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Regulation of microglial chemotactic responses during neuroinflammation

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By

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An abstract of
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Abstract

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Cell motility drives a variety of biological processes, including inflammation, development, and tumor metastasis. In the brain, microglia are phagocytic immune cells that actively survey brain tissue and scavenge sites of injury using elaborate motile processes. Motility of these processes is guided by local release of chemoattractants. Recent studies have shown that acute brain injury induces microglial process extension toward sites of tissue damage, which is thought to serve neuroprotective functions by allowing microglial scavenging of necrotic debris. This chemoattractive response is guided by the release of nucleotides such as ATP from damaged cells, and involves microglial purinergic receptors. Thus, process motility enables microglial monitoring of healthy tissue and clean up of injured tissue. In contrast, prolonged brain damage is accompanied by proinflammatory, or activated, microglia with highly retracted processes. While microglial process retraction has been documented for over 80 years and serves as a hallmark of brain trauma and neurodegeneration, its causes and consequences remain unknown.

Using novel four-dimensional confocal imaging methods, we discovered that ATP triggers process retraction, slowed process motility, and repulsive migration in activated microglia. Our results indicate that while ATP is a factor that attracts naïve microglia, it instead repels activated microglia. Moreover, we found that repulsion from ATP is mediated by $G\alpha_s$-coupled signaling downstream of an upregulated adenosine A_{2A} receptor. In light of evidence that the G_i-coupled $P2Y_{12}$ receptor that mediates chemoattraction to ATP is downregulated upon microglial activation, we propose that a switch from $P2Y_{12}$ to A_{2A} receptor signaling drives the shift in microglial chemotaxis from attraction to repulsion. Our results further suggest that A_{2A} stimulation inhibits phagocytosis by activated microglia and prevents scavenging during injury. Lastly, we present evidence that the A_{2A} receptor is upregulated during neuroinflammation and induces microglial process retraction *in vivo*. Our findings indicate that ATP and its breakdown products provide an opportunity for context-dependent shifts in receptor signaling that can alter the effect of these chemotactic factors. Thus, our investigations have revealed an unexpected chemotactic switch central to CNS inflammation and suggest that other cell motility-driven biological processes may be similarly regulated.

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TABLE OF CONTENTS

FIGURE INDEX

TABLE INDEX

CHAPTER 1: Introduction

1.1. Inflammation

Inflammation is an organism's main defense mechanism to a variety of threats. It is a complex process that involves numerous cell types and a large assortment of signaling factors. An inflammatory response promotes inactivation and removal of toxic agents and invading microorganisms, such as a virus or bacteria, and it allows recovery from physical or hypoxic trauma. Inflammation can also mount in response to threats generated within the organism itself. For example, accumulation of aggregated or abnormally modified proteins, aberrant signals emanating from damaged cells, or imbalances in immune signaling, can each generate inflammatory responses (Wyss-Coray and Mucke, 2002).

While inflammation may not prevent severe impairment, the ultimate outcome of an inflammatory response is most often the successful elimination of a noxious stimulus and recovery from injury. Interestingly, another possible outcome of inflammation involves a chronic non-resolving immune response that generates secondary tissue injury (Wyss-Coray and Mucke, 2002). This outcome is thought to be a common thread linking a variety of neurodegenerative illnesses of the central nervous system (CNS), including Alzheimer's and Parkinson's diseases.

In the brain, inflammatory responses are driven by a multi-component system which includes microglia, the resident macrophage-like cells, and immune cells infiltrating from the periphery, such as T cells. Non-immune CNS cells, including astrocytes and neurons, can also contribute to and modulate immune responses in the CNS. However, while these cells can influence neuroinflammation, this process is initiated and executed primarily by microglia, the only resident hematopoietic cells in the CNS and the first responders to all types of brain insults. Indeed, most structural or functional disturbances in brain homeostasis

trigger a response by microglia, which have been termed the “pathology sensors” of the CNS (Kettenmann, 2006; Hanisch and Kettenmann, 2007).

The ability of the immune system, and indeed of microglia, to sense the presence of aberrant conditions within a complex milieu is critical for initiating a proper inflammatory response. In order to mount an immune response that is appropriate and targeted to a specific pathological insult, immune cells possess the ability to distinguish various foreign or toxic substrates from self constituents, or abnormal conditions from the normal homeostatic state. This capability is afforded by a large array of surface receptors, many of which are expressed exclusively by macrophage-like cells such as microglia. Upon agonist binding, these receptors initiate complex intracellular signaling cascades that influence a variety of cell functions, including the release of soluble factors that signal to other cells, uptake of extracellular matter, cellular proliferation, migration, and changes in gene expression. An inability or error in the recognition of extracellular substrates or improper downstream signaling can lead to an autoimmune response or otherwise inappropriate inflammatory reactivity that can result in damage to healthy tissue and irresolution of an insult.

Both chronic and acute injury to the CNS triggers microglial transformation from a resting (or, naïve/surveying) state to an activated (or, proinflammatory/reactive) state. Interestingly, recent advances in positron emission tomography imaging shows correlations between neurodegenerative disease severity and microglial proinflammatory status (Ouchi et al., 2005; Pavese et al., 2006). Because these cells are the main determinants of neuroinflammation and have a profound influence over CNS injury and disease processes, identification of novel regulators of microglia is relevant for various neuropathological conditions and may offer promising therapeutic targets. Thus, in order to understand and control neurological diseases, we must first understand the mechanisms regulating resting and activated microglia.

1.2. Resting microglia

a. Origin

Microglial cells were first described by Del Rio Hortega in 1919. Unlike neurons, astrocytes and oligodendrocytes, which have a neuroepithelial origin, microglia are derived from the myeloid lineage of bone marrow cells. Originating from circulating monocytes that infiltrate the CNS during development, microglia represent approximately 12% of all cells in an adult brain. The colonization of the CNS by monocytic microglial precursors occurs in two phases, the first taking place during fetal development and the second wave during the early postnatal period (Chan et al., 2007). While microglia are ubiquitous in the CNS, certain brain regions display higher densities of microglia, including the substantia nigra and the hippocampus (Lawson et al., 1990). In adulthood, microglial cell number may be replenished by infiltration of circulating monocytes, but the extent of this recruitment is thought to be minimal under normal conditions due to presence of the blood-brain barrier (Simard et al., 2006; Ajami et al., 2007; Mildner et al., 2007). In pathological contexts, microglia may expand in number by undergoing rapid proliferation. The extent of microglial renewal by invading monocytes during disease and breakdown of the blood-brain barrier remains controversial (Flugel et al., 2001; Davoust et al., 2008).

b. Characterization

In a healthy adult, microglia assume a resting state characterized most readily by the presence of numerous elaborately branched processes. As recently shown *in vivo*, these processes are highly dynamic and undergo continuous cycles of rapid protrusion and retraction at an average rate of 1.5 μm per minute (Nimmerjahn et al., 2005). This branched and motile phenotype of resting microglia is thought to reflect constitutive microglial surveillance and maintenance of the surrounding microenvironment (Nimmerjahn et al.,

2005; Davalos et al., 2006). Indeed, Nimmerjahn et al. (2005) observed that microglial processes seem to actively sample brain parenchyma by forming bulbous endings upon process extension. Interestingly, local application of a γ-aminobutyric acid (GABA) receptor antagonist enhances microglial process motility, suggesting that synaptic activity may regulate the rate of local microglial surveillance (Nimmerjahn et al., 2005). Based on this evidence, resting microglia are thought to serve a protective role by actively surveying the CNS and maintaining brain tissue integrity. However, the extracellular signaling factors and mechanisms promoting this resting phenotype remain poorly understood.

A candidate factor that may keep microglia in a resting state is tumor growth factor-β (TGF-β), which is present at high levels in the CNS under normal conditions and is thought to exert anti-inflammatory effects on microglia (Kim et al., 2004; Le et al., 2004; Hinkerohe et al., 2005; Boche et al., 2006; Qian et al., 2008). Indeed, TGF-β1 knockout animals exhibit microglial process retraction, a characteristic feature of activated microglia (Makwana et al., 2007). However, it is not known whether the associated demyelination and astrocytic reactivity observed in TGF-β1 knockout animals triggered the changes in microglial structure. There may also be constitutive microglial stimulation via the chemokine (fractalkine) receptor CX3CR1 that quells microglial activation (Cardona et al., 2006). Moreover, the membrane molecule CD200 may help to maintain microglia in a resting phenotype and suppress inflammation (Hoek et al., 2000). Lastly, neurotransmitter levels may inform microglia of normal CNS activity and thereby downregulate microglial inflammatory reactivity. Indeed, a variety of neurotransmitter receptors are expressed by microglia (Pocock and Kettenmann, 2006).

1.3. Activated microglia

a. Characterization

Microglial activation, or differentiation from a resting into a proinflammatory phenotype, is a set of responses that takes place in microglia following a disturbance in CNS homeostasis. Traditionally, microglial activation has been viewed as an all-or-none effect with little distinction between diverse activating conditions. However, this simplified view of microglial activation has been re-evaluated recently (van Rossum and Hanisch, 2004). Recognizing that responses by activated microglia are often context-specific and show variability depending on concomitant regulatory inputs, microglia are now viewed as versatile immune effectors in the CNS (Streit, 2002; Biber et al., 2007). Indeed, microglia can exhibit dual roles as defenders and attackers. The reader is referred to excellent reviews by Hanisch and Kettenmann (2007) and Schwartz et al. (2006).

Microglial responses upon activation often include cell proliferation and upregulation of inflammatory, and often neurotoxic, secretory factors. Activation also causes microglia to upregulate immunomodulatory surface molecules, including major histocompatibility complex II, certain cytokine and chemokine receptors, and adhesion proteins (Hamill et al., 2005). The neurotoxic effects of proinflammatory microglia have been attributed largely to the release of reactive oxygen species and proinflammatory cytokines. Specifically, activated microglia upregulate inducible nitric oxide synthase (iNOS) and activate nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which produces the highly reactive free radical superoxide. These enzymes are commonly associated with increased neuronal damage during neurodegenerative diseases and traumatic insults (Chao et al., 1992; Bal-Price and Brown, 2001; Marin-Teva et al., 2004; Qin et al., 2004 and 2006; Wilkinson and Landreth, 2006). Moreover, microglia are the primary source of proinflammatory cytokines, such as tumor necrosis factor- α (TNF-α) and interleukin-1ß,

which can contribute to brain injury by activating caspase-3 in neurons, an intracellular pro-apoptotic factor (Hanisch, 2002; Kim and de Vellis, 2005). Activated microglia also secrete proteinases, including cathepsin B and L (Banati et al., 1993) and tissue plasminogen activator (tPA, Tsirka et al., 1995), as well as excitatory amino acids, such as glutamate (Takeuchi et al., 2008), which can further contribute to disease progression. Alternatively, microglia may also promote neurogenesis and repair after injury by releasing a variety of neurotrophic factors, including insulin-like growth factor (Streit, 2002; Schwartz et al., 2006).

b. Triggers of activation

Diverse endogenous and exogenous cues can lead to microglial activation. These stimuli include cell surface markers on necrotic or apoptotic cells, abnormally processed or aggregated proteins such as fibrillary amyloid-β, high neurotransmitter levels, microbial pathogens, and environmental toxins. Many of these stimuli are recognized by constitutively-expressed pattern recognition receptors (PRRs, Block et al., 2007) that are classically known to bind conserved and invariant microbial motifs known as pathogen-associated molecular patterns. Activation of these receptors initiates a rapid innate immune response that helps to contain an insult or infection and to activate the adaptive immune response. The large family of PRRs includes complement receptors, mannose receptors, Fc receptors that recognize antibodies, and toll-like receptors (TLRs) that bind a range of substrates. Among the many molecules that stimulate PRRs, lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, stimulates TLR4 and induces microglial secretion of reactive oxygen species and proinflammatory cytokines, and leads to progressive neuronal degeneration (Lehnardt et al., 2002 and 2003). Since neurons do not

express TLR4, LPS is not directly neurotoxic, indicating that microglia are capable of initiating brain damage upon activation.

Interestingly, it has been proposed that PRRs, including TLR4, are involved in the pathogenesis of peripheral inflammatory diseases independent of microbial pathogens, including Crohn's disease and arthrosclerosis. A number of studies have suggested that peripheral phagocytes use PRRs to recognize endogenous substrates (Gordon et al., 2002; Rifkin et al., 2005). In the CNS, it has similarly been put forward that PRRs expressed by microglia may recognize host-derived substrates and consequently trigger microglial activation and lead to neurotoxicity in the absence of pathogen invasion. Indeed, TLR4 is upregulated during normal aging and upon brain injury (Letiembre et al., 2007a and 2007b) and contributes to CNS pathogenesis following spinal cord transection (Tanga et al., 2005) and cerebral ischemia (Caso et al., 2007; Hua et al., 2007). Furthermore, while TLR2 and TLR9 are known to recognize components of bacteria and initiate inflammation, recent evidence suggests that these receptors may also play important roles in cerebral ischemia, traumatic brain injury, and Alzheimer's disease (Babcock et al., 2006; Lehnardt et al., 2007; Richard et al., 2008; Stevens et al., 2008).

Several endogenous candidate ligands have been proposed to activate PRRs. Sialic acid-containing glycosphingolipids, termed gangliosides, are concentrated on neuronal cell membranes and have been reported to stimulate microglia via TLR4 (Jou et al., 2006). Notably, TLR4 knockout animals exhibit enhanced amyloid-β deposits as compared to wild-type animals, suggesting that TLR4 might aid in the clearance of plaques observed in Alzheimer's disease (Tahara et al., 2006). Indeed, aggregated amyloid-β has been proposed to stimulate TLR2 and TLR4 receptors by binding to the accessory protein CD14 (Udan et al., 2008), which has also been shown to mediate phagocytosis of fibrillary amyloid-β (Fassbender et al., 2004; Liu et al., 2005). Moreover, TLR2, a receptor known to recognize

bacterial lipoproteins, may recognize an endogenous peptidoglycan, a component of the extracellular matrix, as well as heat shock proteins, fatty acids, and necrotic cells (Asea et al., 2002; Lee et al., 2004; Tsan and Gao, 2004; Rifkin et al., 2005). TLR2 has been found to increase expression in the hippocampus and modulate neuroimmune responses following axonal transection (Babcock et al., 2006). Together, these findings suggest that while the macrophage-like microglia are well-equipped for recognizing invading pathogens using TLRs, these receptors may also be utilized for recognition of certain endogenously generated substrates, which may similarly trigger proinflammatory pathways in microglia.

Interestingly, there are also constitutive signals that, when turned off, can trigger microglial activation. These downregulatory inputs include TGF-ß, glucocorticoids, and interleukin-10 (Lindholm et al., 1992; van Rossum and Hanisch, 2003). These agents may promote microglial activation upon a decline in their level or activity.

c. Intracellular signaling

Microglial transformation from a resting to a proinflammatory state often involves the transcription factor nuclear factor-κB (NF-κB). As a key regulator of inflammatory responses throughout the body, NF-κB is activated by diverse pathogenic signals and triggers rapid expression of many immunologically relevant proteins (Baeuerle and Henkel, 1994). The wide range of NF-κB-activating stimuli include bacterial products that stimulate TLRs, such as LPS and viral products, as well as inflammatory cytokines and oxidative cellular stress. Indeed, LPS, amyloid-β, leptin, zinc, acute brain trauma, as well as the presence of oxygen- and glucose deprived neurons, are all potent inducers of NF-κB activity in microglia (Combs et al., 2001; Ito et al., 2005; Kurpius et al., 2006; Tang et al., 2007; Kauppinen et al., 2008; Kaushal and Schlichter, 2008; Woo et al., 2008). Moreover,

microglial NF-κB activation is evident in animal models of acute or chronic injury, including stroke and multiple sclerosis (Bonetti et al., 1999; Mattson and Camandola, 2001).

Although NF-κB is expressed ubiquitously, it can impart cell type- and context-specific changes in gene expression. This capability is partly provided by the five distinct DNA binding subunits that differentially combine to form the NF-κB heterodimer. Specificity in the actions of NF-κB is further afforded by regulatory factors, including specific gene elements and NF-κB-binding and accessory proteins. While NF-κB is constitutively present in the cytoplasm, it is inactive due in part to the binding of the inhibitory protein IκB to the nuclear localization sequence of NF-κB. Upon phosphorylation and dissociation of IκB by IκB kinase, NF-κB translocates to the nucleus and binds to specific gene elements to induce transcriptional changes. While NF-κB has received the most attention, many other factors are similarly involved in initiating and regulating gene expression during microglial activation, including p38 mitogen-activated protein kinase and JAK2-STAT3 (Huang et al., 2008; Kobayashi et al., 2008).

1.4. Cell chemotaxis

Cell chemotaxis, or the directional migration of a cell in response to an extracellular signaling factor, is a coordinated process involving cell membrane extension at the front edge and displacement of the cell body. Chemotaxis is known to drive immune cell homing to injured or diseased regions, and is therefore a vital part of immune responses. This complex cellular process is driven by dynamic regulation of cell adhesion to the extracellular matrix, remodeling of cytoskeletal elements, and signaling by a myriad of intracellular pathways. Rho-family GTPases, including Rho, Rac1, and Cdc42, are key regulators of cell adhesion and remodeling of the actin cytoskeleton, and trigger changes in cell polarity, morphology, and motility in various cell types, including fibroblasts, macrophages, and

neurons (Allen et al., 1997; Fukata et al., 2003). Activated Cdc42 and Rac1 both localize to the leading edge of motile cells, and have been shown to trigger membrane extension in the form of thin filopodial protrusions, or wide lamellipodial extensions and ruffling, respectively. Cdc42 and Rac1 are also thought to be involved in a positive feedback loop with phosphatidylinositol 3-kinase (PI3K), a signaling factor that produces phosphatidylinositol 3,4,5-trisphosphate (PIP_3), modulates motility, and indirectly regulates Rho GTPase activity (Fukata et al., 2003). In contrast, the GTPase Rho triggers the formation of contractile actin-myosin filament bundles, or stress fibers, and the associated focal adhesion complexes that allow cell attachment to extracellular substrates (Hall, 1998). In neurons and other cell types, Rho has been shown to trigger process retraction and cell rounding (Hall, 1998; Gallo, 2006). Interestingly, Rho GTPases are also involved in mounting an immune response in leukocytes by regulating phagocytosis, cytokine release, and the production of reactive oxygen species (Bokoch, 2006).

Numerous factors stimulate microglial chemotaxis and process motility, including purinergic agonists (Honda et al., 2001; Haynes et al., 2006), complement proteins (Yao et al., 1990; Nolte et al., 1996), epidermal growth factor (Nolte et al., 1997), cannabinoids (Franklin and Stella, 2003), macrophage inflammatory protein-1 (MIP-1, Schmidtmayerova et al., 1996; Cross and Woodroofe, 1999), macrophage chemoattractant protein-1 (MCP-1, Calvo et al., 1996), and various other chemokines (Cross and Woodroofe, 1999; Rappert et al., 2002; Carbonell et al., 2005). Chemokines are well-known for triggering migration of immune cells (Fernandez and Lolis, 2002). The majority of chemokine receptors are G protein-coupled and exert their effects through $G\alpha_i$-coupled signaling. While astrocytes and microglia are the primary source of chemokines in the CNS, neurons can also secrete these chemotactic factors and thereby affect microglial activation and recruitment toward sites of neuronal injury. For example, Rappert (2004) found that CXCL10 is expressed in neurons

upon injury and loss of its receptor, CXCR3, prevents microglial recruitment to areas of axonal degeneration and results in continued presence of denervated dendrites. Likewise, the chemokine CCL2 (or, monocyte chemotactic protein-1, MCP-1), which is the main ligand for the CCR2 receptor, is upregulated in neurons within two hours of ischemia and is also expressed by microglia (Che, 2001; Banisadr, 2005; El Khoury et al., 2007). Notably, using a transgenic animal model of Alzheimer's disease, El Khoury et al. (2007) demonstrated that the absence of CCR2 results in a decline in microglial accumulation within the hippocampus, as well as a rise in amyloid-β levels and a rise in animal mortality. Additionally, stimulation of CCR2 induces microglial activation and is sufficient for the development of mechanical allodynia, or pain hyperexcitability, following peripheral sciatic nerve injury (Zhang et al., 2007). Fractalkine is another chemoattractant primarily expressed by neurons and released upon neuronal excitotoxicity (Chapman et al., 2000). By stimulating CXCR1 receptor expressed predominantly on microglia, fractalkine is another recruiter of microglia upon brain injury. Thus, various chemotactic signaling factors play a role in microglial response to CNS injury.

1.5. Microglial morphology and process motility

As discussed in greater detail below (see Section 1.8), resting microglia exhibit a chemotactic response to brain injury. Specifically, microglia are known to extend their elaborate processes towards damaged tissue within minutes following an acute insult (Nimmerjahn et al., 2005). Because microglia are highly phagocytic immune cells, such early motility and homing of microglial processes towards injured neurons is thought to promote rapid clearance of toxic debris and dying cells and thereby limit secondary injury following an insult. In contrast, a key feature of microglial activation is the dramatic retraction of microglial processes within 24 hours following an insult. Specifically,

neuroinflammation during most types of brain damage is marked by loss of the elaborately branched microglial processes and consequent presence of rounded microglia with few membrane protrusions. Indeed, microglia are often characterized as activated within injured or diseased brain tissue based solely on the morphological criteria of shortened processes and an enlarged cell body. This phenomenon has been noted for over 80 years (Jacob, 1927) and serves as a hallmark of inflammation and injury in the brain. It is hypothesized that this structural remodeling reflects changes in the chemotactic and phagocytic properties of microglia upon activation (Petersen and Dailey, 2004). However, both the causes and consequences of microglial process retraction remain a mystery.

Although it is generally assumed that retracted activated microglia are more motile and phagocytic than resting cells, which are highly ramified and thought to be fixed in their position, Petersen and Dailey (2004) reported that approximately 43% of activated microglia in hippocampal slices are stationary with short and immotile cell processes. This observation suggests that activated microglia, while exhibiting the characteristic retracted morphology in fixed brain tissue, may display diverse motile behaviors in living tissue. Indeed, microglia are thought to exhibit several transitional stages of process morphology and motility, with process withdrawal serving as the initial step toward the activated phenotype (Stence et al., 2001). Interestingly, Stence et al. (2001) have proposed that there may be a switch mechanism that triggers microglial retraction upon cell activation. However, this switch remains elusive. Moreover, while resting and activated microglia seem to exhibit different morphologies and chemotactic behaviors, it is unknown whether these cells also show different levels of tissue surveillance and scavenging during brain injury.

1.6. Microglial scavenging

A critical aspect of microglial function in both health and disease is the uptake of substances released into the extracellular space within the CNS, including amyloid fibrils, myelin debris, and glutamate, as well as engulfment of apoptotic or necrotic cells (Block et al., 2007; Petersen and Dailey, 2004; Marin-Teva et al, 2004). Mounting evidence indicates that the injured CNS requires intervention by the innate immune system in order to clear apoptotic and necrotic debris, and to eliminate toxic components such as amyloid (Wyss-Coray and Mucke, 2002). A similar conclusion has been drawn from studies in *Caenorhabditis elegans*, where mutation of genes involved in phagocytosis results in persistence of cells otherwise destined for apoptosis (Reddien et al., 2001; Hoeppner et al., 2001). Indeed, defects in phagocytic clearance are implicated in amyloid plaque toxicity in Alzheimer's disease (Wyss-Coray et al., 2001; Streit, 2004; Hickman et al., 2008).

Phagocytosis and macropinocytosis are processes that enable antigen-presenting cells like microglia to actively engulf extracellular substances for degradation and eventual presentation of short peptides derived from those substrates to T cells. In addition to serving a vital role in immune responses to injury and disease, both phagocytosis and macropinocytosis are also involved in the normal development, maintenance, and repair of all types of tissues (Reddien and Horvitz, 2004; Stuart and Ezekowitz, 2008). However, these two uptake mechanisms have an important difference. While phagocytosis is triggered by receptor-mediated recognition of a specific target, macropinocytosis is non-discriminative and allows for non-specific sampling of the extracellular fluid (Swanson, 2008). Microglia may engage in both types of uptake behaviors. Indeed, microglia have been observed to phagocytose injured or dying neurons (Marin-Teva et al., 2004; Kurpius et al., 2007), as well as soluble and fibrillar amyloid-β, a major component of amyloid plaques in Alzheimer's disease (Rogers et al., 2002). In addition, Nimmerjahn et al. (2005) and Honda et al. (2001)

have shown that microglia seem to engage in constitutive uptake of extracellular substrates *in vitro* and *in vivo*, a process reminiscent of macropinocytosis.

Apoptosis, or programmed cell death, allows for rapid recognition and phagocytic removal of damaged or otherwise dysfunctional cells without leakage of the cytosol into the extracellular space, which can result in further tissue damage (Platt et al., 1998). Upon apoptosis, dying cells facilitate phagocytosis by presenting a variety of signals on the cell membrane, including phosphatidylserine, lysophosphatidylcholine, intercellular adhesion molecule-3, oxidized phospholipids, altered carbohydrates, and C1q- and C3b-binding sites (Giles et al., 2000; Savill and Fadok, 2000; Hengartner, 2001; Wyss-Coray and Mucke, 2002). These substrates not only allow cell phagocytosis, but may also trigger the release of microglial anti-inflammatory cytokines, such as interleukin-10 and TGF-β, and thus dampen microglial activation (Voll et al., 1997; Fadok et al., 2000; Magnus et al., 2001; Liu et al., 2006). Indeed, the newly identified triggering receptor expressed on myeloid cells-2 (TREM2) has been found to mediate microglial phagocytosis and downregulate microglial TNF-α and NOS2 expression (Takahashi et al., 2005). In contrast, cells dying by necrosis lose membrane integrity and release their intracellular contents, thereby causing a proinflammatory response (Searle et al., 1982).

In order to reach the target cell or substrate, phagocytic cells like microglia must exhibit a high degree of motility. Indeed, microglial chemotaxis and active membrane protrusion enables particle contact and engulfment. As previously discussed (*see Section 1.4*), these motile behaviors are regulated by chemotactic factors, such as purine nucleotides.

1.7. Purinergic signaling

Nucleotides, such as adenosine triphosphate (ATP), play central roles in physiology and are involved in a vast array of intracellular processes, including energy production, cell

division, and intracellular signal transduction. As proposed by Burnstock (1972), nucleotides are also released or leaked from cells to act as extracellular paracrine and autocrine signaling factors. First recognized for its extracellular effects at the neuromuscular junction (Buchthal et al., 1944), ATP is now known to serve as a ubiquitous transmitter, neuromodulator, and a trophic factor by binding to a family of purinergic receptors, termed P2. Interestingly, rapid ATP degradation can result in the formation of adenosine, which activates a separate group of purinergic receptors, termed P1 (Burnstock, 2007). As detailed below, P1 and P2 receptors are activated by specific nucleotides and trigger a variety of intracellular signaling pathways.

a. P1 receptors

Adenosine activates a group of four G protein-coupled receptors that comprise the P1 family, namely A_1, A_{2A}, A_{2B}, and A_3. While A_1 and A_3 receptors are coupled to pertussis toxin-sensitive $G\alpha_i$, both A_{2A} and A_{2B} receptors are linked to $G\alpha_s$ and trigger cyclic AMP (cAMP) production by adenylyl cyclase and activation of protein kinase A (PKA). Following the recognition that certain adenosine receptors stimulate cAMP signaling (Afonso and O'Brien, 1970), Daly et al. (1983) subdivided A_2 receptors, with A_{2A} having higher adenosine affinity than A_{2B} based on the binding properties of the adenosine derivative [^{3}H]NECA ([^{3}H]^5'-*N*-ethylcarboxamidoadenosine). All four P1 receptors have an extracellular N-terminus, seven putative α-helical transmembrane domains that are crucial for agonist specificity, and a cytoplasmic C-terminus. Both A_1 and A_{2A} receptors exhibit high expression in the CNS (Rivkees et al., 1995; Ochiishi et al., 1999; Rosin et al., 1998) and are expressed by neurons, astrocytes, and microglia *in vitro* (Nishizaki, 2004; Wittendorp et al., 2004; Rebola et al., 2005; Saura et al., 2005). Interestingly, A_{2A} is co-

localized primarily with dopamine D2 receptors on striatopallidal neurons, but also exhibits lower expression in other brain regions (Rosin et al., 1998).

b. P2 receptors

P2 receptors are classified into ionotropic P2X receptors and metabotropic G protein-coupled P2Y receptors, which will be the focus here. The eight known P2Y receptors ($P2Y_{1, 2, 4, 6, 11, 12, 13, 14}$) contain glycosylated N-terminus domains, seven transmembrane regions which include the agonist-binding pocket, and a high degree of diversity in the C-terminus that determines receptor coupling to various G proteins. While many P2Y receptors couple to $G\alpha_{q/11}$ and trigger calcium release from intracellular stores ($P2Y_{1,2,4,6}$), $P2Y_{12, 13, 14}$ bind preferentially to $G\alpha_i$ and $P2Y_{11}$ binds to both $G\alpha_{q/11}$ and $G\alpha_s$. Notably, P2Y receptors show diversity in their downstream signaling, including activation of adenylyl cyclase, PI3K, phospholipases, MAP kinase, and Rho-dependent kinase (ROCK).

