Published in final edited form as:

Glia. 2015 October ; 63(10): 1797–1808. doi:10.1002/glia.22845.

Alkylindole-sensitive receptors modulate microglial cell migration and proliferation

Susan Fung^{1,2}, Allison E. Cherry¹, Cong Xu¹, and Nephi Stella^{1,2,3}

¹Department of Pharmacology, University of Washington, 1959 NE Pacific Way, Seattle, WA 98195

²Graduate Program in Neurobiology and Behavior, University of Washington, 1959 NE Pacific Way, Seattle, WA 98195

³Department of Psychiatry and Behavioral Sciences, University of Washington, 1959 NE Pacific Way, Seattle, WA 98195

Abstract

Ligands targeting G protein-coupled receptors (GPCR) expressed by microglia have been shown to regulate distinct components of their activation process, including cell proliferation, migration and differentiation into M1 or M2 phenotypes. Cannabinoids, including the active component of the *Cannabis* plant, tetrahydrocannabinol (THC), and the synthetic alkylindole (AI) compound, WIN55212-2 (WIN-2), activate two molecularly identified GPCRs: CB₁ and CB₂. Previous studies reported that WIN-2 activates an additional unknown GPCR that is not activated by plant-derived cannabinoids, and evidence indicates that microglia express these receptors. Detailed studies on the role of AI-sensitive receptors in microglial cell activation were difficult as no selective pharmacological tools were available. Here, three newly-developed AI analogues allowed us to determine if microglia express AI-sensitive receptors and if so, study how they regulate the microglial cell activation process. We found that mouse microglia in primary culture express functional AI-sensitive receptors as measured by radioligand binding and changes in intracellular cAMP levels, and that these receptors control both basal and ATP-stimulated migration. AI analogues inhibit cell proliferation stimulated by macrophage-colony stimulating factor (M-CSF) without affecting basal cell proliferation. Remarkably, AI analogues do not control the expression of effector proteins characteristic of M1 or M2 phenotypes; yet activating microglia with M1 and M2 cytokines reduces the microglial response to AI analogues. Our results suggest that microglia express functional AI-sensitive receptors that control select components of their activation process. Agonists of these novel targets might represent a novel class of therapeutics to influence the microglial cell activation process.

Keywords

microglia; cannabinoid; GPCR; cell migration and cell proliferation

Introduction

Microglia play a central role in the brain's response to a wide range of injuries, including injuries induced by changes in local metabolic supplies and homeostasis, infections and pathological processes (Kettenmann et al., 2011). The microglia phenotype is generally accepted to be both highly plastic and influenced by specific mediators produced by each type of brain injury (Hanisch and Kettenmann, 2007; Ousman and Kubes, 2012). Thus, microglia express membrane receptors that sense these various mediators, and activation of these receptors triggers changes in microglia phenotype that tailors cellular function. Among the different classes of membrane receptors expressed by microglia, G protein-coupled receptors (GPCR) play important roles in modulating distinct components of the microglial cell activation process. Several GPCRs control various microglial mechanisms: purinergic P2Y₁₂ receptors modulate the movement of microglial cell processes that survey the brain parenchyma (Haynes et al., 2006; Miller and Stella, 2009; Lambert et al., 2010); cannabinoid CB₂ receptors control microglia cell proliferation, migration and the induction of M1 phenotype (Walter et al., 2003; Carrier et al., 2004; Correa et al., 2009; Luongo et al., 2010); GPR55 and GPR18 stimulate cell migration (Kohno et al., 2006; Oka et al., 2007; Ryberg et al., 2007; McHugh et al., 2010; Kallendrusch et al., 2013); and mu opioid receptors inhibit cell migration (Chao et al., 1997). Thus, GPCRs expressed by microglia represent promising targets for therapeutics designed to control distinct components of their activation process.

Recent evidence suggests that microglia express a novel GPCR subtype that remains uncharacterized with regard to its pharmacological profile, coupling mechanism and molecular identity (Stella, 2010). Specifically, microglia express cannabinoid-like receptors that are activated by the prototypical AI synthetic CB₁/CB₂ receptor agonist WIN-2 and not by the phytocannabinoid ⁹-tetrahydrocannabinol (THC), or by the AC-bicyclic analogue of THC, CP55940 (Pertwee et al., 2010). These receptors are commonly referred to as AI-sensitive receptors and are thought to couple to G proteins and are likely to regulate specific cellular functions (Abood et al., 2012). Thus, ligands acting at these novel targets might represent promising therapeutics to treat a variety of brain insults (Kozela et al., 2010, 2013). We recently developed a new series of AI analogues that activate AI-sensitive receptors with nanomolar potencies and exhibit reduced affinity to CB₁/CB₂ receptors, providing novel chemical tools that allow for the study of the coupling mechanism and biological role of these receptors in various model systems (Stella and Kline, 2012, 2013). Here, we sought to utilize these tools to determine if AI-sensitive receptors are expressed by mouse microglia in primary culture and whether they control various aspects of their activation process.

