surveying synapses and either releasing growth factors, breaking down extracellular matrix to allow for synapse growth, or pruning synapses that are no longer functioning properly. These behaviors may occur so sporadically that they would be hard to observe unless a largescale event like monocular deprivation created a particularly pronounced set of behaviors like we have demonstrated here.
However, it is possible that microglia respond to monocular deprivation because it represents a large-scale homeostatic challenge and the bulk of synaptic rearrangement, learning, and memory that occur on smaller, incremental scales do not require microglial involvement. The fact that P2Y12 is not necessary for basal microglial motility, but is absolutely critical for microglial laser ablation response, suggests that its purpose may be to detect sudden perturbations in homeostasis and produce a rapid response. If this is the case, then we may find that P2Y12 is not necessary for the fine-tuning of the nervous system in adulthood, but mediates microglial responses to large scale remodeling events such as following stroke or seizures. In fact, a recent study has emerged that linked seizure induced neuronal release of ATP through NMDA channels to attraction of microglial processes via P2Y12. Disrupting P2Y12 in this response was deleterious to neurons indicating that contacts with neurons mediated by P2Y12 may be neuroprotective.

It seems likely that given the broad range of functions that purinergic signaling plays within different tissues and cellular compartments that P2Y12 acts as a ‘sentinel receptor’ that mediates further signaling. If this is the case in ODP, then other downstream signaling pathways should be explored. As discussed earlier with the terbutaline experiments, pseudopodia formation may indicate cellular adhesion and cross-membrane communication between neurons and microglia. P2Y12 may be necessary to facilitate the honing of microglial processes to a cellular compartment undergoing homeostatic challenges and then allow for signaling more specific to the homeostatic perturbation, whether that is a seizure, monocular deprivation, or ischemia. Further work will need to be done looking for new cell-adhesion candidates underlying
ODP specific microglial roles or perhaps revisit known factors implicated in non-pathological microglial functions, such as MHC-I and fractalkine.

What is the source of ATP that drives P2Y12-dependent microglial roles in ODP? This question stands as an obvious future research direction, however purinergic signaling is difficult to study. As a substrate for cellular respiration, it is found at high concentrations within every cell essentially ruling out transcriptome approaches that might have worked to probe for mediators with more selective expression. The next obvious question is whether purinergic release mechanisms display heterogeneity within cellular populations in an informative manner? Although astrocytes are able to release purines in culture, and do so readily, it is unclear how purinergic signaling within astrocytes serves non-pathological roles in the adult cortex. Though some studies suggest ATP is a gliotransmitter released to actively modulate synaptic excitability, these results have been highly controversial and it is still not clear if astrocytic actions occur selectively on individual synapses.

A recent study demonstrated that stimulation of NMDA receptors on a single neuron is sufficient to induce microglial process attraction in a P2Y12-dependent manner. This opens the possibility that neurons themselves release ATP via NMDA under physiological conditions. It is known that ODP involves NMDA-receptor mediated long-term depression. Perhaps one downstream effect is ATP release from insufficiently active neurons, coupled to and facilitated by decreased metabolic load and perhaps a surge of ATP. However, the prospect of testing most of these possibilities in vivo ranges from impossible to non-physiological. With increasingly sensitive optical dyes and indicators, observing point-source ATP release from cortical neurons may be the first
step in understanding whether neuronal release of ATP directly drives microglial process dynamics and behavior during ODP.

Previous work has suggested that extracellular ATP concentrations are highest during periods of slow-wave sleep\textsuperscript{106}. As demonstrated in Chapter IV here, slow-wave sleep may be a critical parameter in governing microglial dynamics. Sleep is restorative for a host of reasons, but it would be interesting if another positive benefit is increased synaptic surveillance of microglial processes, perhaps facilitating homeostatic debris removal roles, or the more interesting role of maintaining network homeostasis by modulating synapses during scaling periods. TNF-\(\alpha\), as discussed in Chapter I, is an interesting classic cytokine that has been shown to facilitate synaptic scaling\textsuperscript{50,51}. It also has been shown to be somnogenic\textsuperscript{115-117}, increasing the delta-wave power of slow-wave sleep oscillations in local cortical columns. It also happens to be expressed by glia including microglia. It would be fascinating if microglia were able to proactively push a microdomain of the cortex into longer periods of slow-wave sleep by release of TNF-\(\alpha\) to drive its own subsequent windows of increased motility. Although highly speculative, this kind of process happens during sickness behavior whereby the immune systems promotes sleep during inflammatory episodes to increase its ability to deal with foreign pathogens. So too, microglia might respond to homeostatic challenges, such as monocular deprivation, with proactive release of factors to promote periods of slow-wave sleep. However, these speculations have little substantive evidence supporting them and more research will need to be done characterizing sleep/wake state changes and microglial behavior before we understand how they might drive their own behavior.