Due to low amino acid sequence homology within the transmembrane regions (19-55% similarity), P2Y receptors exhibit different pharmacological profiles. Thus, while some subtypes are activated by the nucleotide triphosphates ATP and uridine triphosphate (UTP) ($P2Y_{2, 4, 11}$), others are stimulated preferentially by nucleotide diphosphates ADP and UDP ($P2Y_{1, 6, 12, 13}$). $P2Y_{2, 4, 6}$ receptors recognize UTP and UDP, while $P2Y_{1,11,12}$ are specific for ATP and ADP. However, because nucleotides are rapidly hydrolyzed and most cell types express several P2Y receptor subtypes, multiple receptors and downstream signaling cascades may be activated by an agonist within a single cell (Norenberg et al., 1997; Haynes et al., 2006; Light et al., 2006; Kobayashi et al., 2006 and 2008; Koizumi et al., 2007). Specifically, microglia are known to express $P2Y_6$, $P2Y_{12}$ and $P2Y_{13,}$ and may therefore be affected by ATP, ADP, and UDP (Haynes et al., 2006; Koizumi et al., 2007; Light et al., 2006; Kobayashi et al., 2006 and 2008).

c. Adenine nucleotides in the CNS

Accumulating evidence indicates that ATP is released under both normal and pathological conditions by diverse cell types, including neurons, astrocytes, and microglia (Coco et al., 2003; Zhang et al., 2003; Bianco et al., 2005b; Montana et al., 2006; Burnstock, 2007; Fujita et al., 2008). The extracellular ATP concentration is estimated to be approximately 1-10 nM in the normal brain (Melani et al., 2005). Notably, neurons contain approximately 2-5 mM ATP, with up to 100 mM in synaptic vesicles (Kogure and Alonso, 1978; Burnstock, 2007). It is thought that ATP is a common co-transmitter secreted from GABAergic, cholinergic, and noradrenergic neurons in various regions of the CNS and PNS (Salter and De Koninck, 1999; Burnstock, 2007). Local injection of NMDA or kainate increases extracellular ATP and adenosine levels in the rodent striatum and hippocampus (Carswell et al., 1997; Delaney et al., 1998). Moreover, high and low frequency stimulation within hippocampal slices triggers ATP and adenosine release, respectively (Cunha et al., 1996). Astrocytic ATP release has also been reported following exposure to thrombin, lysophosphatidic acid, or bradykinin (Verderio and Matteoli, 2001; Koizumi et al., 2003; Blum et al., 2008).

The nucleoside adenosine is also receiving growing attention for its roles as a neuromodulator (Dunwiddie and Masino, 2001; Latini and Pedata, 2001). Extracellular adenosine levels have been estimated to be 50-120 nM in a normal rodent brain (Phillis et al., 1987; Chen et al., 1992; Pazzagli et al., 1993; Carswell et al., 1997; Latini and Pedata, 2001). Given that adenosine has 70 nM and 150 nM affinity for A_1 and A_{2A} receptors, respectively (Dunwiddie and Masino, 2001), these receptors may be activated by adenosine under normal conditions in the CNS. Moreover, Lloyd et al. (1993) have reported that adenosine increases 10-fold or 16-fold in rat hippocampal slices following electrical field stimulation or energy depletion, respectively. Additionally, adenosine levels can reach 10-

50 μM following 15 minutes of ischemia (Hagberg et al., 1987). These findings suggest that adenosine receptors may play important roles in both physiological and pathological contexts. It was further suggested that most of the adenosine was released by cells and not generated by extracellular ATP breakdown. Indeed, adenosine can be produced intracellularly by dephosphorylation of AMP by 5'-nucleotidases and then transported into the extracellular space (Jonzon and Fredholm, 1985). Intracellular cAMP is another potential source of adenosine, which can be converted to AMP by phosphodiesterases. However, extracellular adenosine can also be generated from the cellular release of ATP, ADP, or AMP. These nucleotides can be degraded to adenosine within hundreds of milliseconds by extracellular or membrane-bound enzymes, including CD39 and CD73 ectonucleotidases (Wang and Guidotti, 1996; Dunwiddie et al., 1997; Zimmermann, 1998). Indeed, synaptic membranes from the cortex and hippocampus exhibit high ecto-nucleoside triphosphate diphosphohydrolase (NTPDase) activity that results in nucleotide hydrolysis (Kukulski et al., 2004).

d. Physiological roles of purines in immune function

In addition to serving as a neuromodulator, adenosine is also a potent regulator of inflammation. Adenosine regulates a variety of immune cell functions and is considered a crucial early negative feedback signal for inflammatory events in the periphery (Ohta and Sitkovsky, 2001; Lukashev et al., 2004; Sitkovsky and Ohta, 2005). For example, adenosine regulates macrophage proliferation, migration, phagocytosis, as well as nitric oxide and cytokine production (Hofer et al., 2003; Hasko et al., 2007).

Adenosine can similarly regulate immune responses in the brain. For example, adenosine and its analogs inhibit cytokine production and neutrophil infiltration following intracerebral hemorrhage (Mayne et al., 2001). A_{2A} receptor activation has been reported to

reduce damage in certain animal models (Mayne et al., 2001; Cassada et al., 2002). However, A_{2A} receptor blockade or genetic inactivation has also been found to protect against a variety of neural insults that involve neuroinflammation (Phillis, 1995; Jones et al., 1998; Monopoli et al., 1998; Chen et al., 1999 and 2001; Ikeda et al., 2002; Popoli et al., 2002; Dall'Igna et al., 2003; Fink et al., 2004). Consistent with these finding, Saura et al. (2005) found that activation of the A_{2A} receptor enhances the release of nitric oxide in mixed glial cultures, further suggesting that adenosine can contribute to neuroinflammation. While adenosine is implicated in various neuropathologies that are exacerbated by microglial inflammatory responses, the effects of adenosine on microglial function remain largely unknown.

e. The effects of purines on microglia

ATP can induce a variety of microglial responses, depending on the agonist concentration and receptor expression, which varies with the state of microglial activation. While ATP can trigger the release of cytokines and growth factors at lower doses, it can induce microglial apoptosis at higher concentrations (Farber and Kettenmann, 2006). $P2X_4$ is upregulated in activated microglia after spinal cord nerve injury and is crucial for induction of neuropathic pain (Tsuda et al., 2003). $P2Y_6$ is similarly upregulated upon kainate-induced excitotoxic injury in the hippocampus and mediates UDP-induced phagocytosis in microglia via the G_q-coupled pathway (Koizumi et al., 2007). Additionally, $P2Y_{12}$ upregulation on spinal microglia is involved in mechanical and thermal pain hypersensitivity upon peripheral nerve injury by activating p38 MAPK (Kobayashi et al., 2008; Tozaki-Saitoh et al., 2008). However, it has also been reported that $P2Y_{12}$ is expressed by resting, but not by activated, microglia both *in vivo* and *in vitro* (Haynes et al., 2006). This $G\alpha_i$-coupled receptor is known to trigger chemotaxis and process motility in

microglia (Davalos et al., 2005; Haynes et al., 2006; Kurpius et al., 2007). As discussed further below, Haynes et al. (2006) have demonstrated that $P2Y_{12}$ drives rapid chemoattraction of microglia toward sites of brain injury.

1.8. Microglial chemotactic response to injury

Similar to peripheral macrophages, microglia are involved in tissue repair following injury. Recent *in vivo* studies using two-photon imaging of resting microglia in anesthetized mice reveal that microlesions in cerebrovasculature induce rapid microglial process extension toward and envelopment of the injured site (Nimmerjahn et al., 2005; Davalos et al., 2006). This directional guidance, or chemoattraction, towards damaged brain tissue takes place within minutes of an insult and involves purinergic receptor signaling in microglia. Specifically, microglial chemoattraction toward the damaged area is triggered in part by the release of intracellular nucleotides, such as ATP, ADP, and UDP, from injured cells into the extracellular space. Consequently, these nucleotides activate purinergic receptors on microglial cell surface, including $P2Y_{12}$ and $P2Y_6$, to initiate process extension and phagocytosis, respectively (Fields and Burnstock, 2006; Haynes et al., 2006; Koizumi et al., 2007).

The intracellular concentration of ATP is estimated to be 2-5 mM (Dubyak and el-Moatassim, 1993), and as demonstrated in isolated cultures, hippocampal slices, and *in vivo*, the release of ATP and other nucleotides is both necessary and sufficient for microglial chemoattraction toward injured tissue and enables phagocytosis of dead or dying cells (Honda et al., 2001; Davalos et al., 2005; Haynes et al., 2006; Koizumi et al., 2007). In support, degradation of ATP and ADP with the enzyme apyrase triggers the collapse of motile branches in resting microglia and blocks directional process movement upon injury (Davalos et al., 2006; Kurpius et al., 2007), indicating that purines are involved in motility of

microglia under normal conditions as well as during injury. Furthermore, $P2Y_{12}$ knockout animals show delayed microglial chemoattraction toward damaged areas (Haynes et al., 2006), suggesting that this purinergic receptor is involved in acute microglial chemotaxis.

Interestingly, $P2Y_{12}$ is dramatically downregulated upon microglial proinflammatory activation *in vivo* (Haynes et al., 2006). This raises the possibility that other purinergic and/or non-purinergic receptor systems may take control over motile responses in activated microglia. If so, alternate receptor involvement may cause a distinct motile response by activated microglia in comparison to resting cells. Consistent with this, Kurpius et al. (2007) demonstrated that while microglia within hippocampal slices exhibit directional migration toward injured areas during the first 1-3 hours post-injury, activated microglia at later time-points (3-7 hours) showed no directional bias toward injured tissue. These observations highlight the existence of temporal differences in microglial motility during brain injury and suggest that activated microglia may not exhibit the same chemotactic response during brain damage as resting microglia. However, chemotactic responses to purines released during injury have not been examined in the context of inflammation. Given that most neuropathologies are characterized by ongoing neuronal damage in the presence of inflammation and activated microglia, the study of purinergic responses by microglia in their activated state would aid in the understanding of various CNS diseases and may reveal novel therapeutic strategies for preventing or delaying these conditions.

1.9. Summary

Microglia are highly motile and phagocytic CNS-specific immune cells that represent the first line of defense during brain injury or disease (Hanisch and Kettenmann, 2007). Under normal conditions, microglia are in a resting or naïve state and actively survey brain tissue using highly ramified and motile processes (Davalos et al., 2005; Nimmerjahn et

al., 2005). Constitutive process extension and retraction by microglia mediates sampling and surveillance of healthy brain tissue. Moreover, recent studies have shown that acute brain injury induces process extension by naïve microglia toward sites of tissue damage (Davalos et al., 2005; Nimmerjahn et al., 2005). This chemoattractive response to injury is guided by local release of nucleotides such as ATP from damaged cells, and involves microglial purinergic receptors. Indeed, genetic removal of the $G\alpha_i$-coupled purinoreceptor $P2Y_{12}$ inhibits microglial attraction toward brain damage (Haynes et al., 2006). Microglial process convergence onto sites of injury is thought to serve neuroprotective functions by creating a physical barrier around the injury site and allowing microglial scavenging of necrotic debris. Thus, microglial process motility plays roles under both normal and pathological contexts by enabling microglial monitoring of healthy tissue and clean up of injured tissue.

In contrast, prolonged brain damage is accompanied by activated microglia with highly retracted processes and an amoeboid appearance (Kreutzberg, 1996; Stence et al., 2001; Block et al., 2007). Microglial process retraction upon proinflammatory activation has been documented for decades (Jacob, 1927). While the morphological transformation of microglia into amoeboid cells is a hallmark of brain trauma and neurodegeneration, its causes and consequences remain unknown.

$P2Y_{12}$, a receptor that mediates chemoattraction of resting microglia toward injury, is downregulated upon microglial activation (Haynes et al., 2006). This finding suggests that activated microglia may sense acute brain damage using a different receptor. However, this possibility remains unexamined. Given that activated microglia affect the course of brain injury and neurodegeneration, and that chemotaxis is a vital aspect of microglial function, the study of chemotactic responses by activated microglia may be critical for understanding healthy brain function and for treating neuropathological conditions.

CHAPTER 2: Materials and Methods

2.1. Animals and reagents

All procedures using animals were approved by the Emory University IACUC. Transgenic mice expressing enhanced green fluorescent protein (eGFP) driven by an actin promoter (a gift from M. Okabe, Osaka University, Japan) were used to culture microglia for confocal imaging experiments. Transgenic mice encoding eGFP in place of exon 2 within the *Cx3cr1* gene, and thus exhibiting microglia-selective eGFP expression (Davalos et al., 2005), were obtained from the Jackson Laboratory. *Adora2a-eGFP* bacterial artificial chromosome (BAC)-transgenic mice were obtained from Mutant Mouse Regional Resource Centers (MMRRC). These BAC-transgenic mice express an eGFP reporter gene, which is inserted upstream of the BAC A_{2A} coding sequence, and thus exhibit eGFP fluorescence that correlates with changes in A_{2A} expression levels. *Adora2a-eGFP* mice were genotyped using the strain-specific primer set, as specified by the MMRRC: TGCCCAAGTGTGGCTTCT and TAGCGGCTGAAGCACTGCA.

Cx3cr-eGFP mice (1-3 month old) received a single intraperitoneal (IP) injection of either sterile PBS or LPS (2 mg/kg body weight, 100 ul total volume). After 48 hours, PBS and LPS-injected animals were anesthetized with a mixture of 2,2,2-tribromoethanol and tertiary amyl alcohol (Avertin) at 17.5 µl/gram body weight. Mice were then placed into a stereotaxic apparatus for an intracortical injection of vehicle (50% DMSO in PBS, 2 ul) or A_{2A} antagonist SCH-58261 (1 mM in 2 µl) at 400 nl per minute at the coordinates 1.0 mm caudal, 2.0 mm lateral, and 1.0 mm ventral from bregma. The needle was left in place for 5 minutes. After another 25 minutes, the brain tissue was removed and drop-fixed overnight in 4% paraformaldehyde with 0.1% glutaraldehyde and then cryopreserved in 15% sucrose for 24 hours and 30% sucrose for an additional 24 hours. *Adora2a-eGFP* mice received similar

PBS or LPS injections and were sacrificed after 48 hours.

Adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP), 5'-(N-ethylcarboxamido)adenosine (NECA), adenosine, A2a agonist CGS-21680, A2a antagonist SCH-58261, lipopolysaccharide (LPS), lipoteichoic acid from *Staphylococcus aureus* (LTA), Gαs inhibitor NF449, and a protein kinase A (PKA) inhibitor N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89) were all purchased from Sigma. Nuclear factor-κB (NF-κB) inhibitors 6-Amino-4-(4-phenoxyphenylethylamino)quinazoline (QNZ) and SN50, forskolin (FSK), adenylate cyclase inhibitor 2',5'-dideoxyadenosine (ddAdo), Gαi inhibitor pertussis toxin (*Bordetella pertussis,* PTX), Gαs activator cholera toxin (*Vibrio cholerae,* CLX), Rho GTPase inhibitor exoenzyme C3 (*Clostridium botulinum),* Rho kinase (ROCK) inhibitor Y27632, and adenosine deaminase (ADA) were all purchased from Calbiochem. We also utilized recombinant mouse complement component C5a, recombinant mouse tumor necrosis factor-α (TNF-α, R&D Systems), and unmethylated CpG motif-containing oligonucleotides (CpG, InvivoGen). Isolectin GS-IB_4 (IB_4) from *Griffonia simplicifolia* and human β-amyloid 1-42 peptide were obtained from Invitrogen. β-amyloid was aggregated in PBS according to manufacturer instructions. Briefly, β-amyloid was dissolved in sterile H_2O at 6 mg/ml, further diluted in filtered PBS to 1 mg/ml, and incubated at 37°C for 48 hours.

2.2. Cell cultures

Cortical tissue from postnatal day 2-3 eGFP or wild-type C57Bl/6 mice (The Jackson Laboratory) was dissociated into a single-cell suspension by trituration. Cells were plated into 6-well plates coated with 0.05 mg/ml poly-D-lysine in DMEM (Gibco #11960) containing 10% heat-inactivated horse serum, 10% heat-inactivated fetal bovine serum, 25 mM glucose, 2 mM L-glutamine, and 1 mM sodium pyruvate. After 2-3 weeks, floating

microglia were isolated by transferring the glial culture supernatant onto either Matrigel matrix-coated or poly-D-lysine-coated glass coverslips. Microglia were confirmed $\geq$ 95% pure based on selective staining with Alexa Fluor 594-conjugated IB_4 (Invitrogen, 1:100, data not shown).

All confocal time-lapse imaging experiments were performed on isolated eGFP microglia cultured in a Matrigel Basement Membrane Matrix (phenol red-free, BD Biosciences, #356237) on 15 mm round glass coverslips (#1.5, Warner Instruments, #64-0713). Coating of coverslips with Matrigel matrix was performed immediately prior to microglial isolation. Briefly, 30ul of ice-cold liquid matrix was spread evenly with pre-cooled pipette tips and brush onto coverslips in 12-well plates on ice and then incubated at 37°C for 20-30 minutes to allow the matrix to gel. This resulted in approximate gel thickness of 0.2 mm. Thereafter, microglia were added and allowed to adhere and migrate into the matrix for 24 hours. Imaging was performed on microglia that have migrated into the Matrigel and displayed process extension in three dimensions. For astrocyte-microglia co-cultures, isolated eGFP microglia were applied onto 50% confluent wild-type astrocytes and allowed to adhere overnight.

Adult human microglial cells were obtained from Emory University Hospital patients (ages 30-45) undergoing hippocampectomy due to seizures or tissue resection due to a subarachnoid tumor. All procedures involving the use of human tissue were approved by the Emory University IRB. Microglia were isolated using a modified protocol established by Klegeris et al. (2005). Briefly, human brain tissue was dissociated into single cell suspension by chopping with a sterile razor, trituration, and incubation in 0.25% Trypsin-LE (Invitrogen, #12605) for 30 minutes at 37°C. Thereafter, cells were centrifuged for 10 minutes at 0.3 rpm, washed in DMEM-F12 supplemented with 10% fetal bovine serum, triturated again, and plated onto 0.05 mg/ml poly-D-lysine-coated 15 mm coverslips. After

2 hours, cells were washed to remove debris and cultured for 5-7 days prior to experiments. Cell cultures were maintained at 37^{o}C in a humidified 5% CO_2-containing atmosphere.

2.3. Four-dimensional confocal imaging

a. Image acquisition

Cells were perfused continuously at approximately 1 ml/minute with imaging buffer containing (in mM): 10 HEPES, 150 NaCl, 3 KCl, 22 Sucrose, 10 Glucose, 1 $MgCl_2$, and 2 $CaCl_2$, pH 7.4. Buffer within the cell chamber was maintained at 32-33.5°C using TC-344B temperature controller (Warner Instruments). Image stacks were acquired using IPlab image acquisition software (BD Biosciences), an IX51 microscope fitted with a DSU confocal unit (Olympus), a MFC-2000 z-motor (ASI) and a Uniblitz shutter (Vincent Associates). After setting the exposure time to 70 msec and binning to 2 x 2, the upper and lower z-axis limits of a single microglial cell were established. Image stacks spanning about 25-30 μm were then collected continuously at a rate of approximately 20 seconds per stack during 5 minute periods of buffer perfusion, agonist perfusion, and washout with an ORCA-ER cooled CCD camera (Hamamatsu).

b. Image processing and analysis

Following acquisition, images were compiled and transformed into four-dimensional (4D) renderings throughout the course of a perfusion using Imaris 4.2 imaging software (Bitplane). After background noise subtraction with a 10 μm-width Gaussian filter, image stacks were reconstructed into 3D surfaces at each time-point based on calibrated voxel sizes (x/y: 0.105 μm^2, z: 0.5-2 μm), a signal intensity threshold, and a 0.2 μm-width Gaussian filter. All time-lapse surfaces were then split into objects to enable measurements of cell volume and surface area within each stack or time-point. Cell surface area-to volume

ratio (SA:V) at each time-point was then calculated to assess changes in cell structure. We found that baseline SA:V ratio values in untreated microglia were lower than that of CpG- and TNF-α-treated microglia ($p < 0.01$), but were not different from LPS- and LTA-treated groups.

Microglial cell process motility was assessed by comparing average speed of tracked regions of interest (ROIs) within cell processes during periods of buffer and agonist perfusion. ROIs, or discrete spherical volumes of fluorescence, were tracked objectively based on pre-defined criteria, including minimum ROI diameter, maximum ROI travel distance between consecutive image stacks, and the type of tracking algorithm utilized. We found that the baseline process motility of untreated microglia was lower than that of CpG- and LPS-treated microglia ($p<0.01$), but not different from LTA- and TNF-α-treated microglia.

c. Parameter optimization for tracking regions of interest

In order to measure agonist-induced changes in microglial process motility, 3D tracking of regions of interest (ROI, objectively assigned to cell processes) through time was performed using Imaris 4.2 software (Bitplane). We first defined the parameters that would allow for accurate and reliable ROI recognition and tracking by the software. Using 3D confocal imaging records from untreated and LPS-treated microglia receiving ATP, a range of parameter values were evaluated. A subset of those comparisons is shown in Figures 1 and 2. Specifically, we varied the minimum ROI diameter (the diameter of tracked volumes), and maximum ROI tracking distance (the maximum distance that an ROI can travel between two consecutive time-frames). We then compared how changes in these parameters affected software recognition and tracking of cell processes across time.

When the minimum ROI diameter was defined to be 5 μm or greater, most cell processes remained undetected, resulting in process under-sampling (Figure 1). However, if the minimum ROI diameter was set to 0.5 μm or lower, an excessive number of tracks per process were produced, possibly due to recognition of noise fluctuations, and resulted in process over-sampling. We found that setting the minimum ROI diameter to approximately 2 μm allowed for the most accurate process recognition by Imaris software (Figure 1).

When the maximum ROI tracking distance was set to 1 μm or lower, there was a failure to accurately represent the full extent of cell process motion and resulted in formation of shortened and over-constrained process tracks (Figure 2). However, ROI tracking distance of 5 μm or greater resulted in the formation of errant tracks between two distinct adjacent processes, yielding under-constrained tracks. Thus, we concluded that setting the minimum ROI tracking distance to 3 μm allowed for the most accurate process tracking by Imaris software (Figure 2).

In addition to comparing how the parameters ROI diameter and maximum tracking distance affect cell process recognition, we also assessed how the agonist effect varied with changes in these parameters. For this, we compared agonist-induced changes in track speed and the number of unassigned ROIs (regions of interest not designated by the algorithm to any track, possibly due to rapid noise fluctuations) (Figure 3), as well as changes in the total number of tracks and average track length (Figure 4). We aimed to minimize changes in the number of unassigned ROIs.

Similar to the results discussed above, the minimum ROI diameter of 2 or 3 μm was the most sensitive to ATP-induced increases in cell process motility in untreated microglia, while ROI diameter ranges of 2-4 and 2-5 μm exhibited little sensitivity to changes in process motility (Figure 3A). Furthermore, assessment of agonist-induced changes in the number of unassigned ROIs in LPS-treated microglia demonstrated that minimal changes in

unassigned ROIs occurred when the ROI diameter was set to 2 μm and the maximum tracking distance was set to 3 μm (Figure 3D).

Finally, we also assessed how using different tracking algorithms alters the readout of agonist effects in untreated and LPS-treated cells. Comparison of ATP-induced changes in track speed, number and length, as well as the number of unassigned ROIs, in both untreated and LPS-treated microglia revealed no dramatic differences when comparing Brownian motion and GapClose Autoregressive algorithms (Figures 3-4). While Brownian motion models random motile behaviors, GapClose Autoregressive function models continuous motion by matching predicted ROI positions with actual ROI positions within image data. Therefore, we chose to perform all of our tracking analyses using the GapClose Autoregressive algorithm in order to model directional cellular process movement.

In summary, agonist-induced changes in cell process motility were assessed by tracking regions of interest through time during baseline and agonist application after setting the minimum ROI diameter to 2 μm, the maximum ROI tracking distance to 3 μm, and utilizing the GapClose Autoregressive algorithm. These parameter settings allowed for optimal recognition of cell processes and the modeling of process motion, and provided high sensitivity to agonist-induced effects on process motion. Tracks created in the cell body were omitted from all analyses.

d. Iontophoresis

A glass micropipette (inner diameter: 1.15 $\pm$ 0.05 mm, outer diameter: 1.65 $\pm$ 0.05 mm, glass type 8250, Garner Glass Co.) was pulled to a ~1.4 MΩ tip (measured with 0.9% saline) with a Narishige pipette puller and filled with agonist diluted in imaging buffer. The holding current value (+65 nA) was determined empirically by observing microglial morphological responses (occurrence of membrane ruffling) while in close proximity to an

ATP-containing micropipette with different holding currents, which ranged from +30 nA to +100 nA. To expel ATP, a current of -200 nA was applied for at least 30 minutes. In order to analyze cell chemotaxis in response to iontophoretic agonist application, the cell body was tracked using Imaris 4.2 software by setting ROI diameter to the size of the nucleus.

2.4. Immunohistochemistry

Cryostat-sliced 40 µm-thick brain tissue sections derived from *Cx3cr1-eGFP* mice were washed in PBS three times, placed on Superfront Plus slides, and coverslipped with Vectashield (Vector Laboratories). Brain sections from *Adora2a-eGFP* mice were washed with 0.2% Triton-X for 90 min, blocked with 10% normal donkey serum for 45 min, and incubated in anti-GFP antibody diluted in 0.2% Triton-X for 48 hrs (1:1000, Invitrogen). Brain sections were then washed in PBS and incubated in FITC-conjugated donkey anti-rabbit secondary antibody for 2 hours (1:200, Jackson ImmunoResearch Labs). Tissue fluorescence was detected using IPlab image acquisition software (BD Biosciences), an IX51 microscope fitted with a DSU confocal unit (Olympus), and an ORCA-ER cooled CCD camera (Hamamatsu).

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was derived from homogenized isolated primary mouse and human microglia according to manufacturer instructions using Purelink Micro-to-Midi total RNA purification system (Invitrogen, #12183-018). Briefly, isolated microglia were plated into 6-well plates coated with 0.05 mg/ml poly-D-lysine and allowed to adhere overnight. Following lysis, samples were transferred into homogenizers (Invitrogen, #12183-026). After incubation in DNase I (Invitrogen, #18068-015) for 15 minutes, RNA was washed and eluted with sterile H_2O.

Isolated RNA (50 ng) was reverse transcribed and amplified according to manufacturer instructions using SuperScript III One-Step RT-PCR System with Platinum *Taq* (Invitrogen) and specific primers (*see Tables 1-3*; Haynes et al., 2006; Bystrova et al,. 2006; Bianco et al., 2005; Hoskin et al., 2002; Grijelmo et al., 2007; Chen et al., 2004; Feng et al., 2004; Kyrkanides et al., 2002; Tha et al., 2000; Park et al., 2005). Specifically, cDNA synthesis was carried out at 50ºC for 30 min, followed by pre-denaturation at 94ºC for 2 min, 30-40 cycles of denaturation at 94ºC for 15 sec, annealing at 55ºC for 30 sec (except for mouse $P2Y_4$: 51ºC; human P1 receptors: 58ºC; microglial activation markers: 60ºC), and finally elongation at 70ºC for 1 min. To check for DNA contamination, reverse transcriptase was omitted from some reaction mixtures. For primer pairs that did not produce visible bands using isolated microglial RNA, cDNA encoding each receptor was used to run control reactions. Plasmids for mouse adenosine receptors and mouse $P2Y_2$ were obtained from Open Biosystems, while mouse $P2Y_4$ and mouse $P2Y_6$ were a gift from W. O'Neal at the University of North Carolina, Chapel Hill. PCR reaction products were analyzed using a 2% agarose gel containing ethidium bromide and a 1Kb Plus DNA ladder (Invitrogen, #10787-018).