Materials and Methods

Reagents

ST-11 was synthesized by John W. Huffman at Clement University, and ST-47 and ST-48 were synthesized by Medchem Source (Seattle, WA). SR144528 was provided by the NIDA drug supply program (RTI International, Research Triangle Park, NC). WIN55212-2 and O-2050 were purchased from Tocris (Minneapolis, MN), and ATP and isoproterenol were

purchased from Sigma (St Louis, MO). [^3H]WIN55212-2 and [^3H]thymidine were purchased from Perkin Elmer (Boston, MA). All drug dilutions and experiments were performed using silanized glass test tubes (Alltech, Deerfield, IL) and silanized pipette tips (VWR Scientific, Brisbane, CA) to minimize the loss of hydrophobic compounds.

Cell culture and migration

All experiments were performed in accordance with Institutional Animal Care and Use Committee at the University of Washington. Primary cultures of mouse microglial cells were generated from 1-3 day old C57BL-6 pups as previously described (Walter et al., 2002). Briefly, mixed glial cell cultures were maintained in T75 flasks (BD Biosciences, San Jose, CA) in DMEM supplemented with 10% FBS, 10% F12 media, 5 mM HEPES, 100 IU penicillin and 100 $\mu\text{g/ml}$ streptomycin for up to six weeks. Starting on week 2 and every week thereafter, floating and loosely adherent microglia were harvested by shaking. Cells were used in Boyden chamber (A series, 96-well format) migration assays as previously described (Miller and Stella 2009) with minor modifications. Specifically, polycarbonate filters with a pore diameter of 10 μm (Neuroprobe, Gaithersburg, MD) were incubated in 10 ml of a collagen solution [2.5 mg rat tail collagen I (BD Biosciences, San Jose, CA), 57 μl glacial acetic acid (Fisher, Santa Clara, CA) in 50 ml distilled water] for 30 min at 37°C. Filters were rinsed 3 times with PBS and allowed to dry before use. Cells were allowed to migrate for 3 hrs at 37°C, after which the number of DRAQ5 (BioStatus, Shephed, Leicestershire, UK)-labeled cells was quantified using the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE) with the following scanning settings: resolution, 169 μm ; quality, low; focus off-set, 1 mm; and intensity, 4.0. For experiments testing the effect of cytokines, cells were pre-treated with IL-4 (10 ng/ml) or TNF α (5 ng/ml) and IFN γ (30 U/well) (all from R&D Systems, Minneapolis, MN) for 10 min on ice before loading them in the Boyden chamber.

Radioligand binding

Microglia membranes were prepared by lysing cells with ice-cold homogenization buffer (50 mM Tris, 1 mM EDTA, and 3 mM MgCl_2). Lysed cells were homogenized with a Dounce homogenizer (Wheaton Science Products, Millville, NJ). Homogenates were centrifuged at $200 \times g$ for 10 min at 4°C, and the supernatant was collected and stored at -80°C until further use. On the day of experiments, membranes were thawed and pooled, and the protein concentration was quantified with the DC protein assay (BioRad, Hercules, CA) using BSA as a standard. Binding buffer (pH 7.4) contained the following: 50 mM Tris-base, 1 mM EDTA, 3 mM MgCl_2 , and 1 mg/ml BSA. The following components were added to test tubes placed on ice: 50 μl of binding buffer with compound, 50 μl of binding buffer with radioligand, and 100 μl of binding buffer containing membranes (100 μg of protein). Tubes were then covered and incubated for 1 hr in a 30°C water bath with mild agitation. Reactions were terminated by rapid filtration using a Brandel harvester (Brandel, Gaithersburg, MD), collecting radioactive membranes on Whatman GFB filter strips (Brandel, Gaithersburg MD), and rinsing with ice-cold binding buffer. Filters were transferred to 7-ml glass scintillation vials (VWR Scientific, Brisbane, CA) using the Brandel Manual Deposit System (Brandel, Gaithersburg, MD), and 4 ml scintillation fluid (National Diagnostics, Atlanta GA; Ecoscint XR) was added to each vial. Samples were

vortexed for 10 sec and followed by 18 hrs incubation at room temperature prior to quantifying radioactivity with a scintillation counter (PerkinElmer, Boston, MA).