One important consideration from the results in Chapter IV is the importance of slow-wave sleep during periods of early development and also pathological progression
in the aging, both scenarios where microglia are known to play important roles. Could slow-wave sleep disruption actively inhibit the ability of microglia to fulfill basic homeostatic functions, such as pruning synapses during young synaptic plasticity or clearing the accumulation of protein aggregations in the aged brain? Microglial motility is thought to facilitate microglial signaling with their external environment and I have done a lot of motility characterization here. However, I think future studies will need to go beyond these analyses and really parse out the functional effects that presumably lie downstream of microglia motility. Then, perhaps sleep/wake states will not just signify an increase in the one-dimensional metric that is motility, but open up a host of functional consequences for sleep loss.

Overall, the work done in this thesis has opened up more questions than it has answered. But, it provides compelling information that suggests microglia should continue to be studied outside of their archetypical immune roles and inside the non-pathological brain.
## Appendix

### Table 1: Post-hoc summary for Sholl analysis of different MD durations compared to ND in ND V1 Contra.

<table>
<thead>
<tr>
<th>MD Duration</th>
<th>Distance from Soma (µm)</th>
<th>18</th>
<th>20</th>
<th>22</th>
<th>24</th>
<th>26</th>
<th>28</th>
<th>30</th>
<th>32</th>
<th>34</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 HR MD</td>
<td></td>
<td>-</td>
<td>*</td>
<td>**</td>
<td>***</td>
<td>****</td>
<td>*****</td>
<td>*****</td>
<td>**</td>
<td>***</td>
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<tr>
<td>1D MD</td>
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<tr>
<td>2D MD</td>
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<td>*</td>
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<tr>
<td>4D MD</td>
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*p = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.

### Table 2: Summary of post-hoc comparisons for Sholl analysis in different brain regions compared to ND V1 Contra.

<table>
<thead>
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<th>Condition</th>
<th>Distance from Soma (µm)</th>
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<th>8</th>
<th>10</th>
<th>12</th>
<th>14</th>
<th>16</th>
<th>18</th>
<th>20</th>
<th>22</th>
<th>24</th>
<th>26</th>
<th>28</th>
<th>30</th>
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<td>12HR V1 Contra</td>
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<td>*</td>
<td>**</td>
<td>***</td>
<td>****</td>
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<td>-</td>
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</tr>
<tr>
<td>ND V1 Ipsi</td>
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<td>**</td>
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<tr>
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*p = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.

### Table 3: Summary of post-hoc comparisons for P2Y12<sup>WT</sup> Sholl analysis of different MD durations compared to ND V1 Contra.

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<th>Distance from Soma (µm)</th>
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<th>14</th>
<th>16</th>
<th>18</th>
<th>20</th>
<th>22</th>
<th>24</th>
<th>26</th>
<th>28</th>
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<tr>
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*p = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.
Table 4: Summary of post-hoc comparisons for P2Y12<sup>KO</sup> Sholl analysis of different MD durations compared to ND V1 Contra. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.

<table>
<thead>
<tr>
<th>MD Duration</th>
<th>Distance from Soma (μm)</th>
</tr>
</thead>
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<td>6</td>
</tr>
<tr>
<td>12 HR MD</td>
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<td>7D MD</td>
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</table>

Table 5: Summary of post-hoc comparisons for Sholl analysis of different MD durations in P2Y12<sup>KO</sup> compared to P2Y12<sup>WT</sup>. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.

<table>
<thead>
<tr>
<th>MD Duration</th>
<th>Distance from Soma (μm)</th>
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<tbody>
<tr>
<td></td>
<td>16</td>
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<tr>
<td>12 HR MD</td>
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<td>7D MD</td>
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</tr>
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</table>

Table 6: List of drugs used and relevant pharmacological properties.

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>Targeted Receptor</th>
<th>Type</th>
<th>Blood-Brain Barrier Permeable</th>
<th>Half-Life (~hours)</th>
<th>Dose Used (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clopidogrel</td>
<td>P2Y12</td>
<td>antagonist</td>
<td>Yes (at high dose)</td>
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<td>50</td>
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<td>Ticagrelol</td>
<td>P2Y12</td>
<td>antagonist</td>
<td>No</td>
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<td>10</td>
</tr>
<tr>
<td>Nadolol</td>
<td>Aδβ1, 2, 3</td>
<td>antagonist</td>
<td>No</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Clenbuterol</td>
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<td>agonist</td>
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<td>5</td>
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<tr>
<td>Terbutaline</td>
<td>Aδβ2</td>
<td>agonist</td>
<td>Yes</td>
<td>10</td>
<td>1mM</td>
</tr>
</tbody>
</table>
Figure 34: Molecular structures of relevant drugs
Bibliography

12. Robertson, W. F. *A Textbook of Pathology in Relation to Mental Diseases.* (William F. Clay, 1900).