2.6. *In vitro* assays

a. Calcium imaging

Isolated microglia were plated onto 0.05 mg/ml poly-D-lysine-coated 12 mm coverslips and allowed to adhere overnight. Cells were then incubated with 5 μM Fura-2/AM and 1 μM pluronic acid (Invitrogen) for 30 minutes at 37°C, and subsequently transferred to a microscope stage for imaging. Cells were perfused continuously with imaging buffer containing (in mM): 10 HEPES, 150 NaCl, 3 KCl, 22 Sucrose, 10 Glucose, 1 $MgCl_2$, and 2 $CaCl_2$, pH 7.4. Following excitation at 340 nm and 380 nm, Fura-2/AM

emission intensity at 510 nm was acquired as ratiometric images (340nm/380nm) every 1 second using an Olympus BX51WI microscope, a PTI IC200 intensified camera, and Imaging Workbench 2.2.1 software (INDEC BioSystems). After recording a baseline for at least 1 minute, agonists were applied for up to 30 seconds, followed by a 5 minute buffer wash. Fluorescence intensity was measured within cell bodies and expressed as change above baseline signal prior to agonist application.

b. Cyclic adenosine monophosphate (cAMP) analysis

Agonist-induced release of cAMP was measured using the CatchPoint Cyclic-AMP Fluorescence Assay kit (Molecular Devices) with some modification to manufacturer instructions. HEK 293 cells were plated at 70% confluency onto 96-well plates with DMEM-Glutamax (Gibco #10569) supplemented with 10% fetal bovine serum, 10 U/ml penicillin, and 10 μg/ml streptomycin. Cells were allowed to adhere overnight and transfected with cDNA encoding the mouse A_{2A} receptor (Open Biosystems, clone ID# 30242398, accession #BC110692) at 3:1 ratio using FuGene 6 (Roche). HEK cells were then incubated in 0.75 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma) for 10 minutes to inhibit phosphodiesterase activity. Some wells also received either adenosine deaminase (5 U/ml) or the A_{2A} receptor antagonist SCH-58261 (100 μM) during IBMX pretreatment. Indicated agonists or 30 μM forskolin (activator of adenylate cyclase) were then added for 15 minutes at 37°C. All agonists and inhibitors were diluted in HBSS containing 10 mM glucose (pH 7.4). Cells were then lysed and the lysates transferred onto goat anti-rabbit IgG-coated 96-well plates. Some wells received unconjugated cAMP instead of lysate samples in order to derive a calibration curve for each plate. Lysates and calibrators were incubated with anti-cAMP antibody and horseradish peroxidase (HRP)-conjugated cAMP for 2 hours at room temperature. After washing, the proprietary Stoplight Red substrate supplemented

with 1 mM H_2O_2 was added to all wells for 30 minutes. Fluorescence intensity in each well was detected using the FlexStation II plate reader (Molecular Devices) set to 530 nm excitation and 590 nm emission. In order to derive an approximate cAMP concentration within each cell lysate sample, a standard curve was plotted for each plate using readouts from the calibrator-containing wells.

c. Phagocytosis

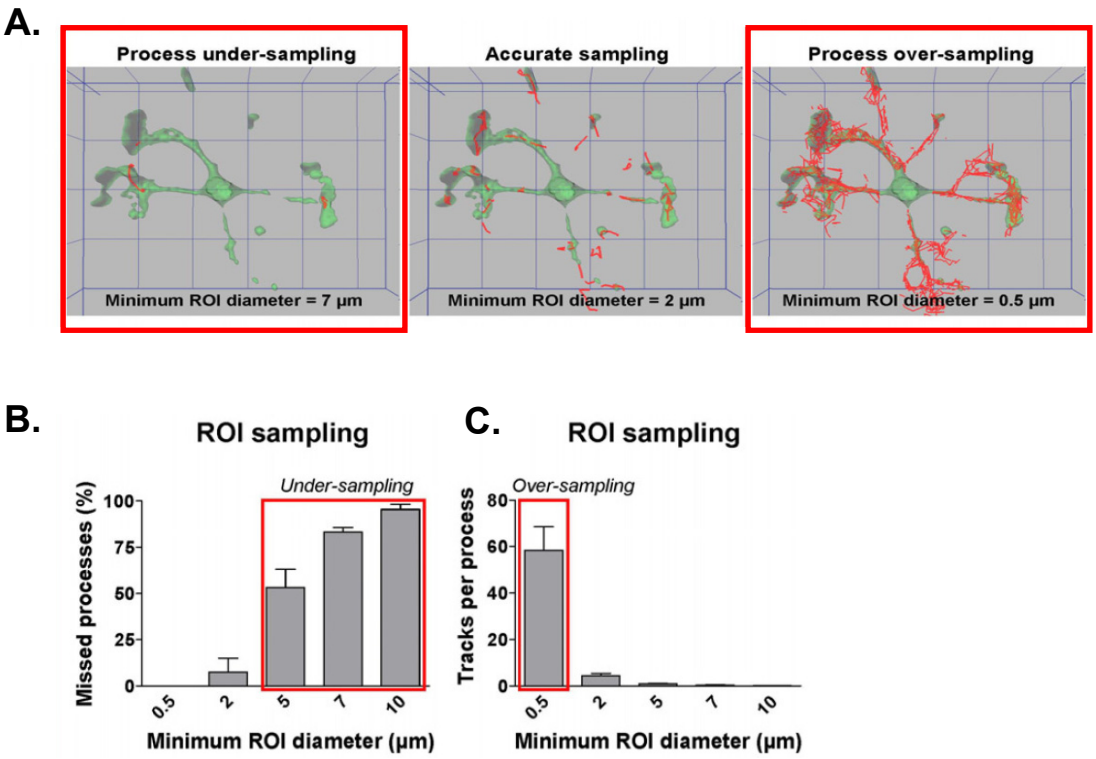
Microglial phagocytosis was analyzed using the Vybrant phagocytosis assay kit (Invitrogen) according to manufacturer instructions. Briefly, primary mouse microglia were isolated, allowed to adhere overnight to 0.05 mg/ml poly-D-lysine-coated 96-well plates, and treated with LPS (100 ng/ml) at 37ºC for 24 hours. After pre-exposure to ATP (50 uM), CGS-21680 (50 μM), or adenosine (50 μM) for 20 min, fluorescein-labeled *E. coli* bioparticles were applied for 2 hours along with agonists. After a wash in PBS, cells were examined using the FlexStation II microplate reader (Molecular Devices) at 480 nm excitation and 520 nm emission. Results were normalized to empty wells and DMEM-treated cells.

d. Actin filament staining

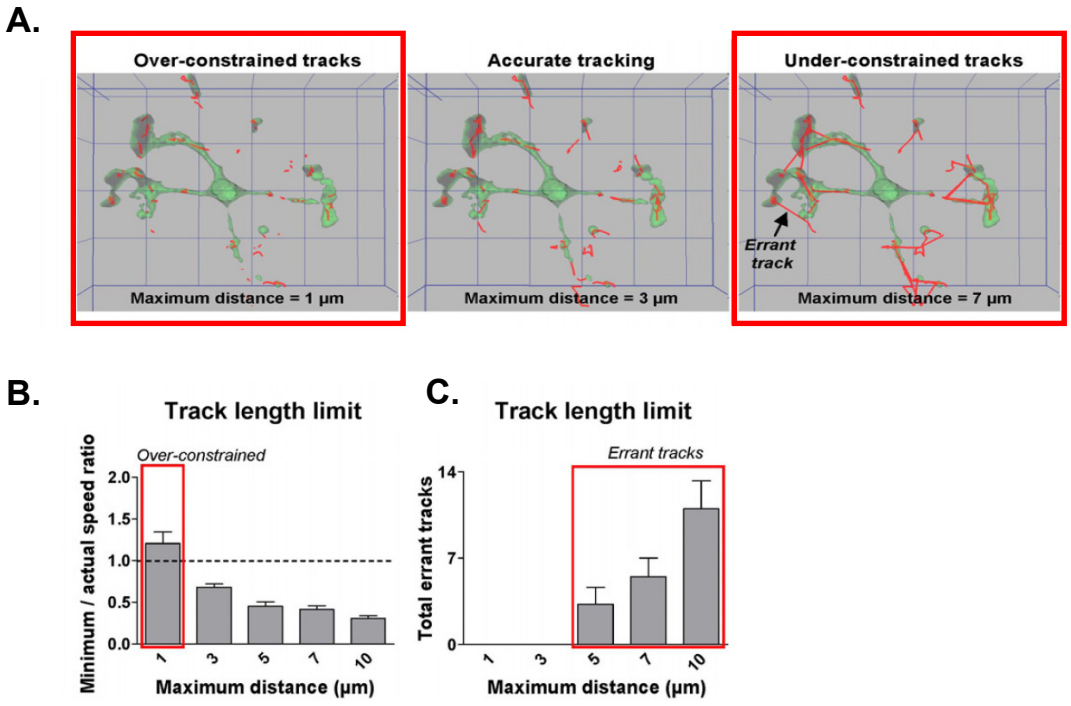
Isolated wild-type microglia plated onto 0.05 mg/ml poly-D-lysine-coated coverslips were treated with indicated agonists, fixed in 4% paraformaldehyde with 0.1% glutaraldehyde for 20 minutes at room temperature, washed in PBS for 10 minutes, and incubated for 30 minutes in 1:100 Alexa Fluor-488-conjugated phalloidin (Invitrogen) and then in 1:100 IB_4 diluted in calcium imaging buffer for 20 minutes to identify microglia. Cells were then washed in PBS and the coverslips placed onto slides with Vectashield (Vector Laboratories).

2.7. Statistics

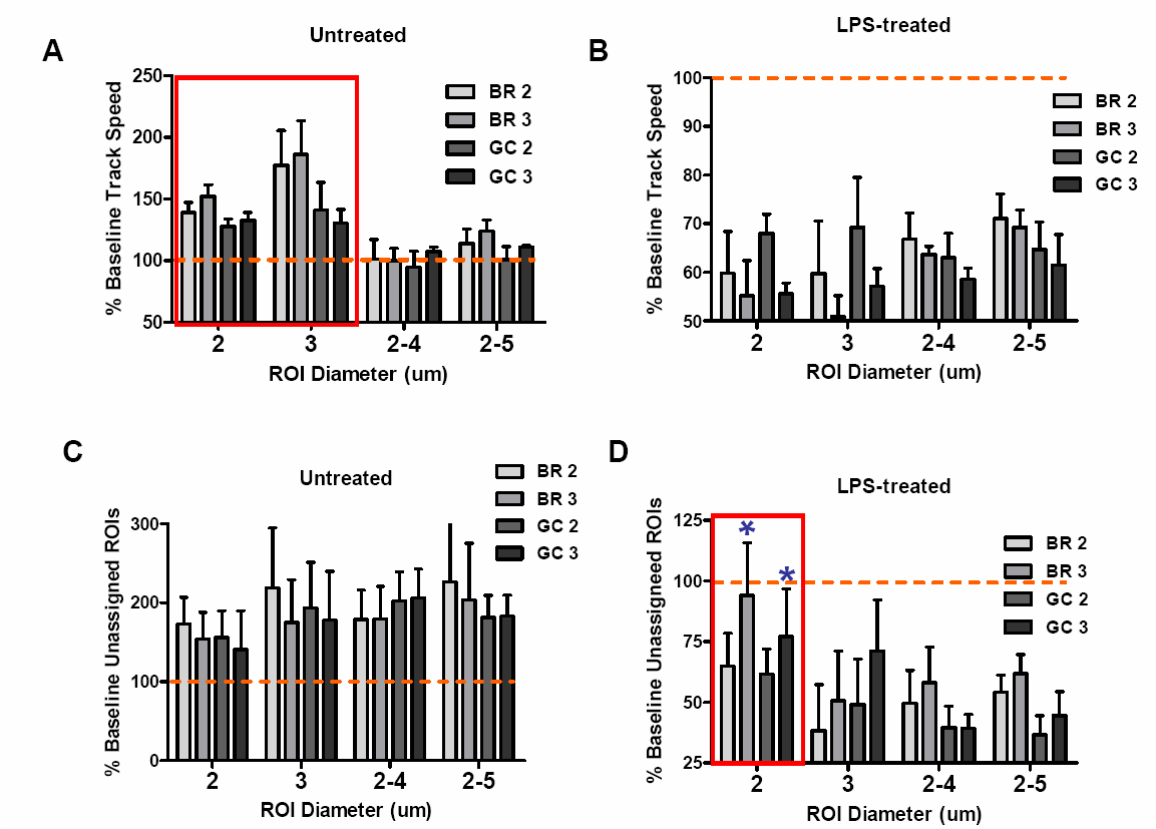
Bar graphs show mean and standard error of the mean (s.e.m.). All statistical tests were performed on raw imaging data. Average cell ramification, process track speed, and migration during baseline were compared to periods of agonist exposure using a paired Student's t-test. Analyses of different pre-treatment conditions or inhibitor effects on agonist responses were performed using repeated-measures two-way ANOVA. cAMP release was analyzed with one-way ANOVA and Dunnet's multiple comparisons post-test. Phagocytosis analysis was done using the Kruskal-Wallis test and Dunn's multiple comparisons post-test.



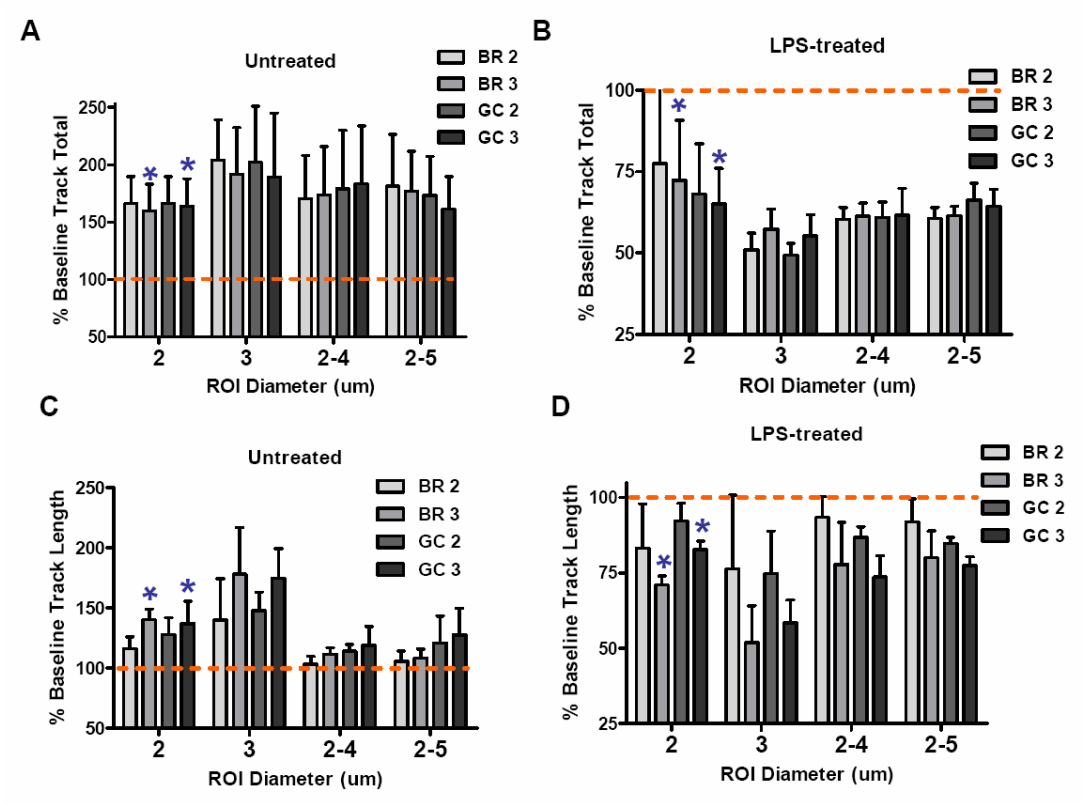
Methods Figure 1. Parameter optimization for ROI tracking I. In order to define the appropriate analysis parameters for region of interest (ROI) recognition and tracking, and evaluate errors in tracking, we evaluated a range of parameter values. A subset of those comparisons is shown. (**A**) A microglial cell is shown at one time-point reconstructed into a 3D structure (ie. isosurface) based on above-threshold fluorescence intensities. Tracks (red) were accumulated during 5 minutes based on movement of ROIs, which were defined to be 0.5 µm, 2 µm, or 7 µm minimum diameter. Setting ROI diameter too high or too low fails to accurately represent cell process motion. Inspection of tracks shows that adjusting ROI diameter to 7 µm fails to include most processes (under-sampling), while ROI of 0.5 µm creates an excessive number of tracks (over-sampling) due to recognition of noise fluctuations. Frame scale: 10 µm. (**B**) During track analysis, increasing the minimum ROI diameter increases the number of neglected cell processes (under-sampling, red box). (**C**) Setting the minimum ROI diameter too low creates too many tracks for each cell process (over-sampling, red box).



Methods Figure 2. Parameter optimization for ROI tracking II. In order to define the appropriate analysis parameters for region of interest (ROI) recognition and tracking, and assess errors in tracking, we evaluated a range of parameter values. A subset of those comparisons is shown. (A) The same cell as in Figure 1A is shown with tracks (red) accumulated over 5 minutes (based on 2 µm minimum ROI diameter). Here, we varied the maximum distance allowed for ROI to travel between consecutive time-points (ie. between two stacks). Setting the maximum travel distance too high or too low fails to accurately represent cell process motion. Inspection of tracks shows that adjusting the distance to 1 µm fails to reflect the full extent of motion (over-constrained), while setting the distance to 7 µm allows formation of errant tracks between adjacent processes (under-constrained). Frame scale: 10 µm. (**B**) During track analysis, setting the maximum ROI travel limit between consecutive time-points too low results in shortened tracks with speeds below the minimum (over-constrained, red box). To estimate the minimum speed of process motion, absolute displacement of a process was calculated manually for four processes in each cell and was divided by time. This minimum speed was divided by the average track speed derived with tracking analysis. Ratios above 1.0 reflect track speeds below the estimated minimum and are deemed over-constrained. (**C**) Setting the maximum ROI travel limit to $\geq$ 5 increases the number of incorrect tracks that form between two adjacent processes (errant tracks, red box). n = 4 for all bar graphs.



Methods Figure 3. Parameter optimization for ROI tracking III. We assessed how the ATP (20 μM) effect in untreated and LPS-treated microglia varied with changes in tracking analysis parameter values. For this, we compared agonist-induced changes in track speed and the number of unassigned ROIs (regions of interest not designated by the algorithm to any track, possibly due to rapid noise fluctuations). We aimed to minimize changes in the number of unassigned ROIs. (**A-B**) The minimum ROI diameter of 2 or 3 μm (red box) was the most sensitive to the ATP-induced increase in cell process motility in untreated microglia, while ROI diameter ranges of 2-4 and 2-5 μm exhibited little sensitivity to changes in process motility. No clear parameter effects were observed in tracking of LPS-treated cells (**B**). Brownian Motion algorithm (BR), GapClose Autoregressive algorithm (GC). Values on the upper right (2 or 3) indicate the value used for the maximum tracking distance. (**C-D**) While no apparent effects in the number of unassigned ROIs were noted in the tracking of untreated cells (**C**), assessment of ATP-induced changes in LPS-treated microglia demonstrated that minimal changes in unassigned ROIs occurred when the ROI diameter was set to 2 μm and the maximum tracking distance was set to 3 μm (red box).



Methods Figure 4. Parameter optimization for ROI tracking IV. We assessed how the agonist effect in untreated and LPS-treated microglia varied with changes in tracking analysis parameter values. Comparing ATP-induced changes in the total number of tracks and average track length in both untreated and LPS treated cells revealed no apparent advantage in the use of Brownian Motion (BR) or GapClose Autoregressive (GC) algorithms. Values on the upper right (2 or 3) indicate the value used for the maximum tracking distance.

Receptor	Primers (forward and reverse)	Size (bp)	Reference
mP2Y1	5'-ACGTCCAATGATTACCTGCG-3' 5'-CCCTGTCGTTGAAATCACAC-3'	289	*Bystrova et al., 2005*
mP2Y2	5'-TTTCCTCTTCTACACCAACCT-3' 5'-CAGCATGACGGAGCTGTAAGC-3'	280	*Bystrova et al., 2005*
mP2Y4	5'-CCTCGTCTACTACTATGCT-3' 5'-GCTACTACCAACCAAACAC-3'	222	*Bystrova et al., 2005*
mP2Y6	5'-TGCTGCCCTTCATAGCCTTAC-3' 5'-TGGAAAGGCAGGAAGCTGATGG-3'	163	*Bystrova et al., 2005*
mP2Y12	5'-CCTCAGCCAATACCACCTTCTCCCC-3' 5'-CGCTTGGTTCGCCACCTTCTTGTCCCTT-3'	1004	*Haynes et al., 2006*
mP2Y13	5'-GGGACACTCGGATGACACAGCTGC-3' 5'-GCCAGAAAGAGAGTTGCTTCTTTAGCAATAAACAGC-3'	799	*Haynes et al., 2006*
mA1	5'-GTGATTTGGGCTGTGAAGGT-3' 5'-CAAGGGAGAGAATCCAGCAG-3'	322	*Hoskin et al., 2002*
mA2a	5'-CACGCAGAGTTCCATCTTCA-3' 5'-AGCAGTTGATGATGTGCAGG-3'	497	*Hoskin et al., 2002*
mA2b	5'-GCGAATAAAAGCTGCTGTCC-3' 5'-AAAATGCCCACGATCATAGC-3'	245	*Hoskin et al., 2002*
mA3	5'-GACTGGCTTCAGAGAGACGC-3' 5'-AGGGTTCATCATGGAGTTCG-3'	202	*Hoskin et al., 2002*
mB-Actin	5'-TGACGGGGTCACCCACACTGTGCCCATCTA-5' 5'-CTAGAAGCATTGCGGTGGACGATGGAGGG-3'	660	*Bianco et al., 2005*

Methods Table 1. Primers used for running RT-PCR reactions. Isolated RNA samples derived from mouse microglial cultures were examined for relative transcript levels of specific purinergic receptors using published primer pairs. Each PCR product band size corresponded to the expected product band size. β-actin mRNA levels were used to confirm equal loading of mouse RNA between samples.

Receptor	Primers (forward and reverse)	Size (bp)	Reference
COX-2	5′-CCGTGGGGAATGTATGAGCA-3′ 5′-CCAGGTCCTCGCTTATGATCTG-3′	530	*Kyrkanides et al., 2002*
TNF-a	5′-CCCCTCAGCAAACCACCAAGT-3′ 5′-CTTGGGCAGATTGACCTCAGC-3′	373	*Tha et al., 2000*
iNOS-2	5′-CAAGAGTTTGACCAGAGGACC-3′ 5′-TGGAACCACTCGTACTTGGGA-3′	653	*Park et al., 2005*
MMP-9	5′-ATGTCACTTTCCCTTCACCT-3′ 5′-TTAGAGCCACGACCATACAG-3'	656	*Kyrkanides et al., 2002*
MCP-1	5′-CAGCAGGTGTCCCAAAGA A-3′, 5′-CTTGAGGTGGTTGTGGAAAAG -3′	234	*Kyrkanides et al., 2002*
IL-1B	5′-AATCTCACAGCAGCACATCAA-3′ 5′-AGCCCATACTTTAGGAAGACA -3′	671	*Tha et al., 2000*

Methods Table 2. Primers used for running RT-PCR reactions. Isolated RNA samples derived from mouse microglial cultures were examined for relative transcript levels of specific markers of microglial proinflammatory activation using published primer pairs. Each PCR product band size corresponded to the expected product band size. β-actin mRNA levels were used to confirm equal loading of mouse RNA between samples.

Abbreviations: COX-2: cyclooxygenase-2, TNF-a: tumor necrosis factor-α, iNOS-2: inducible nitric oxide synthase-2, MMP-9: matrix metalloproteinase-9, MCP-1: monocyte chemoattractant protein-1, IL-1B; interleukin-1β.

Receptor	Primers (forward and reverse)	Size (bp)	Reference
hA1	5'-GCCACAGACCTACTTCCACA-3' 5'-CCTTCTCGAACTCGCACTTG-3'	305	*Chen et al., 2004*
hA2a	5'-CGAATTCAACCTGCAGAACGTCACC-3' 5'-TCGAATTCGCGGTCAATGGCGATG-3'	216	*Chen et al., 2004*
hA2b	5'-CAGACGCCCACCAACTACTT-3' 5'-GCCACCAGGAAAATCTTAATG-3'	513	*Chen et al., 2004*
hA3	5'-ACCACTCACAGAAGAATATG-3' 5'-ACTTAGCCGTCTTGAACTCC-3'	328	*Chen et al., 2004*
hP2Y12	5'-CCGTCGACAATCTCACC-3' 5'-GCCCAGATGACAACAGAG-3'	442	*Feng et al., 2004*
hP2Y11	5'-CTCGGGTGCCAAGTCCTGCCC-3' 5'-CAGTCATGCCTGGGCTGCGTAG-3'	704	*Feng et al., 2004*
hGAPDH	5′- ATCACCATCTTCCAGGAGCG-3′ 5′-CCTGCTTCACCACCTTCTTG-3′	574	*Grijelmo et al., 2007*

Methods Table 3. Primers used for running RT-PCR reactions. Isolated RNA samples derived from human microglial cultures were examined for relative transcript levels of specific purinergic receptors using published primer pairs. Each PCR product band size corresponded to the expected product band size. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels were used to confirm equal loading of human RNA between samples.

CHAPTER 3: The Effects of ATP on Microglial Motility

3.1. Abstract

Recent studies have shown that microglia are highly motile in culture and *in vivo,* and exhibit continuous and rapid process motion within intact healthy brain tissue. This dynamic morphology is mediated in part by chemoattraction towards nucleotides released from surrounding cells, including ATP and ADP, which can activate a variety of purinergic receptors. Microglial chemoattraction toward ATP is mediated by the G_i-coupled $P2Y_{12}$ purinergic receptor in microglia, which triggers directional microglial motility in culture and *in vivo*. However, $P2Y_{12}$ is dramatically downregulated upon microglial proinflammatory activation and the effects of ATP in activated microglia have not been investigated. Using a novel 4D confocal imaging technique, we corroborated previous reports that the chemotactic influence of ATP is dependent on G_i-coupled signaling. We also show that G_s-coupled signaling may inhibit ATP-induced chemotaxis. Surprisingly, we found that ATP triggers process retraction and slowed process motility in activated microglia. Moreover, we observed that localized ATP application induces repulsive migration by activated microglia. Our results indicate that while ATP is a factor that attracts resting microglia, it instead repels activated microglia. To our knowledge, the ability of a chemoattractant to convert to a chemorepellant has not previously been observed in microglia. This dynamic regulation of microglial chemotaxis by ATP could be involved in various neuropathologies that involve activated microglia.

3.2. Introduction

Until recently, resting microglia in healthy brain tissue were considered to be inactive. It was assumed that these cells exhibit motility and cell chemotaxis only upon

proinflammatory activation during brain injury or disease. However, as recently shown by Davalos et al. (2005) and Nimmerjahn et al. (2005), resting microglia are highly motile and exhibit continuous process remodeling under normal conditions. Both of these studies were conducted *in vivo* using two-photon microscopy in transgenic *Cx3cr1-eGFP* mice, which exhibit microglia-specific enhanced green fluorescent protein (eGFP) labeling due to placement of the eGFP reporter gene into the locus encoding the chemokine receptor CX3CR1 (Jung et al., 2000). Transcranial time-lapse imaging within healthy intact brain tissue revealed that microglial processes are remarkably motile and undergo constant extension and retraction at a rate of 1.5 μm per minute. Interestingly, due to such dynamic motility, it was approximated that microglial processes screen the entire brain parenchyma every few hours (Nimmerjahn et al., 2005). In contrast, no rapid morphological remodeling was evident in neurons or astrocytes.

This high rate of basal motility in microglia is thought to be regulated in part by local release of extracellular ATP and/or ADP. Indeed, tissue exposure to apyrase, an ATPase that hydrolyzes both ATP and ADP, inhibits microglial process movement *in vivo* (Davalos et al., 2005). Moreover, ATP and ADP are both potent chemoattractants for microglia in culture and in slice preparations, triggering dramatic membrane remodeling and directional migration (Honda et al., 2001; Haynes et al., 2006; Kurpius et al., 2007). This chemotactic response to purinergic stimulation is mediated by the G_i-coupled $P2Y_{12}$ receptor (Honda et al., 2001; Haynes et al., 2006). However, Haynes et al. (2006) recently established both *in vivo* and in hippocampal slices that $P2Y_{12}$ is dramatically downregulated to undetectable levels upon microglial activation with LPS or upon traumatic tissue injury. Notably, the decline in microglial $P2Y_{12}$ receptor expression correlated with the retraction of microglial processes and adoption of the amoeboid morphology characteristic of activated microglia (Haynes et al., 2006). The basis for this relationship and the triggers of microglial

process retraction have not been revealed. It also remains unknown whether activated microglia, without expression of $P2Y_{12}$, still exhibit a chemotactic response to ATP or ADP. In fact, microglial chemotactic responses to purines have not been examined in the context of inflammation. Given that most neuropathologies are characterized by ongoing neuronal damage in the presence of activated microglia, examination of microglial motility in their activated state may reveal new information about microglial function during neuroinflammation.

Here, we investigated the morphological and chemotactic ATP responses in both resting and activated microglia. Using isolated microglia and time-lapse three-dimensional (3D) confocal imaging, we were able to quantify changes in microglial cell structure in response to agonist exposure and examine the intracellular pathways mediating these effects. We were also able to detect changes in the motile responses by microglial cell processes as well as track microglial migratory activity.

3.3. Results

a. Process ramification in resting microglia

We first established a novel time-lapse 3D confocal imaging method and verified its ability to detect changes in cellular morphology and motility (*see Methods*). For this, we isolated microglia from transgenic *Actin-eGFP* mice, which exhibit widespread eGFP fluorescence in most cell types, and plated the cells in a 3D matrix. This culture technique allowed microglia to adhere, ramify, and migrate within a 3D space. Following a 24-hour incubation of eGFP-positive isolated mouse microglia within the matrix, coverslips was placed in a perfusion chamber for time-lapse 3D imaging, which was performed by continuously acquiring image stacks spanning the entire z-axis of one microglial cell within the matrix.

During image acquisition, perfusion with buffer for five minutes was followed by application of 20 μM ATP for five minutes and then a return to buffer solution.

After image processing and reconstruction of image stacks into 3D cell structures (*see Methods*), we quantified cell surface area and cell volume within each z-stack before, during, and after agonist application. These measurements allowed the study of changes in microglial cell morphology upon agonist exposure. As shown in Figures 1 and 2, microglial exposure to ATP triggered a rapid and reversible increase in cell surface area, as well as a slower decline in cell volume. These quantitative changes are reflective of symmetrical microglial membrane extension upon ATP application. In addition, we measured ATP-induced changes in microglial cell process motility. This was assessed by tracking regions of interest (ROIs) within cell processes and then evaluating changes in the average ROI speed upon agonist application. As shown in Figure 3, microglial cell process speed was enhanced upon ATP exposure.

Next, we examined whether the ATP-induced microglial cell process extension was mediated by $G_{i/o}$-coupled receptor signaling, as reported by Honda et al. (2001). For this, we pretreated microglia with pertussis toxin (PTX) for 24 hours, which is known to ADP-ribosylate the α subunit of $G_{i/o}$ proteins and interrupt their association with G protein-coupled receptors. As expected, following PTX exposure, ATP no longer caused an increase in microglial cell surface area (Figure 4). In fact, there was a moderate, but consistent decline in cell surface area, reflective of process retraction. These results further support that $G_{i/o}$ signaling mediates ATP-induced process extension in microglia.

Given that G_s-coupled signaling activates adenylate cyclase and thereby may antagonize the actions of G_i, we next asked whether G_s is also involved in regulating microglial morphological responses to ATP exposure. Indeed, G_s-coupled signaling can inhibit motility in select cell types (Nagasawa et al., 2005). In order to target $G\alpha_s$, we utilized cholera toxin

(CLX), which is known to ADP-ribosylate and thereby constitutively activate $G\alpha_s$, resulting in intracellular cAMP accumulation. We found that the effect of ATP on microglial morphology was blunted following CLX pretreatment (Figure 5), indicating that $G\alpha_s$ signaling may inhibit ATP-induced microglial process extension upon ATP stimulation.

We also investigated whether microglial pre-exposure to PTX or CLX itself caused morphological changes in microglia. As shown in Figure 6, CLX induced a decline in both surface area and volume in microglia, reflecting an overall cell shrinkage. However, PTX had only a modest effect on these parameters. These findings suggest that activation of $G\alpha_s$ inhibits microglial cell spreading and process ramification.

Together, our initial analyses using four-dimensional imaging corroborate previous reports indicating that ATP triggers microglial process extension and increased motility, and that PTX-sensitive $G_{i/o}$ mediates these effects in microglia (Honda et al., 2001). We also found that $G\alpha_s$ signaling may play an inhibitory role in this response. Importantly, by using ATP, an agonist known to trigger robust morphological changes in microglia, and PTX, which is known to inhibit these responses, we have further confirmed that our four-dimensional image analyses are sensitive to changes in cell morphology and process motility.

b. Characterization of isolated microglia

Most types of CNS injury or disease processes trigger microglia to undergo a transformation from resting to a reactive pro-inflammatory phenotype. This activated microglial state is associated with increased expression of numerous pro-inflammatory factors both in culture and *in vivo*. Importantly, microglia are thought to be uniquely sensitive to changes in the microenvironment, which allows a rapid immune response in the brain upon tissue disturbance or abnormal cellular function. In light of extensive tissue disturbance that takes place during the process of microglial isolation from the brain, we next determined

whether isolated microglia utilized in our experiments exhibited a phenotype that mimics an activated state.