cAMP measurement

cAMP levels were measured as previously described (Franklin et al., 2003). Briefly, microglia were seeded at 5×10^4 cells *per* well in 48-well plates (Corning, Tewksbury, MA) and maintained for 24 hrs in the cell culture conditions described above. When required, PTX (1 μ g/ml) was added to the culture media 24 hrs after cell seeding. To measure cAMP levels, cells were placed in a shaking water bath at 37°C with mild agitation, and culture media were replaced with 500 μ l of assay buffer (20 mM HEPES, 5 mM NaHCO₃, 100 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 1 mM NaH₂PO₄, and 10 mM D-(+)-glucose). After 15 min, the buffer was replaced by a pre-incubation buffer containing non-specific phosphodiesterase inhibitor IBMX (1 mM), and after 10 min, this buffer was replaced with buffer containing both IBMX and the indicated ST-compounds for 15 min at 37°C. The reaction was terminated by the addition of 75 μ l of 0.1% Triton X-100 in NaOH (0.1 M) and 75 μ l of 0.1% Triton X-100 in HCl (0.1 M). Sample lysates were used to quantify cAMP levels using a [¹²⁵I]cAMP radioimmunoassay kit according to the manufacturer's guidelines (PerkinElmer, Waltham, MA).

Cell viability and proliferation

Microglia were plated at a density of 2.5×10^4 cells/well in 48-well plates (Corning, Tewksbury, MA) and maintained in normal cell culture conditions for 24 hrs. Cells were treated with ST-compounds and [³H]thymidine (PerkinElmer, Waltham, MA) for 72 hrs at 37°C. To measure cell viability concomitantly with cell proliferation, cells were incubated with 10 μ l WST-1 (Roche, Indianapolis, IN) for 60 min at 37°C prior to cell lysis. The WST-1 absorbance was measured using a SpectraCount BS10000 (PerkinElmer, Waltham, MA) at a wavelength of 450 nm. Culture media was removed, and cells were rinsed with 250 μ l ice-cold PBS and lysed with 250 μ l NaOH (1 M). Cell lysates were kept on ice for 10 min and then transferred to 7-ml glass scintillation vials (VWR Scientific, Brisbane, CA). Distilled H₂O (250 μ l) was added to each vial followed by 4 ml scintillation fluid (National Diagnostics, Atlanta GA; Ecoscint XR). Samples were vortexed and radioactivity was measured using a scintillation counter (PerkinElmer, Waltham, MA).

Nitric Oxide and IP-10 measurements

Microglia were plated at a density of 2×10^5 cells/well in 24-well plates (Corning, Tewksbury, MA) and maintained in standard cell culture conditions for 24 hrs. Culture media was switched to MEM supplemented with 10% Cellgro, 5 mM HEPES, 100 UI penicillin and 100 μ g/ml streptomycin. Cells were pre-incubated with ST-11 for 24 hrs before treatment with TNF α (5 ng/ml) and IFN γ (30 IU) (R&D Systems, Minneapolis, MN) for 24 hrs. Culture media (100 μ l) was removed from each well and combined with 100 μ l Griess reagent (Sigma, St. Louis, MO) to measure nitrite levels by absorbance at 540 nm. The remaining culture media was removed and saved at -80°C for cytokine measurements. Cytokines were measured via a Luminex multiplex assay according to the manufacturer's instructions (Luminex, Austin, TX).

Quantitative RT-PCR

Microglia were plated at a density of 5×10^5 cells/well in 6-well culture plates (Corning, Tewksbury, MA) and maintained in standard cell culture conditions for 24 hrs. Cells were switched to macrophage-serum free media (Life Technologies, Grand Island, NY) for 24 hrs, and ST-11 pre-treatment occurred for 1 hr before the end of the serum deprivation period. Cells were rinsed and returned to standard cell culture media and treated with IL-4 (10 ng/ml) (R&D Systems, Minneapolis, MN) or vehicle. Twenty-four hours later, cells were rinsed with PBS and frozen at -80°C until further use. RNA was isolated using a PerfectPure RNA Cultured Cell kit according to the manufacturer's instructions (5 PRIME, Gaithersburg, MD). Quantitative RT-PCR was performed with the LightCycler® 480 RNA Master Hydrolysis Probes kit (Roche, Indianapolis, IN) using the following primers: FIZZ1 (probe 3) forward, tatgaacagatgggcctcct and reverse, aggcagttgcaagtatctcca; Ym1 (probe 88) forward, aagaacactgagctaaaaactcct and reverse, gagaccatggcactgaacg; Ribosomal protein S5 (probe 71) forward, cactgcgtcgagtgaatcag and reverse, gctcatctgcaaggcactc; Eukaryotic translation initiation factor 4A2 (probe 53) forward, cgatctactaccaatcgtgaa and reverse, accttctctccaaatcgac; Ubiquitin C (probe 11) forward, gaccagcagaggctgatctt and reverse, cctctgaggcgaaggactaa.