We utilized RT-PCR to examine the mRNA expression level of six factors known to be upregulated upon microglial proinflammatory activation *in vivo,* including inducible nitric oxide synthase 2 (iNOS2), interleukin-1β (IL-1β), cyclooxygenase-2 (COX-2), tumor necrosis factor-α (TNF-α), metalloproteinase-9 (MMP-9), and monocyte chemoattractant protein-1 (MCP-1) (Choi et al., 2003; Hamill et al., 2005). Figure 7 shows that the isolated microglia used in our experiments exhibited negligible levels of mRNA encoding these factors, suggesting that microglia were minimally activated. Importantly, pre-treatment of microglia with LPS for 24 hours triggered robust upregulation of these activation markers, indicating that the cultured cells were effectively transformed into an activated phenotype.

c. Process retraction in activated microglia

We next examined ATP-induced morphological responses in activated microglia. For this, we pretreated isolated microglia for 24 hours with agonists of different pattern recognition receptors, including LPS (activator of TLR4), lipoteichoic acid (LTA, activator of TLR2), or unmethylated CpG motif-containing oligonucleotides (CpG, activator of TLR9). We also examined microglia activated with TNF-α, a proinflammatory cytokine released during most types of brain injuries (Wang and Shuaib, 2002).

Surprisingly, we found that ATP causes process retraction in LPS-activated microglia (Figures 8 and 9), which is a response opposite from that observed in resting cells. Specifically, in LPS-activated microglia, ATP triggered a decline in microglial cell surface area without affecting cell volume (Figure 9). Moreover, microglial activation with the other three activating agents, including LTA, CpG, or TNF-α, triggered a similar switch in the morphological response of microglia to ATP. Namely, while resting microglia showed an

ATP-induced rise in cell surface area, reflecting process extension, microglia activated with LTA, CpG or TNF-α showed an ATP-induced decline in cell surface area (Figures 10-12). Thus, pre-exposure to proinflammatory activators switches microglial morphological ATP response from process extension to process retraction. These results are summarized in Figure 13, where cell surface area-to-volume ratio serves as a measure of microglial ramification. While ATP increases process ramification in control (ie. resting) microglia, ATP causes membrane withdrawal, and thus a decrease in ramification, in activated microglia. Moreover, four-dimensional tracking of processes in activated microglia before and during ATP exposure revealed that the speed of process motion was decreased by ATP application (Figure 14). Thus, similar to the opposing effects of ATP on process morphology, this agonist also had the opposite effect on process motility in activated microglia.

ATP is one of many known chemoattractants that stimulates microglial motility (Honda et al., 2001; Davalos et al., 2005; Haynes et al., 2006). Given that we observed two opposing responses to ATP in resting and activated microglia, we examined whether other chemoattractants known to cause microglial process extension, such as complement 5a (C5a, Nolte et al., 1996), would instead trigger process retraction in activated microglia. As expected, exposure to C5a caused a robust increase in process ramification in resting microglia (Figure 15). However, C5a also enhanced ramification in LPS-activated microglia, suggesting that the shift to retractile responses is specific to purinergic stimulation.

d. Microglial chemotaxis

As mentioned above, ATP is a chemoattractant and thus stimulates not only process extension, but, if applied as a gradient, it can also trigger cell migration in the direction of increasing agonist concentration. Indeed, ATP triggers migration in monocytes, dendritic cells, oligodendrocyte progenitor cells, and microglia (Honda et al., 2001; Idzko et al., 2002;

Agresti et al., 2005; Kaufmann et al., 2005). Because we observed opposite effects of ATP on microglial morphology and process motility, we asked whether the same opposing effects would be evident in microglial migratory responses to ATP.

In order to examine whether untreated and activated microglia migrate differently toward ATP, we again performed 4D confocal imaging in isolated microglia. However, instead of exposing cells to 20 μM bath-applied ATP, which results in roughly uniform agonist distribution, we released the agonist in a localized manner using iontophoresis through a micropipette tip. Iontophoresis is a technique whereby an electrical charge is applied onto a similarly charged substance contained in a micropipette. This creates a repulsive driving force and expels the agonist from the pipette. Moreover, application of an opposite charge creates an attractive force and prevents agonist outflow, which enables control of agonist discharge.

For our studies, the micropipette was filled with 0.5 mM ATP. After a 5-10 minute baseline, during which a positive charge was applied to contain ATP in the micropipette, we applied a negative charge to expel ATP. The agonist was released for at least 30 minutes at a distance of 30-50 μm from a resting microglia. Thereafter, we assessed the migratory direction and displacement of microglia before and during localized ATP release. As expected, application of an ATP gradient onto resting microglia triggered asymmetric process extension and rapid migration of microglia toward the pipette tip (Figure 16a-c). However, local ATP release triggered LPS-activated microglia to migrate away from the pipette (Figure 16d-f), revealing an opposite chemotactic effect of ATP on resting and activated microglia. Thus, proinflammatory microglia exhibit repulsion from ATP, a well-known chemoattractant.

3.4. Discussion

In the CNS, ATP is normally released by various cell types, including neurons, astrocytes and microglia, and thus serves as a ubiquitous extracellular signaling factor

(Fields and Burnstock, 2006). Indeed, astrocytes utilize ATP signaling to propagate intercellular calcium waves (Anderson et al., 2004). Astrocytic ATP release also triggers calcium influx in microglia and can lead to microglial apoptosis with repeated stimulation (Verderio and Matteoli, 2001; Schipke et al., 2002). Moreover, tissue injury or hypoxia can trigger massive nucleotide release in the brain (Zimmermann, 1994; Lutz and Kabler, 1997; Darby et al., 2003; Parkinson and Xiong, 2004; Gourine et al., 2005).

Microglia possess a wide range of purinergic receptors, which can induce a variety of responses, including increases in intracellular calcium (Toescu et al., 1998; Light et al., 2006), induction of potassium currents (Boucsein et al., 2003), and secretion of cytokines (Hide et al., 2000). Indeed, microglia are thought to express several metabotropic purine receptors (Fields and Burnstock, 2006). This suggests that purines play numerous roles in modulating microglial function in health and disease. Here we studied the regulation of microglial morphology, process motility, and chemotaxis by ATP. To our surprise, we found that ATP is a dynamic chemotactic regulator that can affect microglial morphology and motility in opposite ways depending on the activation state of microglia. Specifically, our results indicate that while ATP is a factor that attracts resting microglia, it instead repels activated microglia.

While a large variety of chemoattractants are known to affect immune cell motility, only few chemorepellents have been identified. In the immune system, the known repellants include interleukin-8, which acts on neutrophil chemotaxis via a $G\alpha_i$-coupled pathway (Tharp et al., 2006) and the chemokine stromal-derived factor (SDF)-1, which induces thymocyte emigration from the thymus (Vianello et al., 2005). In the developing CNS, ephrin-A ligands, slits, and semaphorins are agonists known to repel or collapse axonal growth cones (Messersmith et al., 1995; He and Tessier-Lavigne, 1997; Kidd et al., 1999; Toyofuku et al., 2005). Interestingly, netrins are a family of proteins that exhibit

bifunctional effects, serving as chemoattractants or a chemorepellents for developing neurons (Colamarino and Tessier-Lavigne, 1995; Varela-Echavarria et al., 1997; Barallobre et al., 2005). Netrin-1 is also a chemorepellent for oligodendrocyte precursor cells (Jarjour et al., 2003). The effect of netrin-1 on a given cell type may be determined by the specific receptor expressed by that cell (Barallobre et al., 2005). Likewise, it is possible that the opposing chemotactic effects of ATP observed in microglia may be dependent on changes in receptor expression during microglial proinflammatory activation.

We tested a variety of microglial activators to verify that the shift to repulsive responses by microglia was not specific to any one proinflammatory stimulus. Indeed, the effect was translated across all the tested microglial activators. We examined agonists for three of the nine known TLRs expressed by microglia. TLR4, in conjunction with CD14, recognizes LPS, but may also bind non-pathogenic substrates and induce neurotoxicity (Block et al., 2007). Indeed, TLR4 is upregulated in the CNS during inflammation and exacerbates injury (Caso et al., 2007 and 2008; Walter et al., 2007), and may also contribute to neuropathy after nerve transection (Tanga et al., 2005). While it is not yet known how TLR4 contributes to neuroinflammation in the absence of microbial invasion, it has been suggested that TLR4 binds gangliosides, which are components of neuronal cell membranes (Jou et al., 2006). Likewise, TLR2 may promote neuroinflammation and cell loss associated with Alzheimer's disease and cerebral ischemia (Babcock et al., 2006; Ziegler et al., 2007; Richard et al., 2008). Thus, while TLRs are primarily known for their recognition of pathogens, they may play important roles in CNS injury and disease.

We also tested microglial ATP responses after exposure to TNF-α, a pro-inflammatory cytokine released primarily from activated microglia (Hehlgans and Pfeffer, 2005). Serving as a pro-apoptotic cytokine that also stimulates the release of other cytokines, TNF-α is involved in the pathophysiology of numerous conditions, including

multiple sclerosis, epilepsy, Parkinson's and Alzheimer's diseases, as well as ischemic and traumatic brain injury (Turrin and Rivest, 2004; Morganti-Kossmann et al., 2007; Pan and Kastin, 2007; Sriram and O'Callaghan, 2007; Tweedie et al., 2007). Given that TNF-α is upregulated in various brain injury contexts, our discovery of a chemotactic shift in microglia following TNF-α exposure suggests that a variety of neuropathological conditions may involve this alteration of microglial chemotactic function.

While it is expected that microglial activation *in vivo* during neuronal injury or disease progression differs from the responses induced in microglial culture using TLR agonists or cytokines, isolated culture systems are important for understanding the signaling pathways mediating microglial responses to specific agonists. Importantly, by identifying the factors and mechanisms regulating microglia during activation in an isolated system, we begin to understand the mechanisms that regulate neurotoxicity in the brain during injury and disease. We verified that the isolated microglia utilized in our imaging studies indeed undergo proinflammatory activation by assessing whether signaling factors common to brain injury and disease were low at baseline and high following LPS exposure. As expected, we found that several known markers of CNS inflammation were low in untreated microglia, but were increased in LPS-stimulated cells, including iNOS2, IL-1β, COX-2, TNF-α, MMP-9, and MCP-1.

Using 4D confocal imaging, we corroborated previous reports of ATP triggering microglial ramification and process motility through the $G\alpha_i$-coupled pathway (Honda et al., 2001). Moreover, we show that $G\alpha_s$-coupled signaling may antagonize this effect and block motile responses. While the regulation of microglial motility by the $G\alpha_s$ pathway has not been previously examined, it has been shown that elevation in intracellular cAMP levels or activation of PKA modulates cytokine production and proliferation in microglia (Tomozawa

et al., 1995; Zhang et al., 2002; Facchinetti et al., 2003; Woo et al., 2003; Min et al., 2004; Noda et al., 2007).

In summary, using a novel technique involving 4D confocal imaging, we discovered that ATP, a chemoattractant for resting microglia, transforms into a chemorepellent for activated microglia. Specifically, we observed that ATP triggers process retraction, inhibits process motility, and induces repulsive chemotaxis in activated microglia. However, the signaling mechanisms mediating this chemotactic switch and the possible consequences of this phenomenon remain unexamined.

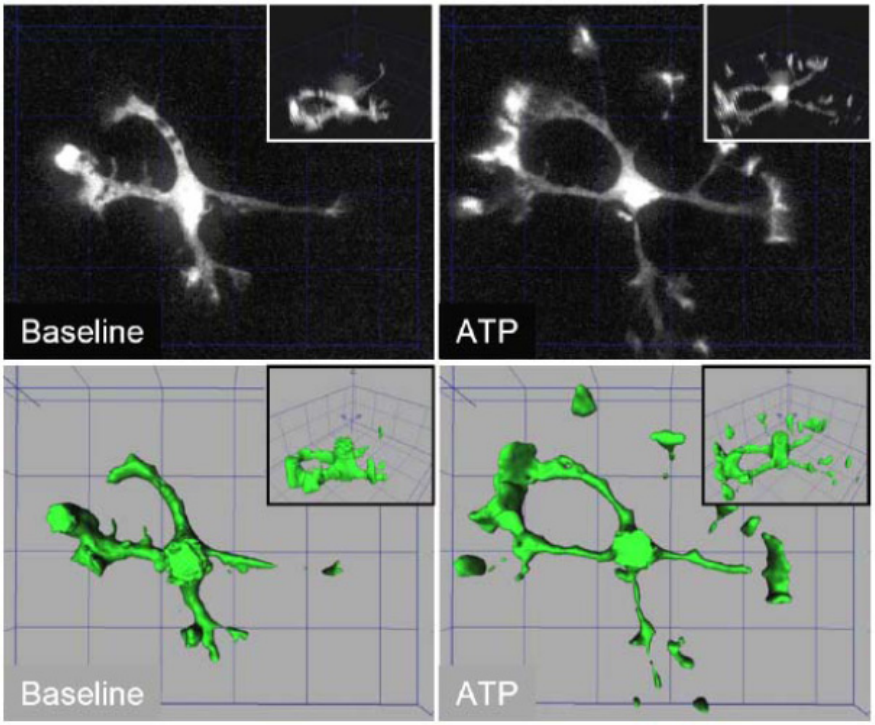


Figure 1. Time-lapse three-dimensional (3D) confocal imaging of microglial morphological responses to ATP. Isolated microglia from transgenic *Actin-eGFP* mice were plated in a 3D matrix. Microglial cell volume (*top row*) and reconstructed surface (*bottom row*) during baseline and bath application of ATP are shown (ATP: 20 μM, 5 min, grid square = 10 μm^2). Insets show side views.

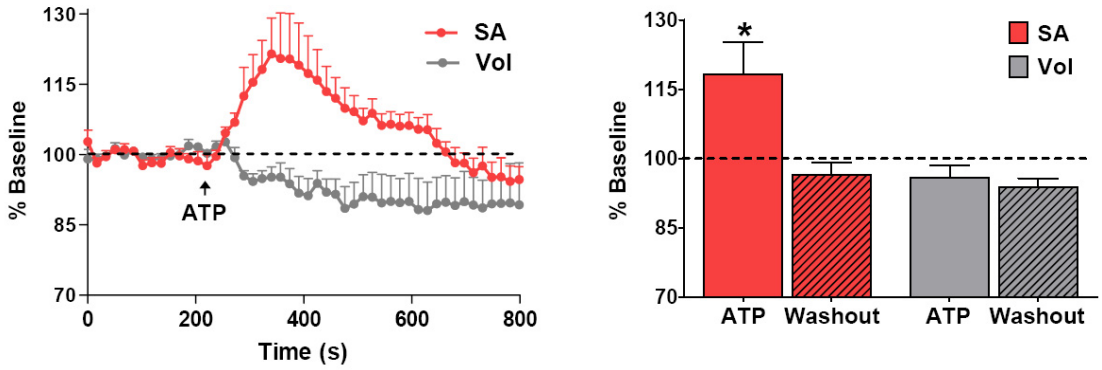


Figure 2. ATP-induced cell process extension in isolated microglia. ATP triggered an increase in microglial cell surface area (SA), but not cell volume (Vol). Following time-lapse 3D imaging, microglial cell surface area and cell volume within each z-stack before, during, and after ATP (20 μM, 5 min) application was measured. Average values during agonist application and washout are shown at right. *$p < 0.05$ vs. baseline. Graphs show mean + s.e.m.

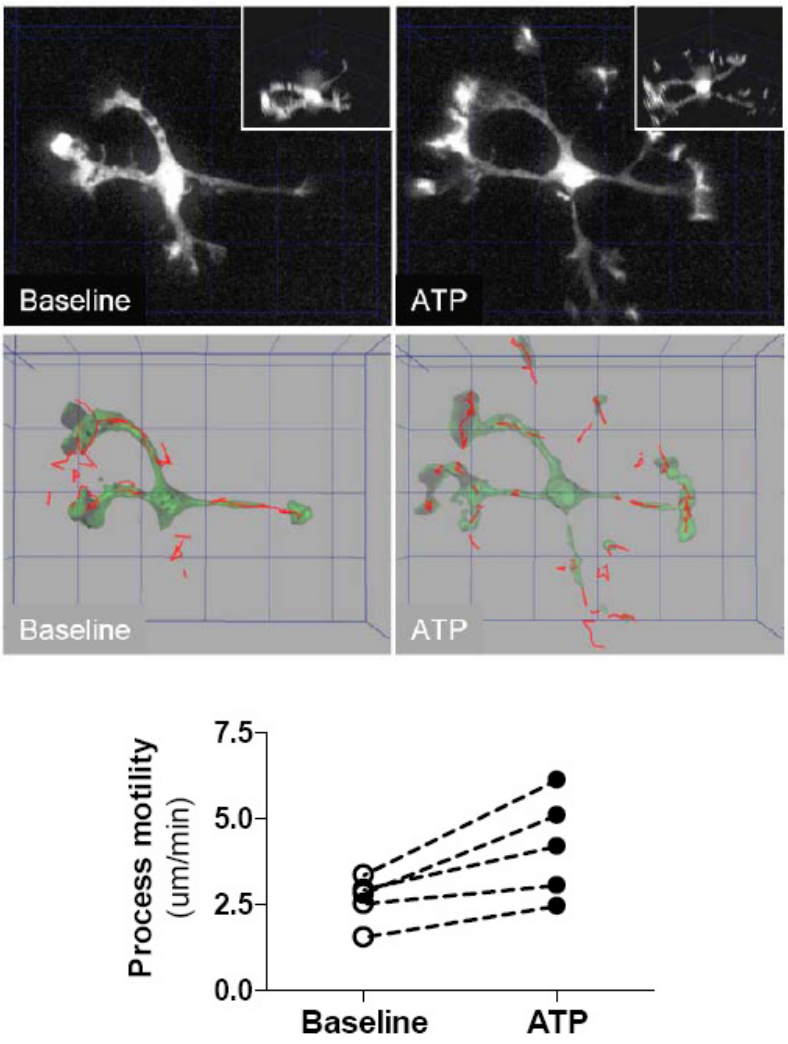


Figure 3. ATP-induced increase in cell process motility in isolated microglia. Images show isolated microglia from transgenic *Actin-eGFP* mice plated in a 3D matrix. Microglial cell volume (*top row*) and tracked process movement (*bottom row*) during baseline and bath application of ATP are shown (ATP: 20 μM, 5 min, grid square = 10 μm^2). Insets show side views. *Bottom graph*: Following time-lapse 3D imaging, average microglial cell process speed before and during ATP application is shown ($n = 5$, $p < 0.05$).

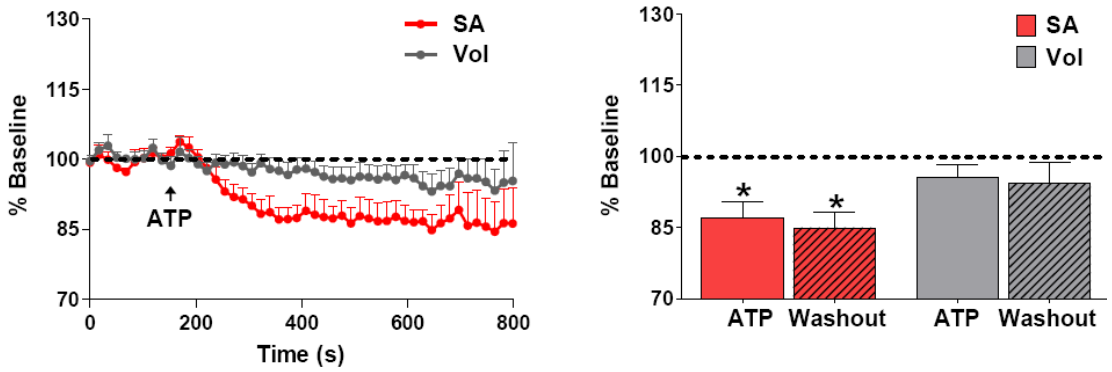


Figure 4. Inhibition of $G\alpha_{i/o}$ signaling suppresses ATP-induced process extension in microglia. Isolated microglia pre-treated with pertussis toxin (PTX, 100 ng/ml, 24 hrs) showed a decline in cell surface area (SA) upon exposure to 20 μM ATP, with no change in cell volume (Vol). Average values during agonist application and washout are shown at right. n = 5; *p < 0.05 vs. baseline. Graphs show mean + s.e.m.

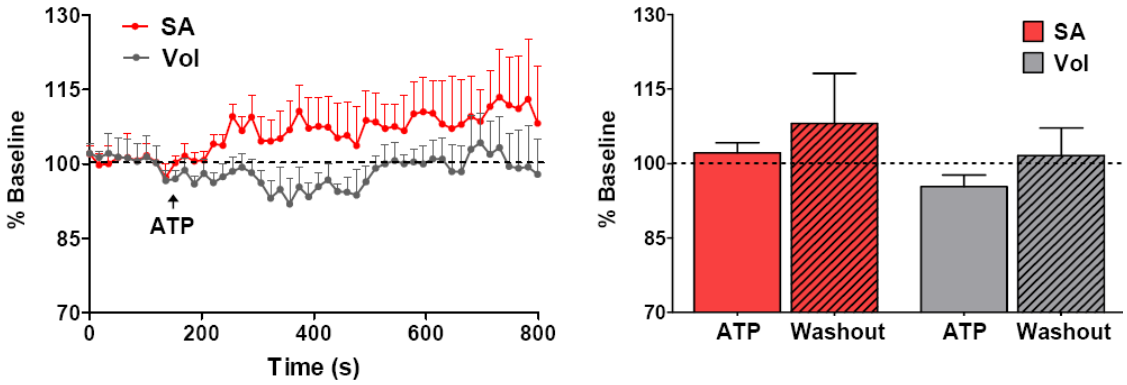


Figure 5. Activation of $G\alpha_s$ signaling suppresses ATP-induced changes in microglial cell morphology. Isolated microglia pre-treated with cholera toxin (CLX, 100 ng/ml, 24 hrs) showed minimal change in surface area (SA) and volume (Vol) in response to 20 µM ATP. Average values during agonist application and washout are shown at right. n = 5. Graphs show mean + s.e.m.

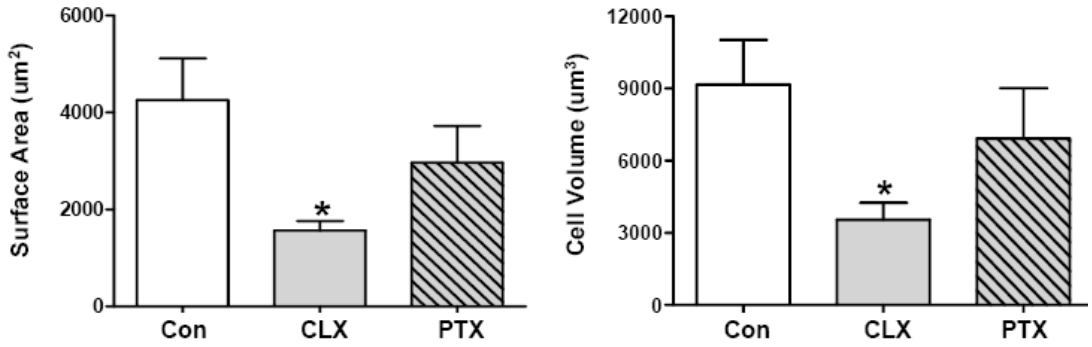


Figure 6. The effects of cholera toxin (CLX) and pertussis toxin (PTX) treatment on microglial cell morphology. CLX (100 ng/ml, 24 hrs) induced a decline in both surface area and volume in microglia, reflecting overall cell shrinkage, as compared to untreated microglia (Con). However, PTX (100 ng/ml, 24 hrs) had no effect. *$p < 0.05$ vs. untreated cells. Graphs show mean + s.e.m.

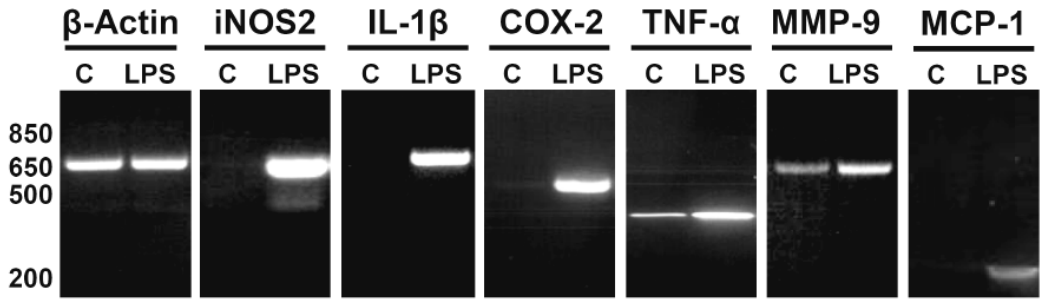


Figure 7. Untreated microglia exhibit low expression of activation markers. RT-PCR analysis of isolated mouse microglia shows that control microglia (C) exhibit low mRNA levels for known proinflammatory factors, as indicated. Upon LPS treatment (100 ng/ml, 24 hrs), microglia show robust upregulation of these factors.

Abbreviations: inducible nitric oxide synthase-2 (iNOS2), interleukin-1β (IL-1β), cyclooxygenase-2 (COX-2), tumor necrosis factor-α (TNF-α), matrix metalloproteinase-9 (MMP-9), monocyte chemoattractant protein-1 (MCP-1).

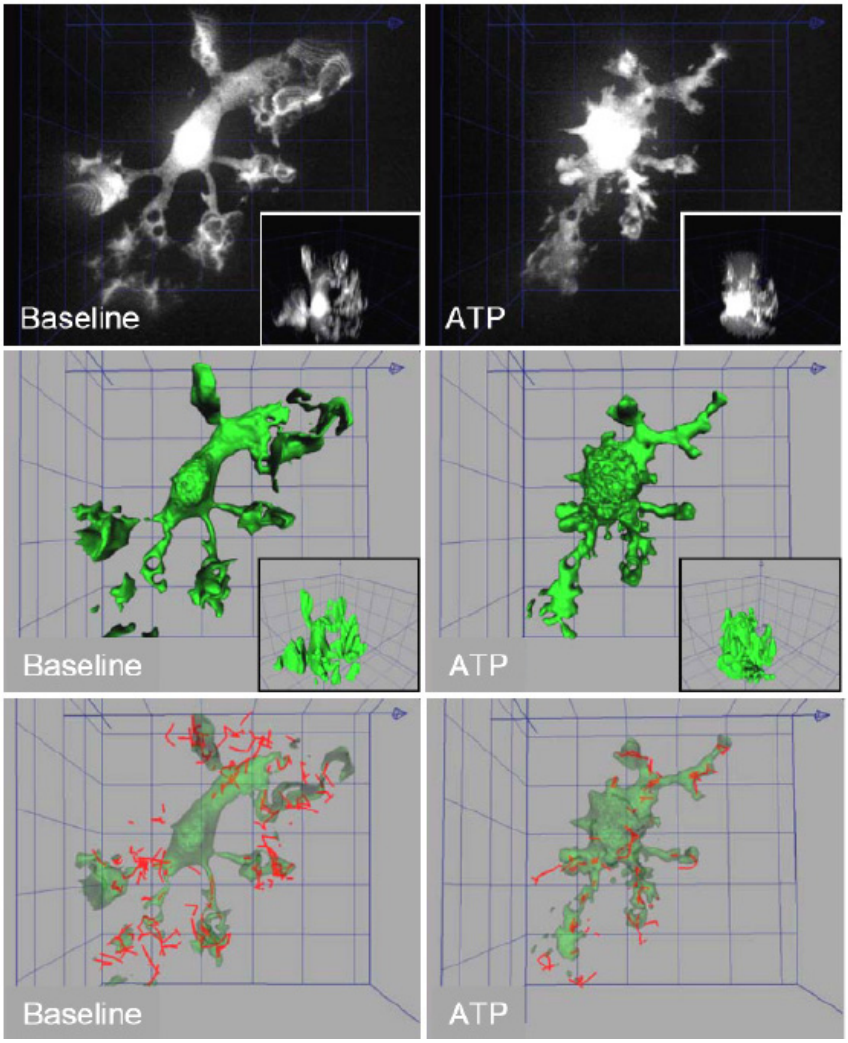


Figure 8. Three-dimensional imaging of LPS-treated microglia. ATP (20 μM) triggers retraction of cell processes. Microglial 3D volume (*top row*), reconstructed surface (*middle row*), and tracked process movement (*bottom row*) during baseline and during ATP exposure (grid square = 10 μm^2). Insets show side views.

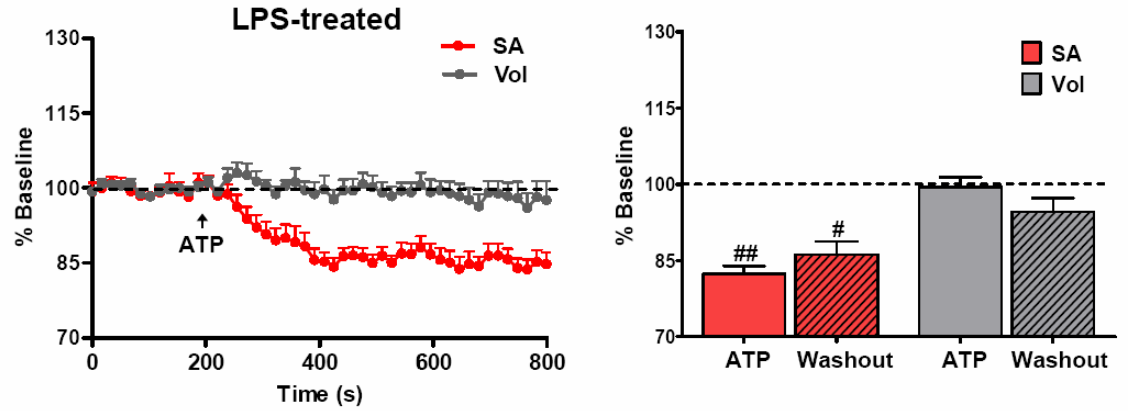


Figure 9. LPS-treated microglia exhibit process retraction in response to ATP. Isolated microglia pre-treated with lipopolysaccharide (LPS, 100 ng/ml, 24 hrs) showed a decline in cell surface area (SA) upon exposure to 20 μM ATP, with no change in cell volume (Vol). Average values during agonist application and washout are shown at right. n = 7; #p < 0.01, ##p < 0.001 vs. baseline. Graphs show mean + s.e.m.

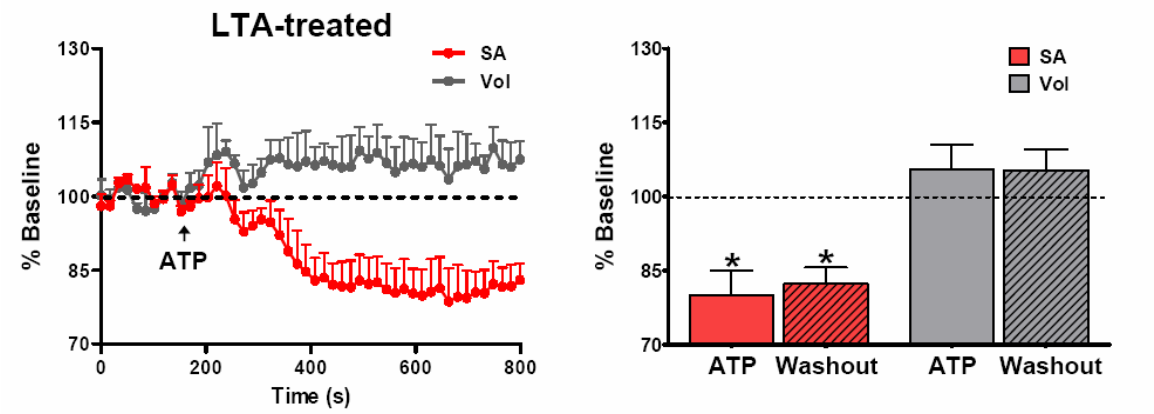


Figure 10. LTA-treated microglia exhibit process retraction in response to ATP. Isolated microglia pre-treated with lipoteichoic acid (LTA, 10 µg/ml, 24 hrs) showed a decline in cell surface area (SA) upon exposure to 20 µM ATP, with minimal change in cell volume (Vol). Average values during agonist application and washout are shown at right. n = 3; *$p < 0.05$ vs. baseline. Graphs show mean + s.e.m.

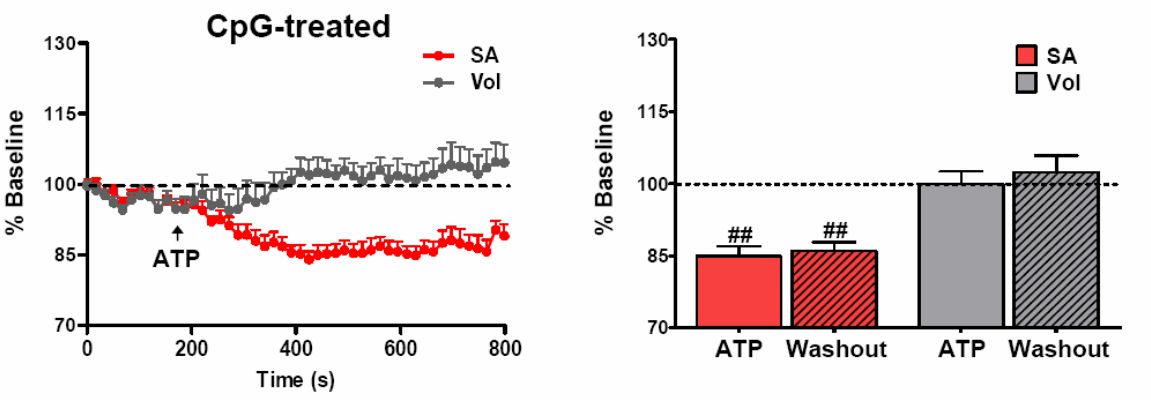


Figure 11. CpG-treated microglia exhibit process retraction in response to ATP. Isolated microglia pre-treated with unmethylated CpG motif-containing oligonucleotides (CpG, 10 µM, 24 hrs) showed a decline in cell surface area (SA) upon exposure to 20 µM ATP, with minimal change in cell volume (Vol). Average values during agonist application and washout are shown at right. n = 9; ##p < 0.001 vs. baseline. Graphs show mean + s.e.m.

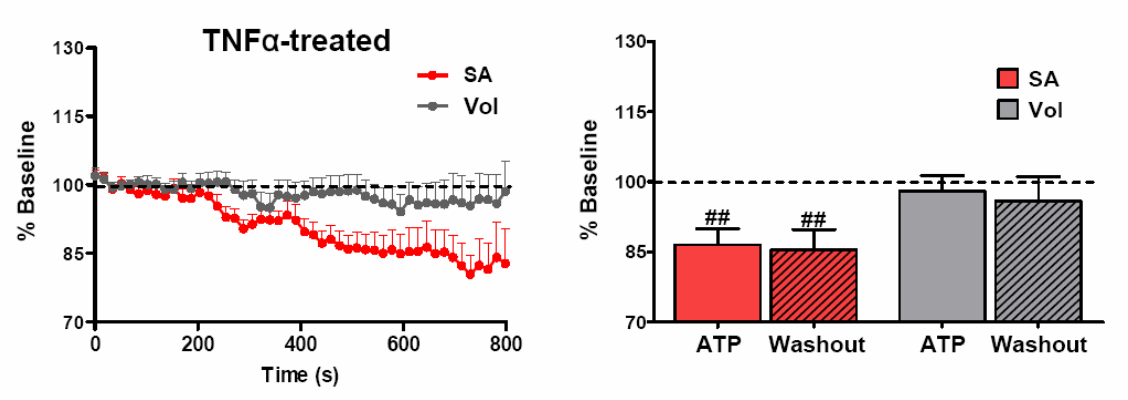


Figure 12. TNF-α-treated microglia exhibit process retraction in response to ATP. Isolated microglia pre-treated with tumor necrosis factor-α (TNF-α, 20 ng/ml, 24 hrs) showed a decline in cell surface area (SA) upon exposure to 20 μM ATP, with minimal change in cell volume (Vol). Average values during agonist application and washout are shown at right. n = 9; ##$p < 0.001$ vs. baseline. Graphs show mean + s.e.m.

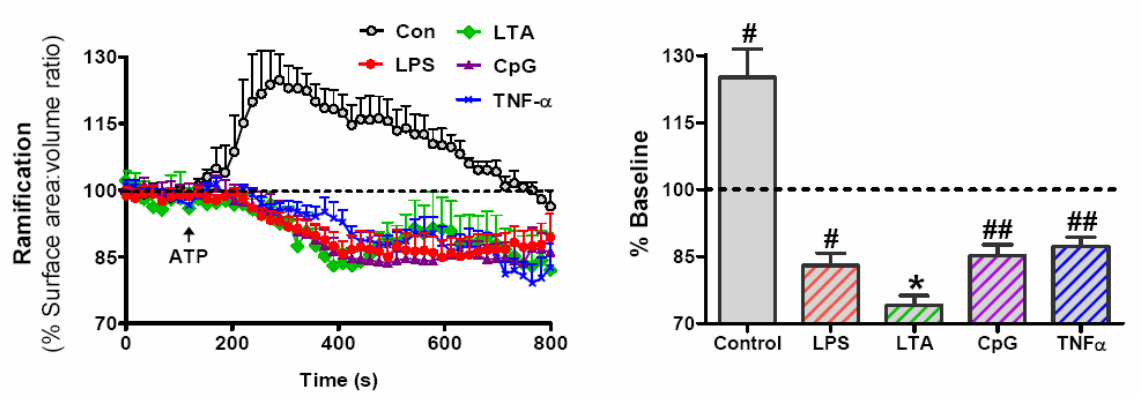


Figure 13. ATP induces process retraction in activated microglia. ATP (20 μM) increased process ramification (quantified as the ratio of cell surface area-to-cell volume) in untreated microglia (Con, n = 6), but caused process retraction in microglia pre-treated with various activators (LPS: 100 ng/ml, n = 7, LTA: 10 μg/ml, n = 3; CpG: 10 μM, n = 9; TNF-α: 20 ng/mL, n = 10). *p < 0.05, #p < 0.01, ##p < 0.001 vs. baseline. Graphs show mean + s.e.m.

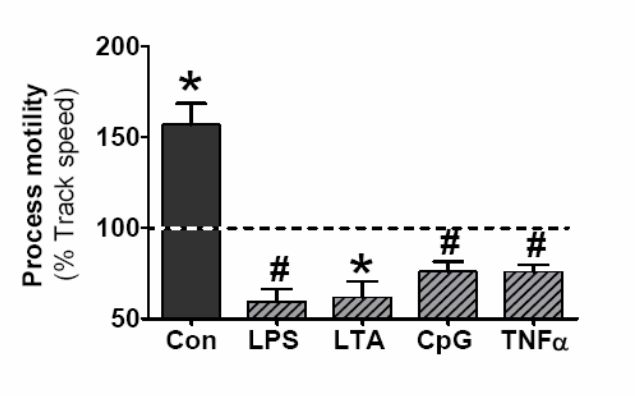


Figure 14. ATP inhibits process motion in activated microglia. While ATP (20 μM) increased process motility in untreated microglia (Con), it decreases process motility in microglia pre-treated with the indicated activators for 24 hrs (LPS: 100 ng/ml, LTA: 10ug/ml, CpG: 10 μM, TNF-α: 20 ng/ml, n = 6-8). *$p < 0.05$, #$p < 0.01$, vs. baseline. Graph shows mean + s.e.m.

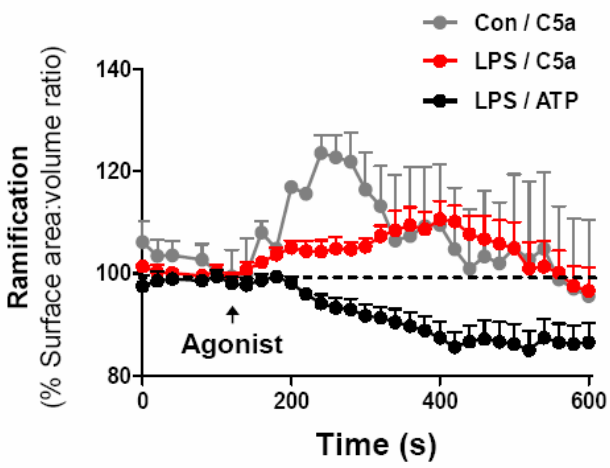


Figure 15. Microglial shift to retractile responses is specific to purinergic stimulation. While ATP (20 μM) triggers a decline in process ramification in LPS-treated microglia (LPS/ATP, n = 7), complement factor 5a (C5a, 20 nM) increases ramification in both untreated (Con/C5a: n = 3, $p < 0.05$, compared to baseline) and LPS-treated microglia (LPS/C5a: n = 8, $p < 0.05$, compared to baseline). Graph shows mean + s.e.m.

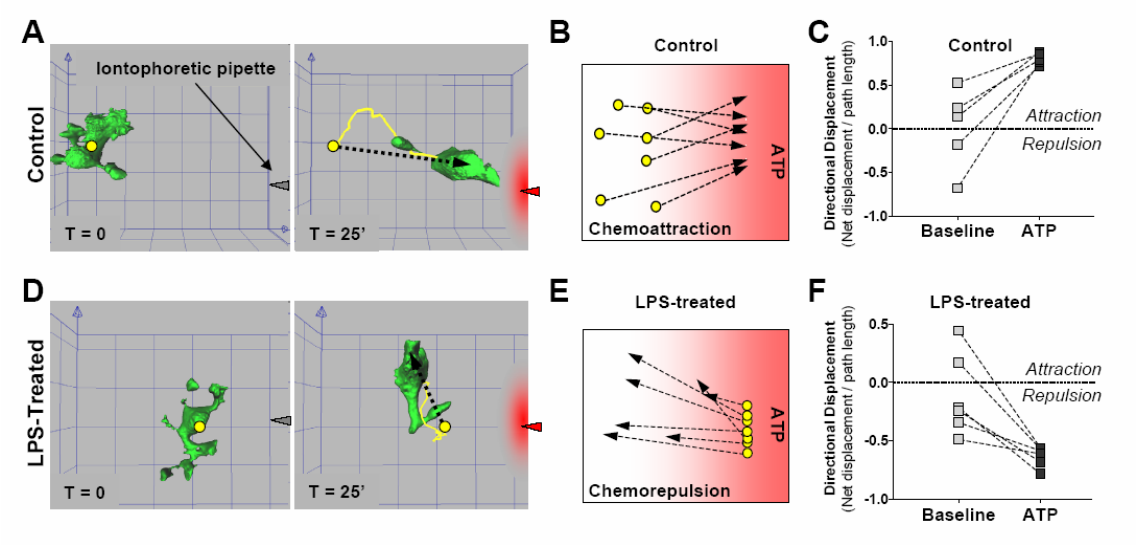


Figure 16. ATP induces migratory repulsion in activated microglia. (**A, D**) Three-dimensional reconstruction of a microglial cell shown before and after 25 minutes of local ATP exposure (0.5 mM, yellow dot marks starting position of cell nucleus, yellow line shows migratory path, dashed arrow indicates net displacement of cell nucleus). (**B, E**) Migrogram showing vector displacement of microglia during ATP ejection for < 1 hour, as plotted after X-axis alignment to pipette tip location. (**A-C**) Microglia exhibit enhanced migration toward ATP source ($n = 5$, $p < 0.05$, compared to baseline). Negative values were assigned for net migration away from pipette. (**D-F**) LPS-activated microglia migrate away from ATP (LPS: 100 ng/ml; $n = 6$, $p < 0.01$, compared to baseline). Pipette tips were positioned 30-50 µm away from a cell.

CHAPTER 4: The Intracellular Mechanisms Mediating Microglial Motile Responses to ATP

4.1. Abstract

In Chapter 3, we described our findings that activated microglia exhibit an opposite chemotactic response to ATP than resting microglia. Specifically, while resting microglia were attracted toward ATP, activated microglia were repelled by this nucleotide. Here, we investigated the intracellular mechanisms mediating this switch to repulsive responses by activated microglia. We report that microglial repulsion from ATP is mediated by $G\alpha_s$-coupled signaling downstream of an upregulated adenosine A_{2A} receptor. While Rho GTPase, Rho kinase and elevation in intracellular calcium did not mediate ATP-induced microglial process retraction, we found that the $G\alpha_s$-coupled pathway, involving cAMP and PKA, was necessary for this chemotactic effect. We show that microglial process retraction in response to ATP is suppressed by inhibition of the $G\alpha_s$-coupled pathway, blockade of the A_{2A} receptor, or by removal of extracellular adenosine. Conversely, stimulation of the $G\alpha_s$-coupled pathway or application of A_{2A} receptor agonists mimics ATP-induced process retraction. In light of evidence that the G_i-coupled $P2Y_{12}$ receptor that mediates chemoattraction is downregulated upon microglial activation, we propose that a switch from $P2Y_{12}$ to A_{2A} receptor signaling drives the shift in microglial chemotaxis from attraction to repulsion in response to ATP. We confirm this finding in microglia isolated from human patients and predict that A_{2A}-mediated microglial repulsion may play an important role in diverse CNS diseases that have a neuroinflammatory component.

4.2. Introduction

Within minutes of brain damage, microglial processes rapidly converge onto newly injured areas in order to shield and/or scavenge the affected sites (Davalos et al., 2005). This chemoattractive response is due partly to activation of the purinergic $P2Y_{12}$ receptor by ATP, which is released from injured cells (Haynes et al., 2006). Thus, ATP serves as a chemotactic factor to attract microglia to sites of brain damage. In contrast to these findings, we have discovered that proinflammatory, or activated, microglia are repelled by ATP (*see Chapter 3*). Specifically, while resting microglia are attracted to ATP, we discovered that activated microglia exhibit migration away from ATP, and show process retraction and slowing of process motility. The effectors of this switch in microglial chemotactic behavior remain entirely unknown. Likewise, the intracellular signaling mechanisms leading to microglial process retraction require investigation.

It is well-established that exposure to a chemoattractant gradient triggers motile cells to assume a polarized morphology, which allows directional migration toward an agonist source. Cell polarity establishes an orientation for migration by creating a leading edge and a trailing edge. In most motile cells, the leading edge is the site where Rac activation, PI3K accumulation, and actin polymerization drives rapid membrane protrusion. In contrast, the trailing edge is the site of activated Rho, Rho kinase and actin-myosin stress fibers, which together allow cell contraction, de-adhesion, and membrane retraction (Hall, 1998; Fukata et al., 2003; Gallo, 2006). Cdc42, the third member of the Rho GTPase family, regulates thin filopodial extensions and may also play a dominant role in cell motility (Nobes and Hall, 1995). Integrin-dependent adherence of the cell membrane to the extracellular matrix is also vital for cell movement as it allows for the formation of anchor points for generating tension within the cell. Indeed, Rac and Rho trigger the assembly of integrin-based focal complexes or focal adhesions, respectively (Ridley and Hall, 1992).

Both Rac and Rho are activated maximally within several minutes of agonist exposure (Xu et al., 2003). However, while Rac drives cell motility, activation of Rho can prevent it. Indeed, blockade of Rho activity with C3 transferase, which disassembles stress fibers in fibroblasts (Machesky and Hall, 1997), can enhance motility in response to a chemoattractant (Koch et al., 1994). Interestingly, Rac and Rho can antagonize each other in various cell types, which may help to stabilize cell polarity and regulate motility (Leeuwen et al., 1997; Hirose et al., 1998; Rottner et al., 1999; Sander et al., 1999). These opposing actions may create a dynamic equilibrium that maximizes cell responsiveness to chemotactic agents.

In neutrophils, G_i-coupled signaling can activate Rac and PI3K at the front edge, while the $G_{12/13}$-coupled pathway can trigger Rho, Rho kinase, and myosin at the rear (Xu et al., 2003). Thereby, the two opposing G protein-coupled pathways are able to generate structurally different force-generating systems: actin polymerization-driven extension and actin-myosin-driven contraction. Interestingly, activation of sphingosine-1-phosphate receptor $S1P_3$ is known to activate both G_i- and $G_{12/13}$-coupled pathways (Sugimoto et al., 2003). While $S1P_3$ stimulation normally triggers Rac activation and cell chemotaxis, PTX-treated cells exhibit S1P-induced inhibition of both Rac and cell chemotaxis, suggesting that suppression of the G_i pathway unmasks $G_{12/13}$-mediated inhibitory effects of $S1P_3$ on cell motility. As discussed in Chapter 3, PTX similarly blocks microglial chemotactic responses to ATP. However, $G\alpha_{12/13}$-coupled signaling has not been investigated in microglia.

Based on this evidence, we hypothesized that while ATP-induced membrane extension in resting microglia is driven by $G\alpha_i$-coupled Rac signaling (Honda et al., 2001), ATP-induced membrane retraction by activated microglia may be driven by Rho and Rho kinase downstream of $G\alpha_{12/13}$ signaling (Figure 17A-B). Specifically, we proposed that microglial proinflammatory activation may lead to inactivity of the $G\alpha_i$-coupled pathway with concomitant disinhibition of the $G\alpha_{12/13}$-coupled pathway involving Rho and Rho kinase

signaling (Figure 17B). Supporting this concept, Haynes et al. (2006) recently found that the $G\alpha_i$-coupled $P2Y_{12}$ receptor that mediates chemoattraction is dramatically downregulated upon microglial proinflammatory activation. This finding also suggests that altered protein expression may be a key component of the mechanism by which chemotaxis is reversed in microglia. Here, we investigated these hypotheses regarding the intracellular signaling that enables activated microglia to switch from chemoattraction to chemorepulsion in response to ATP.

4.3. Results

a. Requirement for protein synthesis

In Chapter 3, we described our observations of a shift in microglial morphological and chemotactic responses to ATP. These studies involved microglia that were activated by pre-treatment with various agents for 24 hours. We next studied the time-course of the chemotactic switch in microglia. We hypothesized that the shift from attraction to repulsion is dependent on changes in gene expression. Therefore, we predicted that ATP-induced process repulsion would only become evident after prolonged exposure to an activating agent. To test this, we varied the duration of microglial LPS pre-exposure and then performed similar four-dimensional analyses to assess microglial morphological dynamics upon ATP application. Specifically, we tested microglial responses after 0, 1, 6, 12, 24, or 48 hours of LPS exposure. As predicted, we found that the shift in the chemotactic response to ATP required a prolonged time period (>12 hours, Figure 18). These results suggest that the switch to chemorepulsion may be dependent on changes in protein expression.

NF-κB is a transcription factor involved in pro-inflammatory signaling cascades throughout the body and drives the expression of inflammatory genes, including cytokines and chemokines. In light of our findings that it takes microglia over 12 hours to switch to a

repulsive ATP response, we asked whether NF-κB is involved in this phenomenon. Thus, we blocked NF-κB activity in microglia during LPS pre-treatment and then examined whether this affected the repulsive response to ATP. We used two different NF-κB inhibitors, namely SN50, which is a cell-permeable 26-amino acid peptide that prevents nuclear translocation of the active NF-κB (Lin et al., 1995), and 6-Amino-4-(4-phenoxyphenylethylamino)quinazoline (QNZ), which prevents NF-κB activation (Tobe et al., 2003). As expected, we found that inhibition of NF-κB with either agent prevented microglial process retraction and low motility upon ATP exposure (Figure 19). Notably, these two different NF-κB inhibitors were equally effective in preventing ATP-induced morphological responses in LPS-activated microglia. Together with the time-course data, these results indicate that NF-κB-dependent changes in gene expression induced during proinflammatory activation are required to shift microglial ATP responses from attraction to repulsion.

b. Emergence of G_s-coupled signaling

We next shifted our focus to investigating the intracellular signaling mechanisms driving microglial process retraction upon ATP application. It is known that resting microglia are attracted to ATP and ADP due to Rac GTPase-driven actin polymerization downstream of the G_i-coupled $P2Y_{12}$ receptor (Honda et al., 2001; Davalos et al., 2005; Nasu-Tada et al., 2005; Haynes et al., 2006). However, $P2Y_{12}$ is rapidly downregulated upon microglial proinflammatory activation (Moller et al., 2000; Haynes et al., 2006). This suggests that a different receptor system mediates ATP responses in activated microglia. While the signaling mechanisms driving microglial retraction are unknown, other cell types undergo retraction due to remodeling of the actin cytoskeleton, which often involves $G_{12/13}$-coupled activation of Rho and Rho kinase (Mitchison and Cramer, 1996; Burridge and Wennerberg, 2004). Therefore, we

tested whether Rho and Rho kinase are similarly involved in microglial retraction (Figure 17B-C). For this, LPS-activated microglia were pretreated for one hour prior to confocal imaging with the Rho inhibitor exoenzyme C3 transferase, an ADP ribosyltransferase from *Clostridium botulinum*, or with the Rho kinase inhibitor Y27632 (Uehata et al., 1997). Rho or Rho kinase inhibition resulted in the appearance of long trailing membrane tails (*data not shown*), similar to previously observed effects of these inhibitors in neutrophils and monocytes (Alblas et al., 2001; Worthylake et al., 2001). Surprisingly, we found that inhibition of either Rho or Rho kinase did not attenuate ATP-induced microglial process retraction (Figure 20), indicating that an alternative mechanism may drive this response.

Numerous reports have shown that ATP and its breakdown products stimulate intracellular calcium release in numerous cell types (Ralevic and Burnstock, 1998; Fields and Burnstock, 2006). Notably, calcium signaling is known to trigger a variety of downstream effects, including changes in cell motility (Zheng, 2000). Therefore, we investigated whether influx of intracellular calcium is involved in ATP-induced changes in microglial morphology. To detect influx of intracellular calcium, we loaded isolated wild-type microglia with Fura-2, a ratiometric calcium-sensitive fluorescent dye that shifts its maximum excitation wavelength upon calcium binding. We imaged the dye-loaded cells continuously before and after exposure to uridine triphosphate (UTP), an agonist for the G_q-coupled $P2Y_2$ and $P2Y_4$ receptors that trigger an increase in intracellular calcium upon activation (Burnstock, 2007). Indeed, we discovered that UTP induces a calcium influx in both resting and LPS-activated microglia (Figure 21A). However, we found that UTP did not have a detectable effect on the morphology of either resting or activated microglia (Figure 21B). Thus, our findings indicate that while calcium flux may be necessary, it is not sufficient for induction of microglial morphological changes.

Interestingly, $G\alpha_s$-coupled signaling can suppress motility in select cell types, including vascular smooth muscle cells, endothelial cells, and hepatic stellate cells (Howe et al., 2004; Nagasawa et al., 2005; Hashmi et al., 2007). This pathway involves activation of adenylate cyclase, which produces cAMP and thereby activates PKA. Indeed, we have previously observed that constitutive activation of $G\alpha_s$ inhibits microglial process extension and induces cell rounding within 24 hours (*see Chapter 3*). To assess whether ATP regulates microglial motility via $G\alpha_s$-coupled signaling, we inhibited $G\alpha_s$, or its downstream effectors adenylate cyclase and PKA, using NF449, 5'-dideoxyadenosine, or H89, respectively. We found that inhibition of $G\alpha_s$, or factors downstream of $G\alpha_s$, attenuated ATP-induced retraction in activated microglia (Figure 22). Moreover, inhibition of the $G\alpha_s$-coupled pathway also prevented ATP-induced decline in microglial process motility. However, blockade of Rho or Rho kinase did not have a significant effect on these responses (Figure 23). To further investigate the involvement of $G\alpha_s$-coupled signaling in microglial process retraction, we asked whether stimulation of this pathway is sufficient for induction of this morphological effect. Therefore, instead of applying ATP, we exposed microglia to forskolin, a direct activator of adenylate cyclase, during confocal imaging and assessed changes in microglial morphology. As shown in Figure 24, forskolin induced dose-dependent process retraction in both resting and LPS-activated microglia. Together, these results indicate that $G\alpha_s$-coupled signaling is both necessary and sufficient for microglial process retraction.

Thus far, all imaging experiments in isolated microglia were carried out on microglia cultured within a 3D basement membrane matrix, which contains a mixture of laminin, collagen and growth factors, such as TGF-β. To determine whether the chemotactic responses we observed in microglia were an artifact specific to the 3D matrix, we examined ATP-induced morphological changes in microglia grown on poly-D-lysine-coated coverslips. After treating the cells overnight with LPS, some cultures received the $G\alpha_s$

inhibitor NF449 or the PKA inhibitor H89 prior to ATP exposure for 5 minutes. Thereafter, microglia were fixed in paraformaldehyde and the actin cytoskeleton was visualized with fluorescently-labeled phalloidin, a toxin from the *Amanita Phalloides* mushroom that binds selectively to actin filaments. As shown in Figure 25A, LPS caused microglia to assume a rounded flat shape and exhibit several actin-rich membrane ruffles. Similar to our previous findings, exposure of LPS-activated microglia to ATP triggered process retraction (Figure 25B). Moreover, inhibition of $G\alpha_s$ or PKA blocked the effect of ATP on the morphology of activated microglia (Figure 25C-D). These observations indicate that the reversal in the chemotactic effect of ATP is independent of the culture method utilized in our studies.

c. Regulation of purinergic receptor expression

Together, our data implicate a $G\alpha_s$-coupled receptor in the chemorepulsion of activated microglia by ATP. While there are over a dozen known purinergic receptors, there is no known mouse G_s-coupled P2Y receptor that could potentially mediate microglial repulsion from ATP. Therefore, we considered other receptor classes that are sensitive to purines.

Upon release in the brain, ATP is rapidly broken down to adenosine by extracellular nucleotidases (Fredholm et al., 2001). Adenosine is also released in the brain and serves as an important regulatory factor in peripheral immune responses (Riberio et al., 2003; Hasko and Cronstein, 2004; Sitkovsky et al., 2004). Interestingly, two of the four adenosine receptors (A_{2A} and A_{2B}) are G_s-coupled (Ongini and Fredholm, 1996). Therefore, we next addressed whether adenosine receptors are expressed by resting and activated microglia. As shown in Figures 26 and 27, reverse transcription-polymerase chain reaction (RT-PCR) revealed that while $P2Y_{12}$ mRNA is downregulated, the high-affinity G_s-coupled adenosine receptor A_{2A} is selectively upregulated upon microglial activation with pathogen-derived factors, including LPS, LTA, or CpG, or with the proinflammatory cytokine TNF-α. Indeed, A_{2A} mRNA

upregulation has previously been noted in microglia treated with LPS, but not functionally studied (Wittendorp et al., 2004). Moreover, RT-PCR revealed that the expression of both A_{2A} and $P2Y_{12}$ is similarly altered by microglial exposure to amyloid-β (Aβ), a main component of extracellular amyloid plaques in Alzheimer's disease (Figure 28). Together, our data indicate that a shift from $P2Y_{12}$ to A_{2A} receptor expression takes place during microglial activation.

Thus far, we have shown that activated microglia can shift their chemotactic ATP response from chemoattraction to chemorepulsion, and that this shift is dependent on NF-κB-mediated changes in protein expression. Moreover, we have shown that microglial chemorepulsion from ATP is driven by $G\alpha_s$-coupled intracellular signaling. Lastly, we discovered a switch in the expression of two purinergic receptors. While resting microglia express $P2Y_{12}$, activated microglia downregulate this receptor and upregulate A_{2A}, reflecting a switch from G_i-coupled to G_s-coupled signaling. Collectively, these findings suggest that a shift in the expression of two purinergic receptors may account for the dichotic effects of ATP in microglia. To confirm this, we next examined the functional role of the A_{2A} receptor in mediating microglial repulsion from ATP.

As shown in Figure 29A, process ramification of control microglia was dramatically enhanced by 2-methylthio-ADP (2-MeSADP), a potent agonist for $P2Y_{12}$ (Kügelgen, 2006), supporting previous studies that this receptor induces process extension and mediates microglial chemoattraction toward ATP (Haynes et al., 2006). Interestingly, 2-MeSADP did not affect the morphology of LPS-activated microglia, corroborating findings that this receptor is downregulated upon microglial activation, thus making the agonist ineffective (Figures 26 and 27; Haynes et al., 2006). Conversely, we found that a nonselective adenosine receptor agonist 5'-(N-ethylcarboxamido)adenosine (NECA) and an A_{2A}-selective agonist (CGS-21680; Ongini and Fredholm, 1996; Fredholm et al., 2001) both mimicked ATP by triggering retraction in activated microglia (Figure 29B). However, these two agonists had no impact on

untreated microglia. Thus, we observed that the morphological effects of $P2Y_{12}$ and A_{2A} receptor agonists in resting and activated microglia correlated with the observed changes in the mRNA levels for these two receptors, strengthening the notion that $P2Y_{12}$ downregulation and A_{2A} upregulation drives the shift in the microglial chemotactic response to ATP. Furthermore, we found that the A_{2A}-selective antagonist SCH-58261 (Ongini and Fredholm, 1996; Fredholm et al., 2001), attenuated process retraction induced by ATP (Figure 30), further suggesting that A_{2A} mediates microglial repulsion from this chemoattractant.

An important feature of adenosine receptors, including $A_{2A,}$ is their insensitivity to ATP. However, as mentioned previously, it is known that ATP is rapidly hydrolyzed into adenosine, a potent activator of A_{2A} (Fredholm et al., 2001). We therefore reasoned that ATP degradation into adenosine may be a necessary step leading to A_{2A} receptor activation and consequent microglial repulsion. To test whether microglial repulsion was dependent on ATP degradation, we stimulated cells with ATP in the presence of adenosine deaminase, an enzyme that converts adenosine into inosine. We predicted that removal of adenosine would prevent ATP responses in activated microglia. Consistent with our reasoning, adenosine deaminase inhibited retractile responses (Figure 30), demonstrating that ATP-induced microglial repulsion is mediated by the ATP breakdown product adenosine.

We next verified that the agonists and antagonists utilized in our experiments were indeed acting on the G_s-coupled A_{2A} receptor. Therefore, we tested these agents in a cAMP assay system using A_{2A}-transfected HEK 293 cells. In these experiments, agonist-induced release of cAMP was measured with or without the presence of the A_{2A} antagonist SCH-58261 or adenosine deaminase. Additionally, we also investigated whether each of the breakdown products of ATP were capable of activating the A_{2A} receptor. Our studies revealed that while adenosine and adenosine monophosphate (AMP) were both effective in triggering A_{2A} receptor activation, ATP and adenosine diphosphate (ADP) induced only modest responses (Figure 31).

These results are consistent with previous reports (Fredholm et al., 1994 and 2001). In addition, we confirmed that SCH-58261 and adenosine deaminase were effective blockers of A_{2A} receptor activation by adenosine and AMP (Figure 31). Interestingly, the effect of AMP was inhibited by adenosine deaminase, suggesting that AMP requires hydrolysis to adenosine prior to A_{2A} receptor activation.

Thus far, our findings have been limited to microglia isolated from the mouse brain. Interestingly, A_{2A} upregulation has also been reported in human macrophages and in brain tissue from Alzheimer's disease patients (Angulo et al., 2003; Murphree et al., 2005). Thus, we investigated whether adult human microglia also upregulate A_{2A} upon proinflammatory activation and consequently display chemotactic reversal. Indeed, we observed that LPS-activated adult human microglia exhibited marked A_{2A} upregulation along with a loss in $P2Y_{12}$ expression (Figure 32A). Notably, while unstimulated human microglia showed migration toward and engulfment of an ATP-filled pipette, LPS-stimulated human microglia exhibited repulsion (Figure 32B and data not shown). Thus, a similar chemotactic switch takes place in adult human microglia.

4.4. Discussion

Rho GTPase and its target Rho kinase are known to trigger membrane retraction in diverse cell types. However, our results suggest that these factors are not involved in microglial membrane retraction upon purinergic stimulation. Instead, we show that the $G\alpha_s$-coupled signaling, involving cAMP and PKA, is an important mechanism regulating cell motility and morphology in activated microglia.

cAMP, a ubiquitous second messenger discovered in the 1950s (Sutherland and Rall, 1958), regulates a variety of cellular processes, including metabolism, gene expression, and cell division. Among its many roles in cellular function, cAMP is also known to modulate

peripheral immune responses. For example, increased intracellular cAMP suppresses phagocytosis and inhibits the production of inflammatory mediators, including the cytokines TNF-α and interleukin-12 (van der Pouw Kraan et al., 1995; Aronoff et al., 2006). Interestingly, cAMP also enhances the production of the anti-inflammatory cytokine interleukin-10 (Aronoff et al., 2006), suggesting that this intracellular signaling factor may generally suppress immune responses. Indeed, numerous studies have suggested an anti-inflammatory role of cAMP signaling (Skalhegg et al., 1992; Eigler et al., 1998; Kambayashi et al., 2001). Our findings that proinflammatory microglia upregulate a $G\alpha_s$-coupled receptor that is known to stimulate cAMP production suggests that a similar regulatory pathway may be utilized by reactive microglia in order to regulate brain inflammation. However, further studies are needed to assess whether activation of the $G\alpha_s$-coupled pathway triggers a decline in the reactive properties exhibited by activated microglia, including the production of inflammatory factors and phagocytosis.

Many agonists are known to activate $G\alpha_s$-coupled receptors, including epinephrine, histamine, serotonin, and prostaglandins. Upon ligand binding to a $G\alpha_s$-coupled receptor, GDP is exchanged for GTP on the α_s subunit, which results in the dissociation of α_s from the $\beta\gamma$ subunit complex. The liberated α_s subunit then stimulates membrane-bound adenylyl cyclase to catalyze the conversion of ATP to cAMP (Kamenetsky et al., 2006). In contrast, $G\alpha_i$-coupled receptors are known to inhibit adenylate cyclase activity and thereby reduce cAMP production. cAMP is also tightly regulated by phosphodiesterases, which degrade cAMP to 5'-AMP (Omori and Kotera, 2007). PKA is a major target of cAMP, and is known to phosphorylate and activate various downstream substrates, including the transcription factor cAMP response element binding protein (CREB) (Chin et al., 2002; Serezani et al., 2008). While PKA is thought to be the primary effector of cAMP, other targets of this intracellular mediator are also

known, including the exchange protein directly activated by cAMP (Epac) (De Rooij et al., 1998) and cAMP-gated ion channels (Zagotta et al., 2003).

Our studies have revealed that activated microglia upregulate the G_s-coupled A_{2A} receptor while concurrently downregulating the G_i-coupled $P2Y_{12}$ receptor. This switch in purinergic receptor expression and in intracellular signaling is proposed to drive the shift in microglial chemotaxis from chemoattraction to chemorepulsion in response to ATP exposure. We have shown that activity of $G\alpha_s$, adenylate cyclase, and PKA are necessary for ATP-induced microglial process repulsion. Moreover, elevation of cAMP exogenously with forskolin triggered process retraction in both resting and LPS-activated microglia. Consistent with this, Kalla et al. (2003) have similarly observed a loss of ramifications in microglia following 48-hour exposure of microglia-astrocyte co-cultures to either forskolin or dibutyryl-cAMP, a cell-permeable cAMP analog. However, the downstream mechanisms mediating the effects of cAMP signaling on microglial morphology and motility remain largely unknown.

Previous studies have demonstrated that increased cAMP levels can impair chemotaxis in smooth muscle cells and immune cells (Bornfeldt et al., 1995; Dubey et al., 1995). cAMP has also been reported to induce morphological remodeling in diverse cell types by triggering disruption of the actin cytoskeleton (Egan et al., 1991; Glass and Kreisberg, 1993). Moreover, Pelletier et al. (2005) have shown that PKA downstream of cAMP interferes with Rac activity in smooth muscle cells. Specifically, this group found that the effects of forskolin on cell morphology can be mimicked by inhibition of Rac (either pharmacologically or via transfection of a dominant negative Rac mutant) and that forskolin effects can be blocked by expression of a constitutively active Rac mutant. In support, cAMP elevation has also been shown to inhibit Rac activity in platelets (Gratacap et al., 2001). Our studies using the PKA inhibitor H89 suggest that PKA is necessary for ATP-induced microglial process repulsion. However, the involvement of PKA in microglial motility requires further investigation.

Alternatively, it is possible that cAMP elevation inhibits PI3K (Wang et al., 2001) or regulates integrin-based cell adhesion via Epac (Bos et al., 2001). Interestingly, Bryn et al. (2006) have recently demonstrated that elevation in cAMP modulates monocyte immune functions, including phagocytosis and cytokine production, through activation of PKA. Likewise, PKA-dependent immune suppression has been noted in B cells and natural killer cells (Torgersen et al., 1997; Rahmouni et al., 2001). Thus, in addition to regulating microglial motility through inhibition of Rac, PKA may also play important roles in modulating other facets of microglial immune reactivity.

We show that UTP-induced increase in intracellular calcium is not coupled to induction of detectable changes in microglial cell morphology. From this, we can conclude that while calcium influx may be a necessary event, it is not sufficient to cause morphological remodeling in microglia. Interestingly, it is known that activation of the calcium-binding protein calmodulin stimulates certain isoforms of adenylate cyclase (Defer et al., 2000). Moreover, it is known that ATP elevates intracellular calcium in microglia with an EC_{50} of 9.2 μM by activating both P2Y and P2X receptors (Moller et al., 2000). In light of this, we cannot rule out the possibility that ATP-induced calcium influx in microglia may enhance the morphological effects of A_{2A} receptor activation due to calmodulin-induced adenylate cyclase activity.

In agreement with our findings of A_{2A} receptor upregulation in activated microglia, Wittendorp et al. (2004) have reported that A_{2A} receptor mRNA is expressed by microglia only following LPS exposure. While the expression of this receptor has also been noted in untreated cultured microglia (Fiebich et al., 1996; Heese et al., 1997), these studies may have utilized microglia with an already activated phenotype and therefore elevated A_{2A} receptor expression. Similar to our findings, the A_{2A} receptor is also increased in endothelial cells, macrophages, and monocytic cells following exposure to LPS, IL-1β, or TNF-α (Nguyen et al.,

2003; Murphree et al., 2005). Importantly, the promoter region of the rat A_{2A} receptor gene contains a sequence that is known to bind NF-κB (Chu et al., 1996). Consistent with this, we report that the A_{2A}-driven chemotactic reversal in microglia is induced by proinflammatory stimuli in an NF-κB-dependent manner. These findings suggest that microglial A_{2A} upregulation and activation by adenosine released during injury or metabolic stress may be evident during neuroinflammation.

We corroborate previous studies showing that $P2Y_{12}$ is downregulated upon microglial activation with LPS or following traumatic tissue damage (Moller et al., 2000; Haynes et al., 2006). However, this receptor was recently shown to be upregulated in spinal microglia following partial sciatic nerve transection (Kobayashi et al., 2008) and in hippocampal microglia following kainate injection (Avignone et al., 2008). Thus, microglial expression of this purinergic receptor may vary between different CNS injury models. We show that both $P2Y_{12}$ and A_{2A} receptors are similarly regulated following microglial activation with various proinflammatory stimuli, including LPS, LTA, CpG, TNF-α, and aggregated Aβ protein. TNF-α is a proinflammatory and potentially neurotoxic cytokine that is elevated during various neuropathologies that possess a neuroinflammatory component, including stroke, epilepsy, and neurodegenerative disorders (Wang and Shuaib, 2002; Lambertsen et al., 2007; Sriram and O'Callaghan, 2007; Vezzani et al., 2008). Moreover, we observed similar purinergic receptor expression changes in LPS-treated primary human microglia. Thus, we predict that A_{2A}-mediated microglial repulsion may play a role in diverse CNS diseases. Interestingly, every microglial stimulus utilized in our studies is known to activate NF-κB (Combs et al., 2001; Chen et al., 2005; Sriram and O'Callaghan, 2007), suggesting that regulation of $P2Y_{12}$ and A_{2A} receptor expression may depend on microglial NF-κB-dependent transcriptional activity.

In summary, we have discovered that activated microglia upregulate the adenosine A_{2A} receptor and that this $G\alpha_s$-coupled receptor mediates ATP-induced process retraction and

repulsive migration in activated microglia. Specifically, we have demonstrated that ATP-induced process retraction is suppressed by inhibition of the $G\alpha_s$-coupled pathway, blockade of the A_{2A} receptor, or by removal of extracellular adenosine. Conversely, we also show that ATP-induced process retraction can be mimicked by direct stimulation of the $G\alpha_s$-coupled pathway or application of A_{2A} receptor agonists. Previous studies have shown that activated microglia downregulate the G_i-coupled $P2Y_{12}$ receptor that mediates microglial process extension and directional migration towards ATP. Thus, we propose that a switch in purinergic receptor signaling, from $P2Y_{12}$ to A_{2A}, drives the switch in microglial chemotaxis from attraction to repulsion in response to ATP. However, the downstream consequences and *in vivo* effects of A_{2A} receptor signaling in microglia are still unknown.

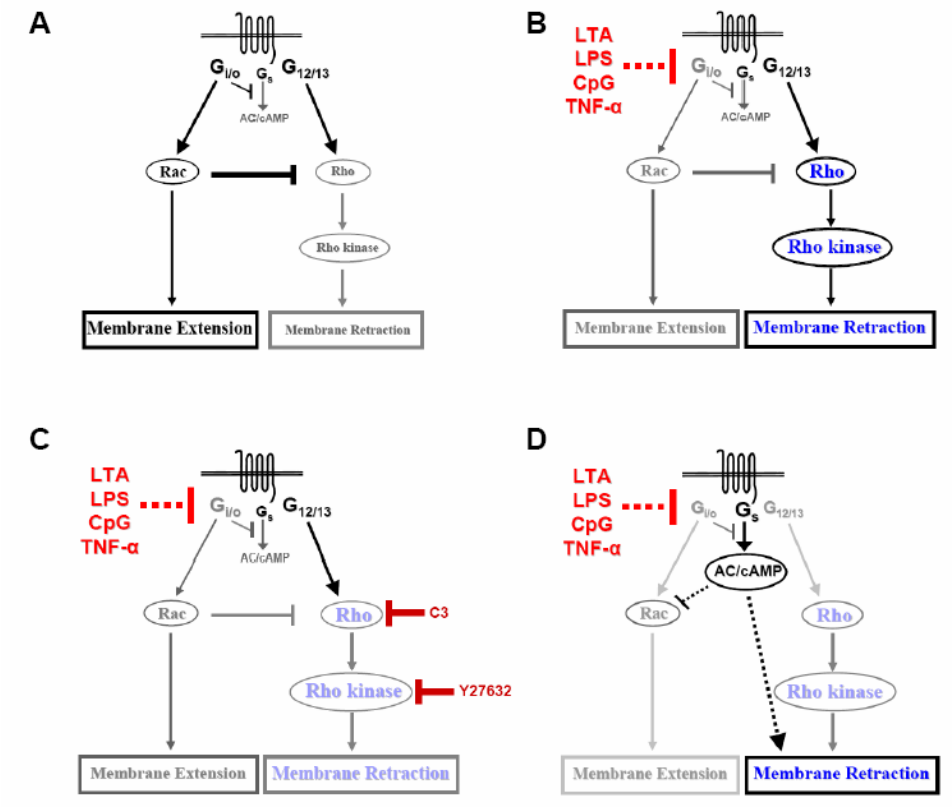


Figure 17. The predicted roles of G protein-coupled signaling in driving changes in microglial morphology. (**A**) In light of previous studies, we proposed that purinergic receptor stimulation in microglia leads to activation of two different G protein-coupled pathways, including $G_{i/o}$-Rac and $G_{12/13}$-Rho-Rho kinase, which lead to microglial membrane extension or retraction, respectively. Under normal conditions, $G_{i/o}$-Rac signaling dominates and allows process extension, as shown previously. (**B**) Upon microglial activation, suppression of the $G_{i/o}$-Rac pathway allows $G_{12/13}$-coupled signaling to become the main mediator of the morphological effects of ATP. (**C-D**) We further predicted that blockade of Rho or Rho kinase with C3 transferase or Y27632, respectively, would inhibit the retractile effect of ATP in activated microglia. However, upon evidence that these blockers had no effect, we examined the role of G_s-coupled signaling in the retractile responses by activated microglia.

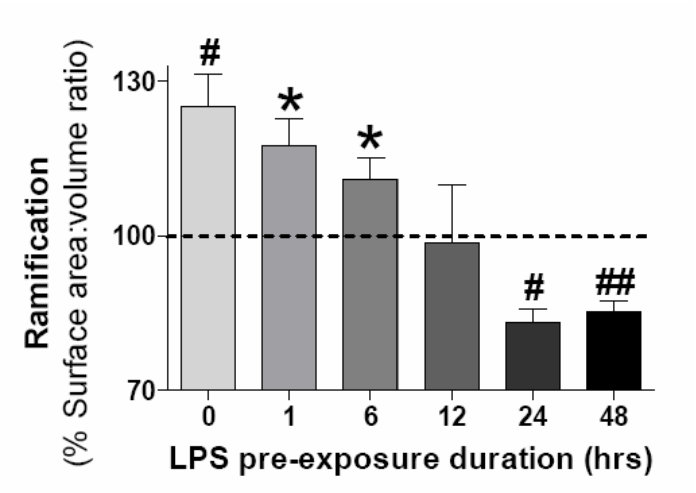


Figure 18. Chemotactic reversal requires over 12 hours. The duration of microglial pre-exposure to LPS (100 ng/ml) was varied as indicated. Microglial morphological response to ATP (20 μM) was then measured with time-lapse 3D confocal imaging. Following 0-6 hours of LPS exposure, microglia exhibited increased process ramification in response to ATP. Although ATP had no morphological effect after 12 hours of LPS treatment, microglia exposed to LPS for 24-28 hours showed ATP-triggered process retraction. n = 4-8, *$p < 0.05$, #$p < 0.01$, ##$p < 0.001$ compared to baseline. Graph shows mean + s.e.m.

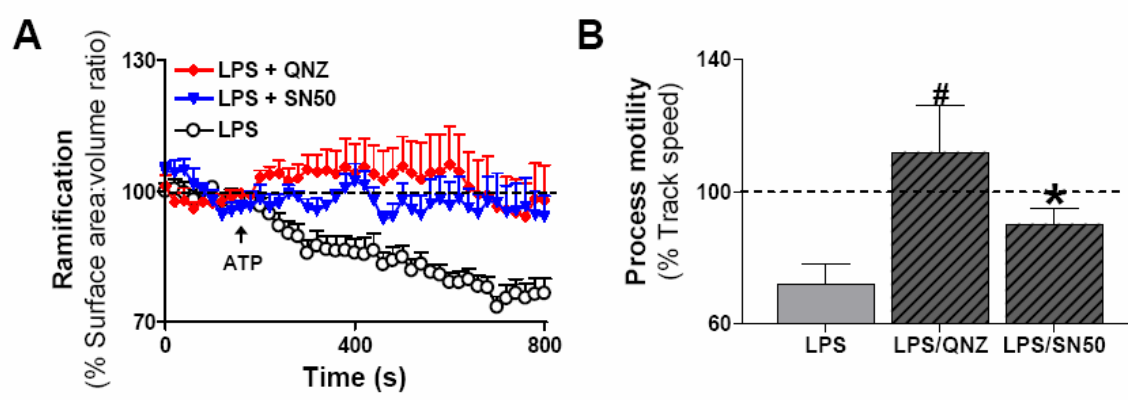


Figure 19. NF-κB involvement in the effects of ATP on process motility in activated microglia. (A) While ATP (20 μM) induces a decline in process ramification in LPS-activated microglia, this effect is blocked by NF-κB inhibitors (1 μM QNZ or 20 μM SN50, applied with LPS for 24 hrs, n = 6-10, $p < 0.05$ compared to LPS-activated cells). (**B**) NF-κB inhibition also prevents the ATP-induced decline in microglial process motility (n = 4-5, *$p < 0.05$, #$p < 0.01$ compared to LPS-activated cells). Graphs show mean + s.e.m.

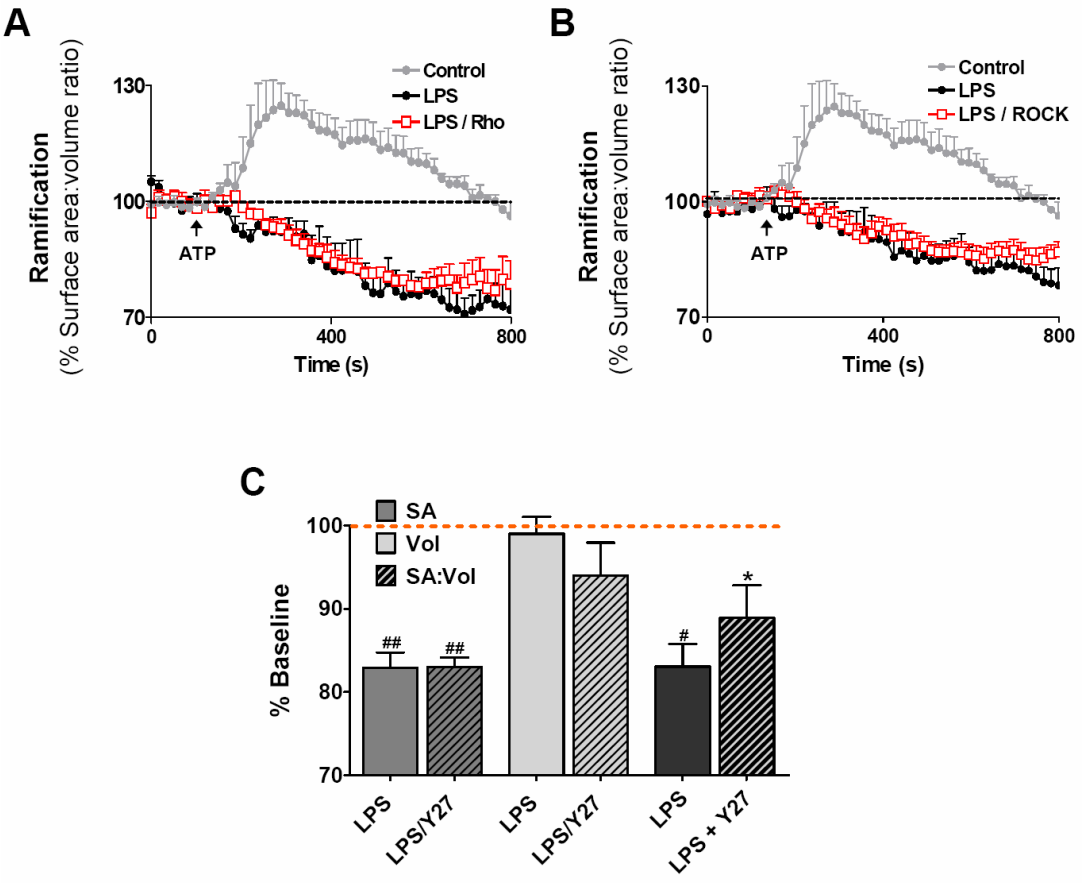


Figure 20. **The lack of Rho and Rho kinase involvement in ATP-induced microglial process retraction.** (**A-B**) Inhibition of Rho with C3 transferase (20 ug/ml, 2 hrs, n = 5, (**A**)) or Rho kinase (ROCK) with Y27632 (10 μM, n = 9, 1 hr, (**B**)) had no effect on ATP-induced process ramification in LPS-activated microglia. Untreated microglia (Con) showed increased process ramification in response to ATP (20 μM). (**C**) Summary of ROCK inhibitor Y27632 (Y27) effects on the cell surface area (SA), volume (Vol), and SA:Vol ratio in LPS-activated microglia. A decline in cell surface area and SA:Vol ratio in response to ATP was similarly evident with or without ROCK blockade. *p<0.05, #p<0.01. Graphs show mean + s.e.m.

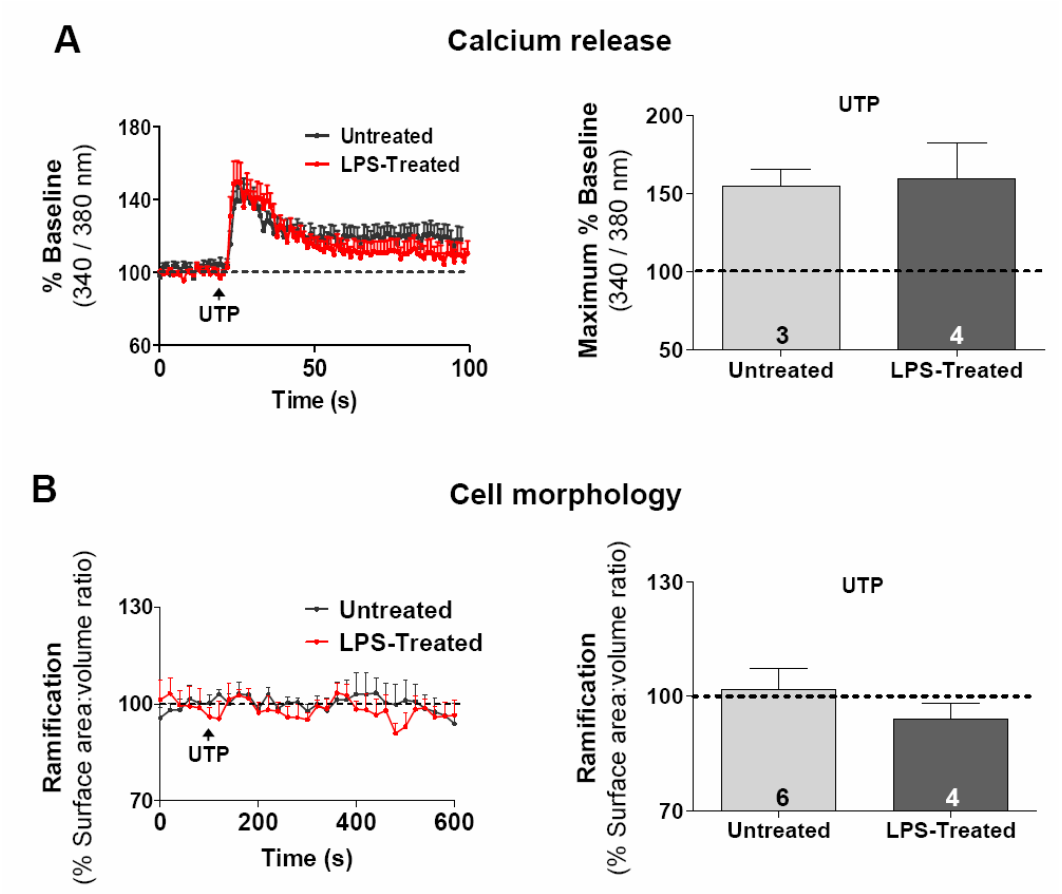


Figure 21. **Increase in intracellular calcium is not sufficient for ATP-induced morphological effects in microglia.** (**A**) Isolated wild-type microglia loaded with Fura-2, a calcium-sensitive fluorescent dye, were imaged continuously before and after exposure to uridine triphosphate (UTP, 20 μM), an agonist for the G_q-coupled $P2Y_2$ and $P2Y_4$. UTP induced a calcium influx in both resting and LPS-activated microglia. (**B**) UTP did not have a detectable effect on the morphology of either resting or LPS-treated microglia. Graphs show mean + s.e.m.

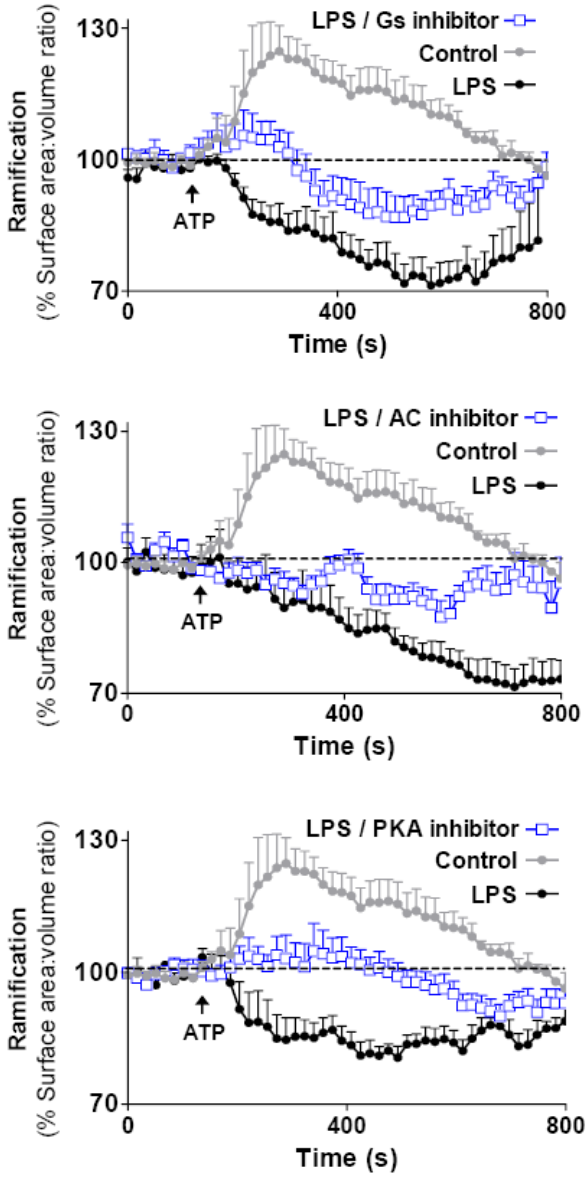


Figure 22. **The involvement of Gs-coupled signaling in process retraction by activated microglia.** ATP-induced process retraction in isolated LPS-treated (100 ng/ml, 24 hrs) microglia was attenuated by inhibition of $G\alpha_s$ with NF449 (50 μM, n = 8), inhibition of adenylate cyclase (AC) with ddAdo (50 μM, n = 6), or by inhibition of protein kinase A (PKA) with H89 (10 μM, n = 8). All inhibitors were applied for 1-2 hrs prior to ATP exposure. Graphs show mean + s.e.m.

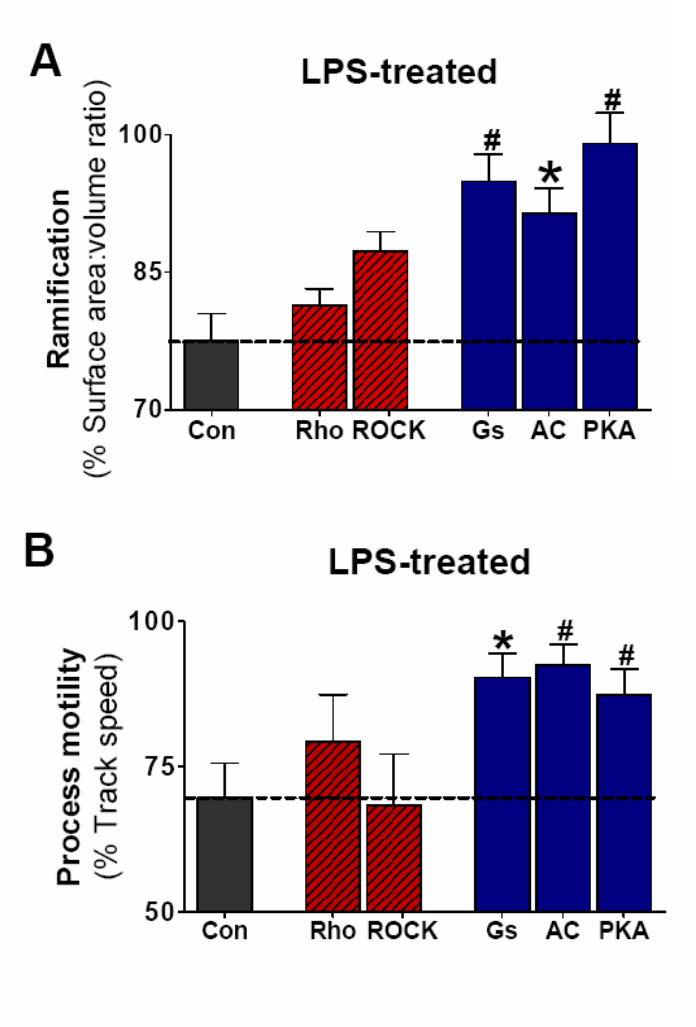


Figure 23. **The involvement of Gs-coupled signaling in process retraction and motility by activated microglia.** ATP-induced decline in process ramification (**A**) and process motility (**B**) in LPS-activated microglia was attenuated with Gαs, AC, or PKA inhibitors (*see also Figure 22*), but not with Rho or ROCK inhibitors (*see also Figure 20*). Rho was inhibited with C3 transferase (20 µg/ml, n = 5, 2 hrs), while ROCK was inhibited with Y27632 (10 µM, n = 9, 1 hr). *$p < 0.05$, #$p < 0.01$. Graphs show mean + s.e.m.

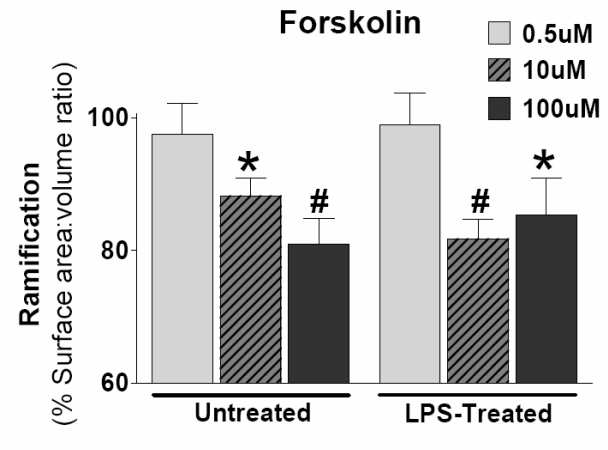


Figure 24. **Gα_s-coupled signaling is sufficient for microglial process retraction.** Untreated or LPS-treated (100 ng/ml, 24 hrs) isolated microglia were exposed to forskolin, a direct activator of adenylate cyclase, at the indicated concentrations during time-lapse confocal imaging. Forskolin induced dose-dependent decline in process ramification in both resting and LPS-activated microglia. *$p < 0.05$, #$p < 0.01$ vs. baseline. Graph shows mean + s.e.m.

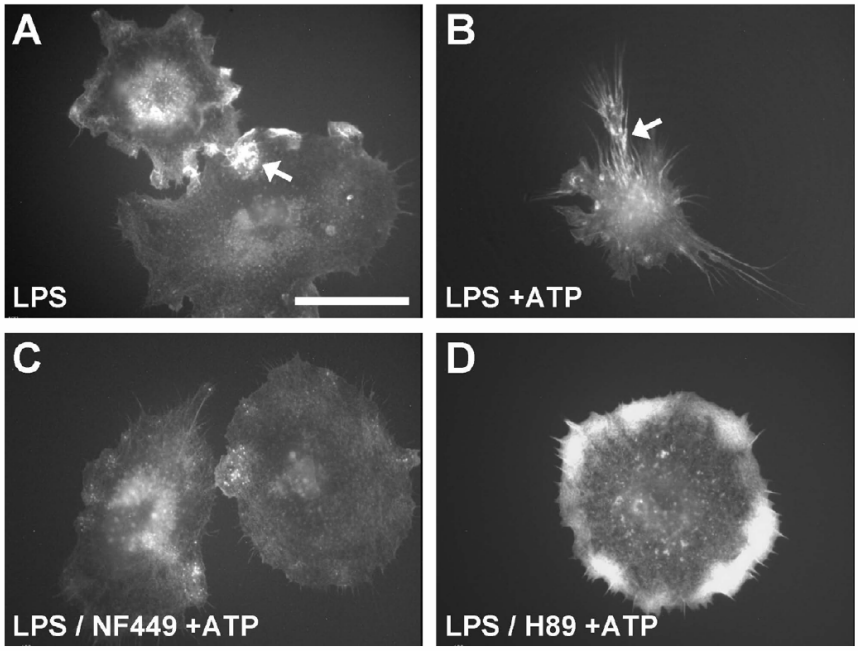


Figure 25. **Actin cytoskeleton in activated microglia undergoing ATP-induced process retraction.** All panels show actin filament staining with phalloidin in fixed mouse microglia pre-treated with LPS (100 ng/ml, 24 hrs). (**A**) At baseline, LPS-activated cells assume a flat and round morphology with regions of membrane ruffling (arrow). (**B**) Upon exposure to ATP (20 μM, 5 min), LPS-activated microglia exhibit process retraction and actin filament bundling (arrow). (**C**) Inhibition of $G\alpha_s$ with NF449 (50 μM, 2 hrs) prevented ATP-induced retraction. (**D**) Inhibition of protein kinase A with H89 (10 μM, 1 hr) prevented ATP-induced process retraction. Images are representative of $\geq$ 3 trials.

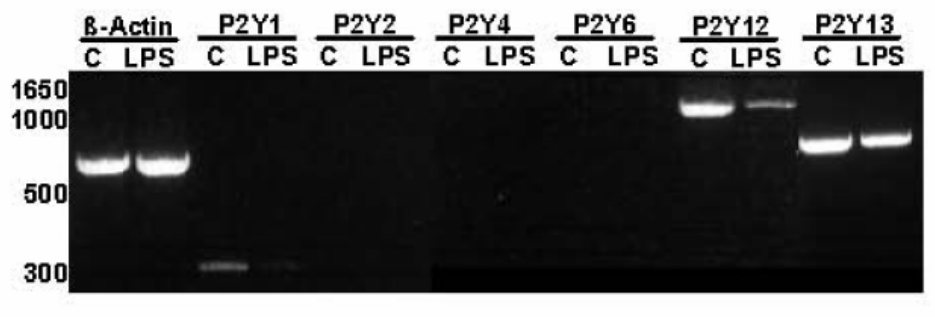


Figure 26. **Downregulation of $P2Y_1$ and $P2Y_{12}$ receptors in activated microglia**. RT-PCR analysis in isolated mouse microglia indicates that $P2Y_1$ and $P2Y_{12}$ mRNA levels decrease upon LPS exposure (100 ng/ml, 24 hrs, n = 3). β-actin mRNA served as a loading control.

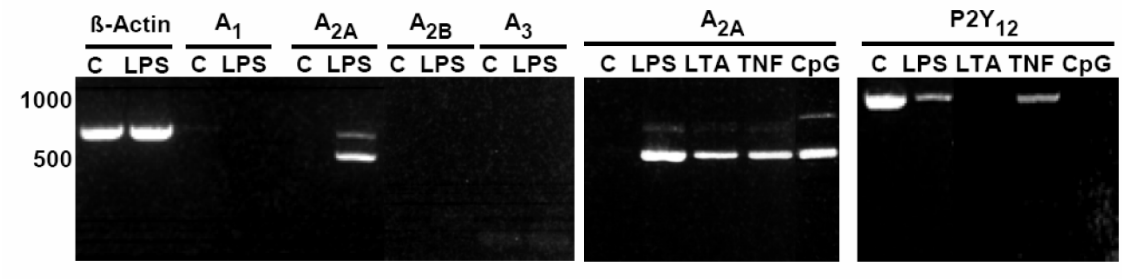


Figure 27. Adenosine A_{2A} receptor upregulation in activated microglia. RT-PCR analysis in isolated mouse microglia revealed that A_{2A} receptor mRNA is selectively increased in microglia following 24-hr exposure to LPS (n = 4, 100 ng/ml), LTA (10 μg/ml, n = 3), TNF-α (20 ng/ml, n = 3), or CpG (10 μM, n = 2). In contrast, $P2Y_{12}$ mRNA is downregulated. β-actin mRNA served as a loading control.

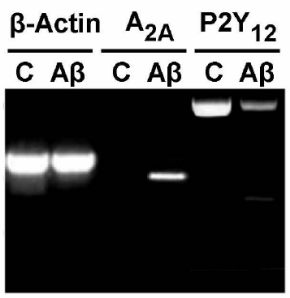


Figure 28. Regulation of microglial purinergic receptor expression by amyloid-β. RT-PCR analysis in isolated mouse microglia revealed that exposure of microglia to aggregated amyloid-β (Aβ, n = 3, 1 μM, 24 hrs) triggers A_{2A} receptor upregulation and $P2Y_{12}$ receptor downregulation. β-actin mRNA served as a loading control.

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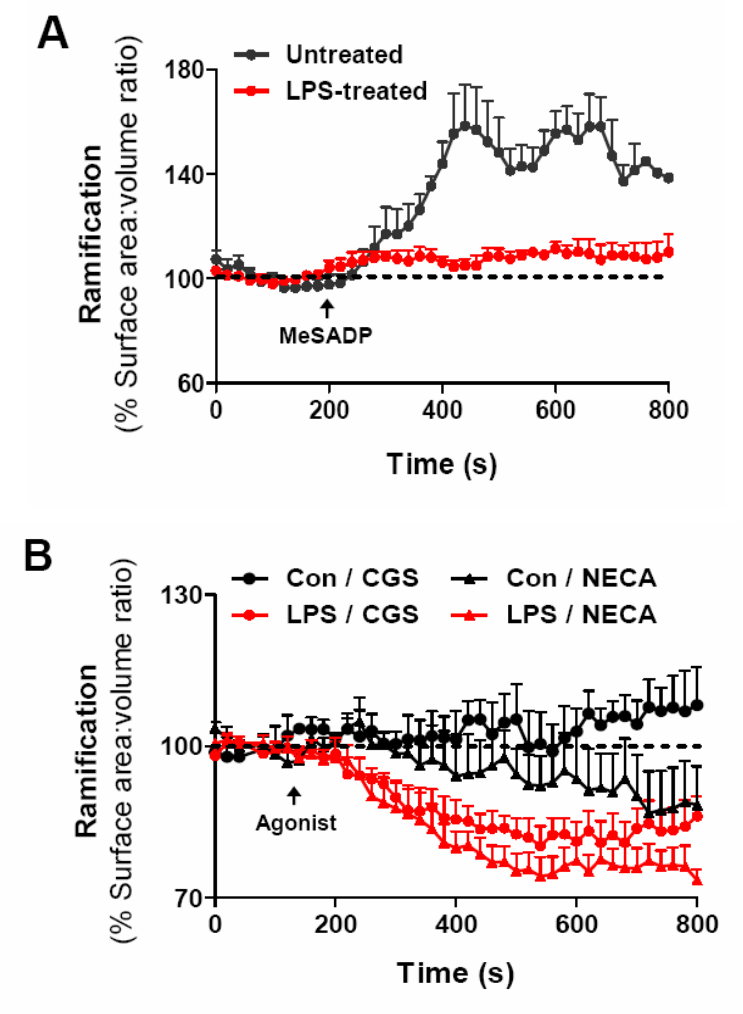

Figure 29. **$P2Y_{12}$ and A_{2A} receptor agonist effects on microglial morphology.** (**A**) MeSADP (20 μM), an agonist for $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$, triggered a robust increase in process ramification in untreated microglia ($n = 4$, $p < 0.05$), but had minimal effect on LPS-treated microglia ($n = 4$, $p < 0.05$ compared to baseline). (**B**) A_{2A} agonist CGS-21680 (CGS, 20 μM) triggered retraction in LPS-treated microglia ($n = 6$, $p < 0.01$ compared to baseline), but not in untreated cells (Con: $n = 3$). Likewise, the nonspecific adenosine receptor agonist 5'-(N-ethylcarboxamido)adenosine (NECA, 20 μM) triggered retraction in LPS-treated microglia ($n = 6$, $p < 0.01$ compared to baseline), but not in untreated microglia ($n = 6$). Graphs show mean + s.e.m.

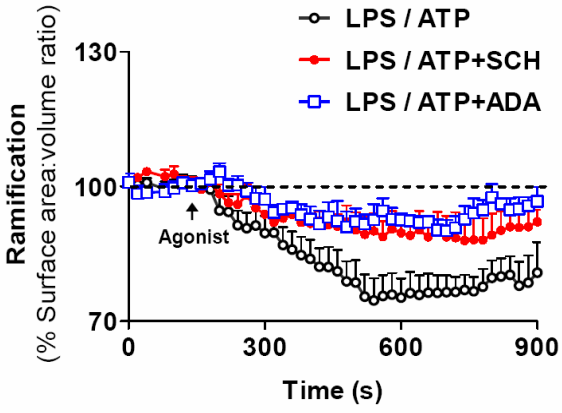

Figure 30. **The adenosine A_{2A} receptor mediates ATP-induced microglial process retraction.** The A_{2A} antagonist SCH-58261 (SCH, 5 μM) and adenosine deaminase (ADA, 5 U/ml) inhibited ATP-induced retraction in LPS-activated microglia (20 μM ATP: n = 5; ATP+SCH: n = 7, $p < 0.05$; ATP+ADA: n = 5, $p < 0.05$ compared to responses to ATP alone). Antagonists were applied for 5 min alone, and then co-applied with ATP. Graph shows mean + s.e.m.

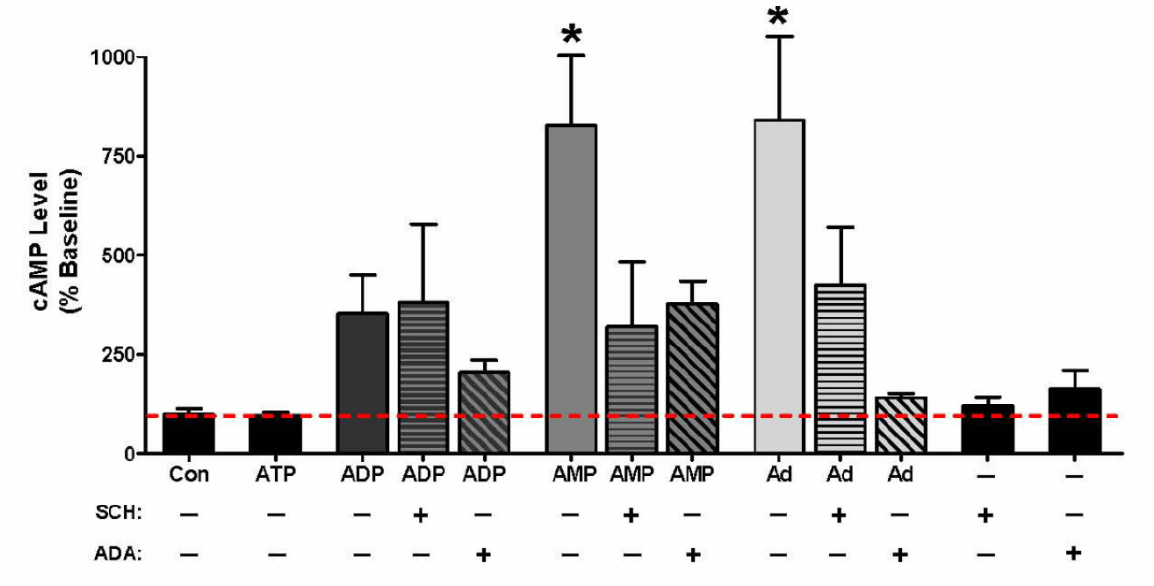

Figure 31. **A_{2A} receptor activation.** Adenosine 5'-monophosphate (AMP, 40 μM) and adenosine (Ad, 40 μM) both increased cAMP levels in HEK 293 cells transiently transfected with the A_{2A} receptor (n = 4, $p < 0.05$ compared to untreated (Con)), while ADP and ATP had minimal or no effect (n = 4-5). A_{2A} receptor antagonist SCH-58261 (SCH, 100 μM) and adenosine deaminase (ADA, 5 U/ml) inhibited AMP and adenosine responses (n = 3-4), but had no effect on their own (n = 4-5). Graph shows mean + s.e.m.

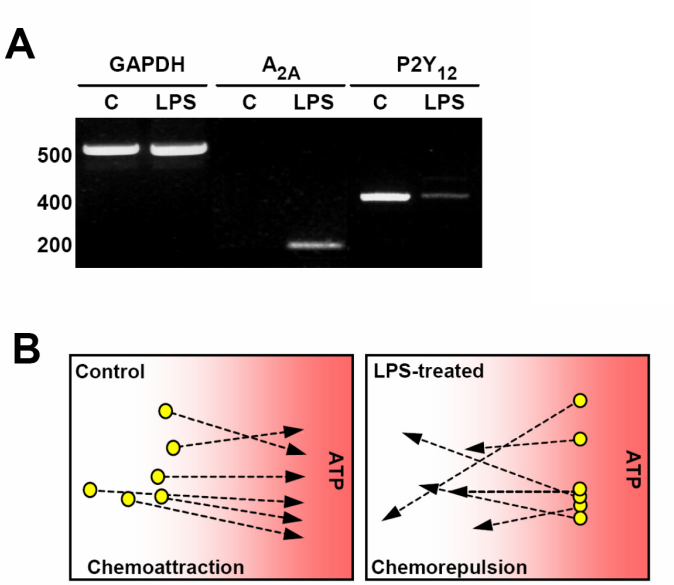

Figure 32. **Chemotactic switch in adult human microglia.** (**A**) RT-PCR analysis revealed that isolated primary human microglia upregulate A_{2A} receptor mRNA and downregulate $P2Y_{12}$ receptor mRNA (n = 4). GAPDH mRNA served as a loading control. (**B**) Migrogram shows directional displacement of microglia in culture in response to ATP gradient application. While untreated primary human microglia migrate toward ATP (Control: n = 6), LPS-treated microglia migrate away from ATP (LPS: 100 ng/ml, n = 6).

CHAPTER 5: Downstream and *In Vivo* Effects of ATP Response Reversal in Microglia

5.1. Abstract

Microglia are known to possess a highly ramified morphology under normal conditions *in vivo*. Recent evidence indicates that microglia extend their processes toward areas of brain injury partly due to ATP release from damaged cells. This chemoattractive response is thought to facilitate microglial engulfment of extracellular debris released during brain injury. However, various neuropathologies involve activated microglia that exhibit an amoeboid morphology with few processes. The causes and consequences of this transformation by activated microglia remain unknown. In previous chapters, we have shown that while resting microglia exhibit chemoattraction toward ATP, activated microglia are repulsed by this nucleotide due to upregulation of the $G\alpha_s$-coupled A_{2A} receptor. Here, we report that A_{2A} receptor activation inhibits phagocytosis by activated microglia and prevents scavenging during acute injury. Moreover, we present evidence that the A_{2A} receptor is upregulated within intact brain tissue during inflammation and that A_{2A} receptor activation induces microglial process retraction *in vivo*. Together, our results suggest that A_{2A}-driven repulsion promotes microglial process retraction *in vivo* and may serve as a regulator of microglial scavenging during brain damage.

5.2. Introduction

As discussed in Chapter 1, Nimmerjahn et al. (2005) and Davalos et al. (2006) have revealed that resting microglia actively sample the extracellular milieu *in vivo* using long ramified processes. This constitutive motile activity has been proposed to reflect microglial surveillance of healthy brain tissue. Upon acute brain injury, rapid motility and homing of microglial processes towards damaged tissue is thought to promote the clearance of toxic debris and dying cells and thereby limit secondary injury following an insult. Thus, process

extension is an important feature of microglial function in both health and disease. In contrast, withdrawal of microglial processes is a characteristic feature of activated microglia and neuroinflammation in diverse CNS diseases. Specifically, a variety of stimuli and neuropathological contexts are known to trigger microglial transformation from a highly branched morphology to rounded cells with little or no processes (Raivich et al., 1999). Although it has been proposed that this dramatic morphological remodeling may reflect changes in the chemotactic and phagocytic properties of microglia (Petersen and Dailey, 2004), the causes and consequences of this phenomenon remain largely unknown.

We have shown that the adenosine A_{2A} receptor emerges upon microglial activation and mediates process retraction and repulsion of microglia from sites of ATP release (*see Chapter 4*). Thus, we hypothesize that the A_{2A} receptor may be involved in microglial retraction *in vivo* during neuroinflammation or brain injury. Indeed, activators of the A_{2A} receptor, including ATP and adenosine, are released under diverse pathological conditions, including trauma, hypoxia, inflammation, or aberrant neuronal activity (Hagberg et al., 1987; Lloyd et al., 1993; Cunha et al., 1996; Verderio and Matteoli, 2001; Koizumi et al., 2003; Blum et al., 2008). ATP and adenosine are also present at lower concentrations in normal brain tissue (Phillis et al., 1987; Chen et al., 1992; Pazzagli et al., 1993; Carswell et al., 1997; Latini and Pedata, 2001). Therefore, it is possible that these endogenously released purines may trigger microglial process retraction by activating A_{2A} receptors that emerge in activated microglia during brain inflammation. However, it is yet unknown whether the A_{2A} receptor expression pattern and effects we observed in cultured microglia are also evident within intact brain tissue. Moreover, it is not known whether repulsion by activated microglia will be evident upon acute tissue injury, which is known to trigger rapid nucleotide release.

Thus, we next investigated whether local tissue damage influences the motility of nearby microglia, and whether A_{2A} receptor activation affects the rate of microglial scavenging

of extracellular matter. Lastly, we examined A_{2A} receptor expression *in vivo*, as well as the effects of A_{2A} receptor blockade on the morphology of activated microglia in a whole-animal model of neuroinflammation.

5.3. Results

a. Functional effects of microglial process retraction

Previous studies have shown that acute injury results in the release of ATP and other nucleotides from damaged cells, which is thought to trigger chemoattraction of microglial processes toward sites of damage (Davalos et al., 2005; Haynes et al., 2006). We therefore asked whether acute tissue damage can repel processes of activated microglia due to the release of intracellular nucleotides into the extracellular space. To address this, we performed time-lapse imaging on $eGFP^+$ microglia co-cultured with wild-type astrocytes that received focal injury with a pipette tip. We observed that acute astrocytic damage triggers adjacent microglia to extend processes toward the injured cells (*data not shown*), corroborating previous reports *in vivo* (Davalos at al., 2005; Nimmerjahn et al., 2005). However, in co-cultures exposed to LPS in order to activate microglia, local cell damage triggered microglial retraction from the injury site (*data not shown*), suggesting that the microglial chemotactic response to acute tissue damage may be reversed during inflammation.

Microglial process extension toward sites of injury is thought to facilitate phagocytic cleanup of debris and thereby help protect surrounding intact tissue (Davalos et al., 2005; Nimmerjahn et al., 2005; Haynes et al., 2006). Since A_{2A} receptor signaling triggers process retraction, we predicted that A_{2A} stimulation would hinder phagocytosis by activated microglia. We therefore examined whether A_{2A} agonists would influence the rate of extracellular substrate uptake by activated microglia. For this, we treated isolated microglia with LPS for 24 hours and then exposed the cells to ATP, CGS-21680 or adenosine for 20

minutes to trigger process retraction. Thereafter, we applied fluorescein-labeled E.coli and allowed phagocytic uptake for 2 hours. As predicted, we observed a decrease in particle uptake by activated microglia in the presence of A_{2A} agonists (Figure 33), suggesting that A_{2A} activation impairs scavenging by activated microglia.

b. A_{2A} receptor upregulation and effects *in vivo*

Lastly, we investigated whether the A_{2A} receptor plays a role in the characteristic retracted morphology assumed by activated microglia during neuroinflammation *in vivo*. For this, we utilized a well-established animal model of neuroinflammation involving systemic LPS exposure (Block et al., 2007). As shown in Figure 34A, LPS-treated animals exhibit microglia with a retracted morphology *in vivo*, which was evident when immunostaining for the microglial marker CD11b. Moreover, the same change in microglial morphology was evident in LPS-treated transgenic *Cx3Cr1-eGFP* mice that exhibit microglia-specific eGFP labeling (Figure 34B; Davalos et al., 2005; Nimmerjahn et al., 2005).

We initially assessed whether the A_{2A} receptor is upregulated during neuroinflammation *in vivo*. For this, we utilized transgenic *Adora-eGFP BAC* mice that express eGFP under the control of the A_{2A} promoter. Figure 35 shows that control animals show highly localized eGFP expression within the striatum, with little or no staining in the cortex, supporting previous findings of striatal A_{2A} localization (Rosin et al., 1998). However, in addition to striatal expression, LPS-treated mice also showed eGFP fluorescence in small cells within the cortex, suggesting that A_{2A} is upregulated in the brain during inflammation. Moreover, in LPS-treated *Cx3Cr1-eGFP* mice, we observed that intracortical injection of the A_{2A}-specific antagonist SCH-58261 resulted in microglial process re-extension, an effect not seen in animals injected with vehicle alone (Figure 36). These observations are consistent with

our *in vitro* data and suggest that activated microglia may assume an amoeboid phenotype *in vivo* due to A_{2A} receptor stimulation by nucleotides released in the brain.

5.4. Discussion

Microglia are thought to scavenge sites of injury by actively engulfing, or phagocytosing, extracellular matter that may have neurotoxic effects on surrounding healthy tissue (Beyer et al., 2000; Nimmerjahn et al., 2005; Davalos et al., 2006; Block et al., 2007; Hanisch and Kettenmann, 2007). This suggests that microglial uptake is a homeostatic process that helps to maintain brain health. A variety of cell surface receptors are involved in the recognition of specific targets and initiation of microglial phagocytosis, including complement, Fγc, and scavenger receptors, which are known to promote amyloid-β (Aβ) clearance (Hickman et al., 2008). Interestingly, while microglia are thought to scavenge Aβ that accumulates early during Alzheimer's disease, it has been proposed that, with disease progression, activated microglia may become ineffective at clearing Aβ (Stalder et al., 2001; Simard et al., 2006; El Khoury et al., 2007). Indeed, it has been suggested that microglia may lose their phagocytic capabilities upon proinflammatory activation (Koenigsknecht-Talboo and Landreth, 2005; Hickman et al., 2008). In support, Fiala et al. (2005) have reported that monocytes and macrophages isolated from Alzheimer's disease patients exhibit diminished phagocytosis of Aβ in comparison to cells from age-matched controls. However, while microglia may exhibit downregulation of phagocytic function during disease, few negative regulators of phagocytosis are known.

Although adenosine enhances phagocytosis in monocytes, it attenuates phagocytosis in differentiated peritoneal macrophages (Leonard et al., 1978; Sung and Silverstein, 1985). To account for these effects, it has been suggested that inhibition of macrophage phagocytosis is mediated by A_2 receptors, whereas augmentation is afforded by A_1 receptor signaling

(Salmon et al., 1993). As monocytes differentiate into macrophages, A_2 receptor expression increases, thus resulting in a switch of the effect of adenosine on phagocytosis. Moreover, the inhibitory effect of adenosine is prevented with PKA blockers, suggesting $G\alpha_s$-coupled signaling downstream of A_2 receptors plays an important role (Eppell et al., 1989).

Here, we found that stimulation of $G\alpha_s$-coupled adenosine A_{2A} receptors suppresses the engulfment of extracellular particles by activated microglia. Consistent with our findings, A_{2A} receptor blockade can promote bacterial clearance in septic animals (Nemeth et al., 2006). Moreover, macrophage phagocytosis is suppressed by prostaglandins via cAMP signaling or by pharmacological agents that elevate intracellular cAMP levels (Newman et al., 1991; Aronoff et al., 2004 and 2005). Makranz et al. (2006) have also reported that while normal cAMP levels in microglia promote phagocytosis of myelin via PKA activation, elevated cAMP production inhibits phagocytic uptake through both PKA and Epac. Indeed, cAMP elevation with PTX, forskolin or phosphodiesterase blockers, or PKA activation with a PKA-specific cAMP analog, 6-Benz-cAMP, is able to block the engulfment of myelin by both microglia and peritoneal macrophages (Bryn et al., 2006; Makranz et al., 2006). cAMP can either promote or prevent F-actin polymerization during phagocytosis (Zalavary and Bengtsson, 1998; Ydrenius et al., 2000). However, little is known about the molecular mechanisms that allow cAMP to regulate phagocytosis. Interestingly, pathogenic microorganisms have evolved mechanisms to exploit the cAMP signaling system. Pathogens such as the human immunodeficiency virus are able to suppress phagocytosis by increasing intracellular cAMP levels, either directly by possessing an adenylate cyclase motif, or indirectly by eliciting the release of mediators that activate $G\alpha_s$-coupled receptors (Thomas et al., 1997; Serezani et al., 2008). These reports further support our findings suggesting that $G\alpha_s$-coupled signaling plays an important role in phagocytosis by microglia, the primary phagocytic cells in the brain.

Using transgenic *Adora-eGFP* mice, we confirmed that A_{2A} receptor upregulation takes place *in vivo* during LPS-induced neuroinflammation, supporting our *in vitro* data (*see Chapter 4*). In the CNS, A_{2A} is thought to be localized primarily in D2 receptor-containing neurons within the striatum (Rosin et al., 1998). However, there has been increasing evidence of A_{2A} receptor expression and function within extrastriatal brain regions and non-neuronal cells (Rosin et al.., 1998). Notably, A_{2A} receptor blockade or knockout is protective in a variety of brain injury models, including transient ischemia and kainate-induced excitotoxicity, suggesting that the A_{2A} receptor plays important roles throughout the brain (Phillis, 1995; Jones et al., 1998; Monopoli et al., 1998; Chen et al., 1999). Consistent with this, A_{2A} receptor expression has been observed in cultured cortical neurons, astrocytes, and microglia (Fiebich et al., 1996; Heese et al., 1997; Nishizaki, 2004; Wittendorp et al., 2004; Rebola et al., 2005; Saura et al., 2005).

Purine nucleotides, including adenosine and ATP, are known to be released in the brain under various physiological and pathological conditions. We found that microglia retract their processes during neuroinflammation *in vivo* at least partly due to A_{2A} receptor activation. Furthermore, we observed that activated microglia retract their processes in response to local acute injury, which is known to trigger purine nucleotide release. Together, our results suggest that both constitutive and injury-triggered release of purines in the brain may induce microglial process retraction under inflammatory conditions. We conclude that A_{2A} receptor activation promotes the characteristic transformation of microglia into amoeboid cells during brain inflammation and may trigger microglial process repulsion from sites of acute injury. Indeed, retracted microglia are observed with most types of neuropathologies and brain injuries that exhibit concomitant inflammation (Kreutzberg, 1996).

Although microglia are thought to accumulate around sites of injury and exhibit increased cell density, we show that the initial chemotactic response by activated microglia is process repulsion from sites of purine nucleotide release. While previous reports have shown increased cell density following brain injury using cell-specific markers, those observations were typically made several days or weeks after an insult, during which time nucleotide release may have subsided, and microglial proliferation and infiltration of peripheral immune cells may also take place. Indeed, Matsumoto et al., (2007) reported that non-microglial immune cells infiltrate sites of damage and express traditional microglial markers. Thus, acute chemotactic behavior of activated microglia shortly after injury cannot be predicted from past studies. Also, our results do not discount the roles of other chemotactic factors that may be involved in attracting microglia toward injury at later time points. In summary, together with our previous *in vitro* data (*See Chapters 3 and 4*), our results suggest that A_{2A}-driven repulsion is an early driving force for microglial deramification and for regulation of microglial motility upon brain damage.

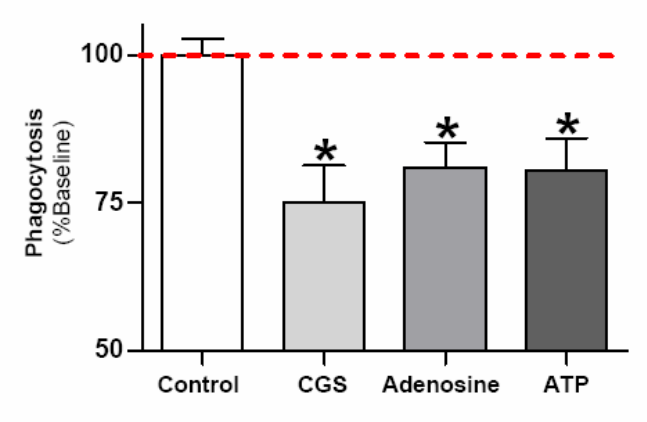

Figure 33. A_{2A} receptor stimulation inhibits phagocytosis by LPS-treated microglia. Treatment with indicated agonists (50 μM) for 20 minutes following microglial exposure to LPS (100 ng/ml) for 24 hours led to a decline in microglial uptake of fluorescein-labeled E.coli, which were applied for 2 hours along with agonists. CGS-21680 (CGS), n > 7, *p<0.05.

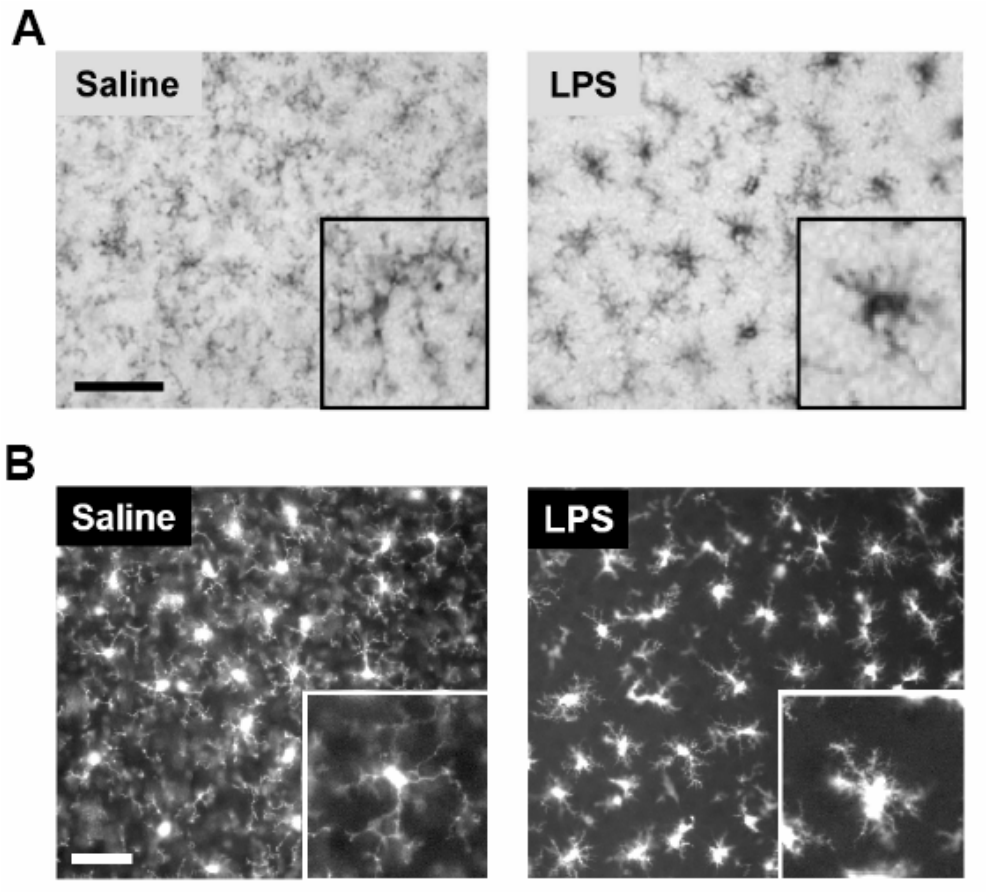

Figure 34. LPS-induced microglial process retraction *in vivo*. Mice injected systemically with LPS (2 mg/kg, 48 hrs) exhibit microglia with highly retracted processes and enlarged cell bodies. Insets show magnified view of a representative cell. (**A**) Fixed cortical brain tissue from wild-type mice was stained with anti-CD11b antibody, a microglia-specific marker. Scale bar: 100 um. (**B**) Fixed cortical brain tissue from transgenic *Cx3Cr1-eGFP* mice that exhibit microglia-specific eGFP expression was immunostained with anti-GFP antibody. Scale bar: 50 um.

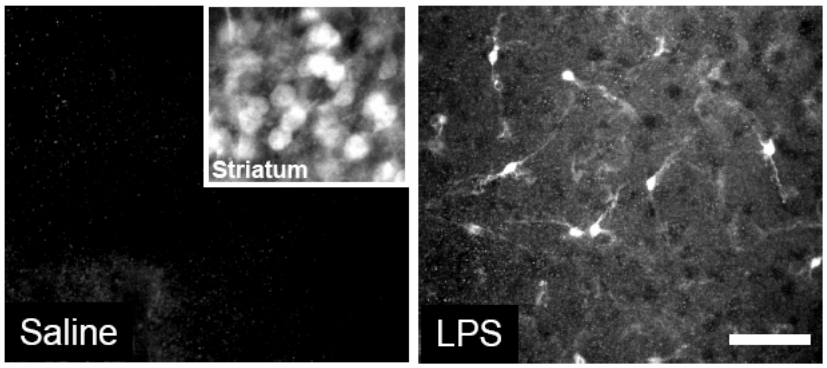

Figure 35. LPS-treated animals exhibit A_{2A} upregulation. Images show fixed cortical brain tissue from BAC-transgenic mice expressing eGFP upstream of BAC A_{2A} coding sequence. Tissue has been stained with anti-GFP antibody. Inset shows constitutive A_{2A} expression within striatal neurons, which served as a positive control for A_{2A} promoter-driven eGFP expression. $n \geq 3$. Scale bar: 50um.

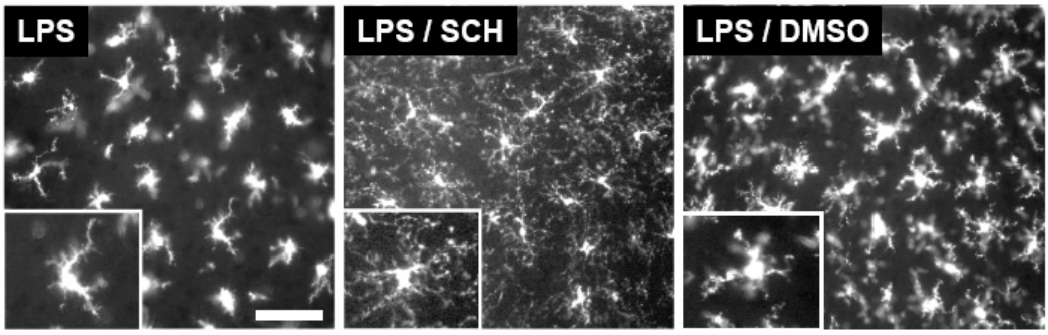

Figure 36. A_{2A} receptor activation promotes microglial process retraction *in vivo*. Intracortical blockade of A_{2A} with SCH-58261 (SCH, 2 nmol) triggered microglial process ramification in LPS-exposed animals (n = 4). Intracortical injection of vehicle (DMSO) has minimal effect on the retracted morphology of LPS-activated microglia. Insets show magnified view of a representative cell. Scale bar: 50 um.

CHAPTER 6: Conclusions and Implications

6.1. Summary

Proinflammatory, or activated, microglia have been implicated as a contributing factor in most neuropathologies. While activated microglia are known to retract into an amoeboid shape during neurological disease or trauma, the cause and significance of this phenomenon remain unknown. We report that the microglial chemotactic response to ATP is reversed upon microglial activation. This reversal, a switch from chemoattraction to repulsion, is driven by upregulation of the G_s-coupled A_{2A} receptor coincident with downregulation of the purinergic G_i-coupled $P2Y_{12}$ receptor. A_{2A} is then activated by the breakdown product of ATP, adenosine. Given that extracellular ATP and its metabolite adenosine are ubiquitous in the brain, our results suggest that activated microglia assume their characteristic amoeboid morphology due to A_{2A}-driven chemorepulsion. We further suggest that this reversal in cell motility may impede activated microglia from performing CNS surveillance and scavenging of injured or diseased brain tissue. Please see Figure 37 summarizing the main results.

Our identification of a signaling pathway that may limit microglial motility and phagocytic uptake provides new rationale for novel therapeutic interventions. Given that A_{2A} gene ablation and A_{2A} receptor antagonists, including caffeine, have shown protective effects in animal models of brain injury and neurodegeneration, it is possible that allowing activated microglia to resume their role as scavengers through blockade of A_{2A} may offer therapeutic benefits. Additional studies into the effects of purinergic receptor agonists on activated microglia are needed to fully elucidate how nucleotides affect various facets of microglial function. Finally, we conclude that G_s-coupled signaling may be an important regulator of microglial function in CNS health and disease. Further investigation of additional G_s-coupled receptors and the effects of this signaling pathway on microglial responses may help to address

the long-standing and fundamental problem in neurobiology regarding the nature of microglial activation as well as reveal novel therapeutic strategies for various CNS diseases.

6.2. Microglial scavenging

Within intact brain tissue, microglia are surrounded by vulnerable cellular structures that possess minimal regenerative capacity. Thus, it is likely that microglial inflammatory responses are tightly regulated *in vivo* in order to preserve neuronal structure and function. Scavenging, or phagocytic uptake, is thought to be a critical aspect of microglial responses during both health and disease. Indeed, it has been shown that microglial cells are able to engulf extracellular debris or degenerating neuronal processes (Bechmann and Nitsch, 1997). As discussed below, phagocytic engulfment can either prevent or promote neuronal loss, and may be under strict regulatory signals from surrounding cells.

It is thought that microglial clearance of extracellular debris may be beneficial for dampening the immune response as well as preventing secondary cell damage. Indeed, degenerating neurons and ongoing cellular distress can promote ongoing inflammation in the CNS (Aldskogius et al., 1999). Likewise, accumulation of aggregated proteins, such as amyloid-β (Aβ), can trigger neuronal damage and inflammation. It is well-known that deposition of aggregated Aβ protein and the formation of senile plaques is a hallmark of Alzheimer's disease (AD), the most prevalent form of dementia. While microglia are known to accumulate around amyloid plaques in human patients and in transgenic mouse models of the disease, their exact role in plaque formation and clearance remains elusive (Frautschy et al., 1998; Wegiel et al., 2001; Simard et al., 2006).

Consistent with the idea that microglial phagocytic responses are beneficial, blocking activation of C3 complement, an inflammatory mediator whose cleavage products promote chemotaxis and phagocytosis, increased Aβ deposition and neuronal degeneration

in hAPP transgenic mice, a model of AD (Wyss-Coray et al., 2002). Moreover, vaccination of PDAPP mice, another model of AD, with Aβ peptides or systemic injection of antibodies against Aβ reduces Aβ burden in the CNS and alleviates neuronal damage and cognitive deficits (Schenk et al., 1999; Bard et al., 2000; Janus et al., 2000). It has been suggested that these effects are mediated primarily by microglial Aβ phagocytosis. To support this, Bard et al. (2000) have shown that while Aβ plaques remained intact after addition of exogenous microglia onto tissue sections of human AD brain, application of anti-Aβ antibodies results in Aβ engulfment by exogenous microglia and elimination of Aβ deposits. Remarkably, this *ex vivo* experiment illustrated that nearly all amyloid was ultimately contained within microglia and was degraded to undetectable levels three days after anti-Aβ antibody application. Thus, although cultured microglia have been observed to engulf Aβ (Kopec and Carroll, 1998; Ard et al., 1996; Bard et al., 2000), these studies suggest that endogenous microglia within intact tissue may be ineffective phagocytes under certain pathological contexts. It is possible that therapeutic enhancement of the rate of microglial scavenging could be an effective strategy for reducing neuronal injury and cognitive decline, especially in neurodegenerative disorders that exhibit extracellular protein deposits that require efficient phagocytic uptake.

Here, we report that Aβ exposure triggers microglia to downregulate $P2Y_{12}$ and upregulate the A_{2A} receptor, which mediates microglial process retraction and repulsion in response to ATP. Moreover, we demonstrate that A_{2A} activation suppresses substrate uptake by activated microglia. These results suggest that Aβ accumulation during AD progression may trigger a reversal in microglial chemotactic response to purines and inhibit microglial tissue surveillance by suppressing process motility. Therefore, we reason that microglia may be ineffective phagocytes during AD due to the repulsive effects of A_{2A} receptor activation, which triggers microglial process retraction and suppression of substrate engulfment. We

further propose that blockade of A_{2A} receptors may help to disinhibit microglial phagocytic responses and improve Aβ clearance, thereby reducing plaque formation and slowing AD progression. Likewise, other neurodegenerative disorders may also benefit from A_{2A} receptor blockade through enhancement of debris clearance and probing of the extracellular space by microglia.

Thus far, microglial phagocytosis has been viewed as a process that benefits cell survival in the context of neurodegeneration. However, phagocytosis can also lead to death of cells otherwise capable of surviving. Indeed, phagocytes in the developing *C. elegans* can promote the apoptotic process by engulfing cells with weak pro-apoptotic activity (Reddien et al., 2001; Hoeppner et al., 2001). These studies revealed that mutation of genes involved in the phagocytic process results in the survival and differentiation of cells that would otherwise die. Thus, while intracellular apoptotic signaling in dying cells is required for engulfment, it is possible that microglial phagocytic functions may favor neuronal degeneration.

In a model of localized anterograde injury, axotomy of the neuronal fibers traveling from the entorhinal cortex to the dentate gyrus results in localized microglial activation in the zone of denervation within the dentate (Rappert et al., 2004). The chemokine CXCL10, a ligand for the CXCR3 receptor, is upregulated in injured neurons within the entorhinal cortex and dentate gyrus. Notably, animals missing the CXCR3 gene, a receptor involved in microglial migration (Biber et al., 2001), exhibit lower dendritic loss than wild-type mice, suggesting that dendrites persist due to ineffective microglial recruitment to the injury site. Moreover, Marin-Teva et al. (2004) have shown that microglia promote developmental Purkinje cell death in the cerebellum by releasing superoxide anions and engulfing neurons containing caspase-3 activity. Notably, elimination of microglia from cerebellar slices rescues these Purkinje neurons, which were able to survive and develop complex dendritic arborizations. Together, these studies suggest that microglia are more than passive scavengers

that engulf debris and dying cells. On the contrary, increasing evidence indicates that microglia may be the perpetrators of neuronal cell death due in part to their phagocytic capabilities.

Interestingly, it has been postulated that neurons with weak pro-apoptotic activity may release regulatory signals that control their own engulfment by either attracting or repelling microglia (Marin-Teva et al., 2004). In light of our current findings, we put forward that neuronal release of nucleotides may help to fulfill this regulatory function. Indeed, the release of ATP during acute cell injury is known to attract naive microglia and promote local scavenging of the injured site (Honda et al., 2001; Nimmerjahn et al., 2005; Davalos et al., 2006; Haynes et al., 2006). In contrast, we have discovered that ATP can also repel microglia under proinflammatory conditions, when microglia are likely to exhibit elevated neurotoxic and phagocytic activity. Furthermore, Koizumi et al. (2007) have recently shown that UDP is also released from injured cells to promote microglial phagocytosis via $P2Y_6$ activation. Thus, nucleotides may serve as an important class of diffusible signals that modulate microglial scavenging.

6.3. Roles of A_{2A} in neuroinflammation

Upon injury in peripheral organs, the release of adenosine and consequent activation of the A_{2A} receptor is thought to regulate inflammatory responses (Hasko and Cronstein, 2004; Sitkovsky and Ohta, 2005; Hasko et al., 2007; Palmer and Trevethick, 2008). The majority of these effects on inflammation are thought to be mediated by PKA-dependent intracellular signaling, which is known to have potent effects on immune cell function (Palmer and Trevethick, 2008). Similarly, we have evidence suggesting that the A_{2A} receptor regulates inflammation in the CNS by modulating microglial morphology and scavenging functions. Consistent with this, it is known that both ATP and adenosine are present in the brain under

normal conditions (Latini and Pedata, 2001; Burnstock, 2007; Fujita et al., 2008), and that these factors are elevated during brain trauma, hypoxia, or increased energy use (Hagberg et al., 1987; Lloyd et al., 1993; Cunha et al., 1996; Pedata et al., 2001; Rivkees et al., 2001). During hypoxia, adenosine can reach high concentrations due to suppression of adenosine kinase, enhanced 5'-nucleotidase activity, as well as increased release of adenine nucleotides and their degradation by a cascade of ectonucleotidases, such as CD39 and CD73 (Dunwiddie and Masino, 2001). Indeed, cerebral ischemia in the rat can trigger increased ecto-5'-nucleotidase expression in astrocytes and microglia surrounding the infarct zone (Braun et al., 1997). Additionally, the proinflammatory cytokine IL-1β has been shown to trigger a rise in extracellular ATP and adenosine levels in hippocampal slices, suggesting that these factors may be elevated during neuroinflammation (Sperlágh et al., 2004). However, the accumulation of adenosine may be temporary due to its uptake via specific transporters and the actions of ecto-adenosine deaminase, which converts adenosine to inosine (Latini and Pedata, 2001; Palmer and Trevethick, 2008). Thus, stimulation of the A_{2A} receptor by adenosine is likely to be a highly regulated event that takes place in a spatially and temporally restricted manner. Therefore, the effects of adenosine on microglia may be constrained to regions of tissue injury or cellular distress.

Caffeine is the most widely consumed psychostimulant and is known to be an adenosine receptor antagonist. Interestingly, the incidence of Parkinson's disease (PD) declines with increased caffeine intake, showing a 5-fold risk reduction with typical caffeine consumption (Ross et al., 2000; Ascherio et al., 2001). Likewise, there is some evidence of a similar inverse correlation between caffeine intake and AD (Maia and de Mendonca, 2002). However, the mechanism by which caffeine affects brain injury is unknown.

While caffeine binds to the adenosine receptors A_1, A_{2A} and A_{2B} with similar potency (K_D (μM): A_1-12, A_{2A}-2.4, A_{2B}-13; Fredholm et al., 1999), the neuroprotective actions of

caffeine can be mimicked with A_{2A}-selective receptor antagonists, such as KW6002 and SCH58261, or by deletion of the A_{2A} receptor gene. Indeed, administration of A_{2A} blockers has been shown to be neuroprotective in various brain injury models, including kainate-induced excitotoxicity (Jones et al., 1998), ischemic injury (Phillis, 1995; Monopoli et al., 1998; Melani et al., 2003), the MPTP model of PD (Chen et al., 2001; Ikeda et al., 2002), and the APPsw mouse model of AD (Arendash et al., 2006). In contrast, A_1 receptor antagonists do not exhibit similar neuroprotective effects (Chen et al., 2001).

It is likely that caffeine and other A_{2A} receptor antagonists may affect various types of brain injury models through a common mechanism. Notably, microglia-driven neuroinflammation is a common thread linking various brain injuries and neurodegenerative disorders. However, the possibility that A_{2A} receptor antagonists may alleviate brain injury by regulating microglial responses has not been explored. Based on our findings, it is possible that inhibition of the A_{2A} receptor expressed by activated microglia may play a vital role in the neuroprotective effects of caffeine and other A_{2A} blockers during brain injury. Indeed, pharmacological agents that target neuroinflammatory responses by microglia, including non-steroidal anti-inflammatory drugs (NSAIDs) and minocycline, often have neuroprotective effects (Wyss-Coray and Mucke, 2000; Yune et al., 2007; Hailer, 2008). We propose that the reported neuroprotection afforded by A_{2A} receptor inhibitors may be due to blockade of the repulsive effects of purine nucleotides on activated microglia. Specifically, caffeine and other A_{2A} receptor antagonists may allow these immune cells to better perform their roles as scavengers and thereby minimize secondary cell loss and ongoing inflammation.

Lastly, our findings imply that while purinergic signaling may promote inflammatory responses by naïve microglia (Nimmerjahn et al., 2005; Inoue, 2008), a switch in microglial purinergic receptor expression may convert purines to suppressors of inflammatory responses by activated microglia. Further studies into the effects of adenosine on the function of

microglia in their activated state, including the release of proinflammatory modulators and cell proliferation, would help to address whether this signaling factor modulates other aspects of microglial reactivity and indeed possesses anti-inflammatory properties.

6.4. Regulation of microglia by G_s-coupled signaling

The most common and salient feature of microglial activation, and hence brain inflammation, is the retraction of microglial processes. Specifically, neuroinflammation during brain damage is typically marked by the loss of the elaborately branched microglial processes and consequent presence of rounded, amoeboid microglia. Indeed, accounts of microglial activation are often based on these morphological criteria. This phenomenon has been noted for over 80 years and serves as a hallmark of inflammation and injury in the brain. While it has been hypothesized that this structural remodeling reflects changes in the chemotactic and phagocytic properties of microglia upon activation (Petersen and Dailey, 2004), both the causes and consequences of microglial process retraction have remained a mystery.

Here, we report that ATP, or its breakdown product adenosine, serves as a potent chemorepellent that triggers activated microglia to assume a retracted morphology. To our knowledge, this is the first microglial chemorepellant to be identified. Moreover, we show that its effects are driven by the G_s-coupled intracellular signaling pathway. Although many agonists are known to activate G_s-coupled receptors, including epinephrine, histamine, serotonin, and prostaglandins, the effects of G_s-coupled receptor activation on microglial function requires further studies. Interestingly, G_s-coupled signaling has been found to either enhance or diminish proinflammatory cytokine release and may thus play a dynamic role in microglial activation (Tomozawa et al., 1995; Woo et al., 2003; Min et al., 2004; Noda et al., 2007). Our results suggest that this signaling pathway regulates microglial

morphology and process motility. Our findings raise the new hypothesis that activated microglia retract and avoid stressed or injured tissue due to emergence of G_s-coupled signaling. However, it is not yet known whether other G_s-coupled receptors trigger similar effects in microglia. Thus, further studies of other receptors coupled to G_s may reveal novel mechanisms by which microglial responses are regulated.

6.5. Final Thoughts

While inflammation is generally thought to protect an organism and promote a return to homeostasis, it possible that the injured or aging brain can also be harmed by certain inflammatory responses. Neuroinflammation, a complex process driven primarily by activated microglia, has been hypothesized to contribute to brain damage. Indeed, most neurodegenerative disorders and acute brain injury involves microglial inflammatory responses, which seem to play an active and contributing role in brain damage. Therefore, in order to better understand and control CNS injury, it is important to investigate which functions of microglia are harmful and which are beneficial, and which signaling mechanisms lead to neuroprotective effects during injury. It is possible that microglia become increasingly dysfunctional with age or repeated insult and gradually lose their neuroprotective properties, which may include tissue surveillance and debris scavenging. Alternatively, changes in CNS tissue could shift microglia into an altered phenotype that prevents these immune cells from carrying out their protective functions. Indeed, aging is associated with microglial activation and increased expression of proinflammatory factors, and is a common risk factor for neurodegenerative disorders (Lee et al., 2000). Thus, in order to understand and control brain injury that occurs with various neuropathologies, we must understand the mechanisms regulating microglial function. Deeper insight into

microglia may offer specific markers that could serve as early indicators of neurodegeneration and aid in diagnosis and prevention of various neuropathologies.

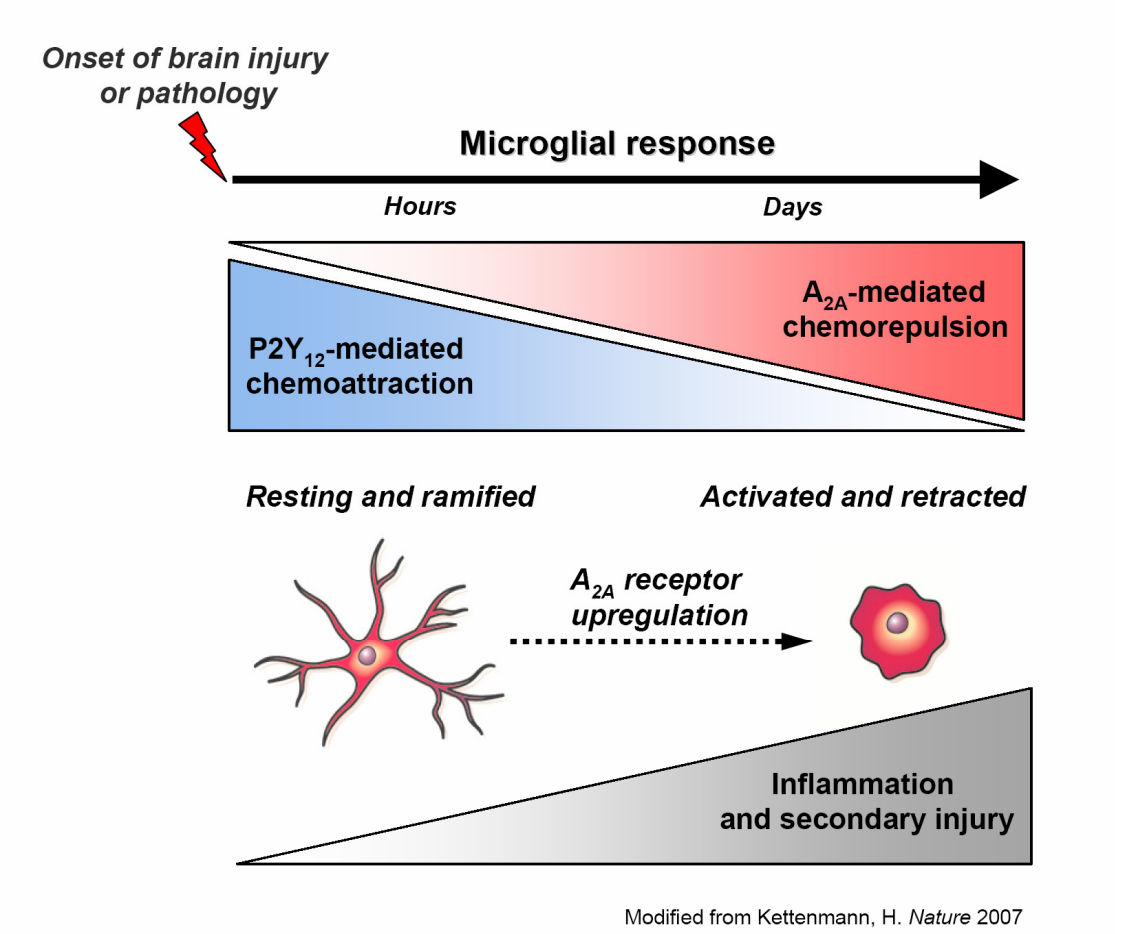

Figure 37. A schematic summarizing the main results. In the short term, resting microglia are attracted to sites of brain injury partly via stimulation of $P2Y_{12}$ receptors. In the long term, however, microglia lose $P2Y_{12}$ expression and assume a characteristic amoeboid morphology that coincides with brain inflammation and continuing tissue injury. During this period, we found that A_{2A} receptors emerge to mediate microglial retraction and chemorepulsion from sites of acute injury. In addition to increased release of neurotoxic factors, this chemotactic switch may be a critical turning point after which microglia become deleterious.

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