Statistics

GraphPad Prism software version 5.01 (La Jolla, CA) was used to conduct statistical analysis. Data are presented as mean $\pm$ S.E.M., and statistical significance was determined with a one-way ANOVA followed by Dunnett's post-test or a two-tailed Student's t-test.

Results

Microglia express functional AI-sensitive receptors

To determine if microglia express functional AI-sensitive receptors, we selected three AI analogues that contain chemical characteristics required for binding selectivity and receptor activation (Figure 1a-c) (Stella and Kline, 2012, 2013). Specifically, ST-11 and ST-48 activate AI-sensitive receptors with nanomolar potency and contain a methyl moiety on the 2 position of the indole to reduce interaction with CB₁/CB₂ receptors (Figure 1a,b) (Huffman and Padgett, 2005). ST-11 represents the basic chemical scaffold used for designing the new series of AI analogues. ST-48 contains a morpholino moiety linked to the indole ring, which increases water solubility. We also selected one AI analogue (ST-47) that does not bind to AI-sensitive receptors despite minimal chemical difference and accordingly represents a useful selectivity control (Figure 1c). Specifically, ST-47 contains a methoxybenzene moiety instead of a 4-methylnaphthoyl moiety that precludes binding to AI-sensitive receptors. Thus, ST-11, ST-47 and ST-48 interrogate critical chemical characteristics required for binding to and activation of AI-sensitive receptors. When measuring [³H]WIN55212-2 radioligand binding in microglia membranes we found that WIN55212-2, ST-11 and ST-48 competed for [³H]WIN55212-2 binding (k_i = 69.5 nM, 32.6 nM and 23.7 nM, respectively), whereas ST-47 did not compete (Figure 1d, e), suggesting that microglia express AI-sensitive receptors. Second, the CB₁ antagonist O-2050 and CB₂ antagonist SR144528 (SR2) did not compete for [³H]WIN55212-2 binding (Figure 1e), suggesting that CB receptors are not detectable in these cells.

Several studies have suggested that AI-sensitive receptors couple to G proteins (Breivogel et al., 2001; Monory et al., 2002) (manuscript in preparation). Therefore, we tested whether AI analogues control microglial cAMP levels as an index of GPCR coupling to G_s and/or $G_{i/o}$ proteins. We treated microglia in primary culture with ST-11, ST-47 and ST-48 and measured changes in intracellular cAMP levels using a radioimmunoassay. We found that both ST-11 and ST-48 increased cAMP with maximal responses of $303 \pm 81\%$ and $206 \pm 33\%$ of basal levels when used at 30 and 300 nM, respectively (Figure 1f), and ST-47 produced no effect (Figure 1g). In support of a signaling amplification mechanism mediated by GPCR coupling to effector proteins, the potencies of these responses ($EC_{50} = 2$ nM and 11.3 nM for ST-11 and ST-48, respectively) were lower than the binding affinities of these compounds (compare Figure 1d and 1f) (Falkenburger et al., 2013). These results suggest that AI-sensitive receptors couple to G_s , although with somewhat low efficacy, since by comparison the β -adrenergic receptor agonist isoproterenol increased microglial cAMP levels by $2569 \pm 912\%$ over basal ($P < 0.001$, $n = 20$ independent experiments performed in triplicate). To test for the involvement of $G_{i/o}$ proteins, we incubated microglia for 24 hrs with pertussis toxin (PTX) to uncouple $G_{i/o}$ proteins and determined if this treatment affected the responses induced by ST-11 and ST-48. As expected, PTX incubation did not modify the ST-11- and ST-48-stimulated increases in cAMP levels, suggesting that AI-sensitive receptors couple to G_s proteins (Figure 1g). Together, these results show that primary mouse microglia in culture express functional AI-sensitive receptors and support previous studies reporting the absence or very low expression of CB_1/CB_2 receptors in this model system (Carlisle et al., 2002; Miller and Stella, 2009).

AI-sensitive receptors control microglial cell migration and proliferation

GPCRs regulate distinct aspects of cell migration, including basal and stimulated migration (Miller and Stella, 2008). Specifically, basal cell migration can be categorized as the random motility of the cell body occurring in the absence of a chemical stimulus. Stimulated migration encompasses two main mechanisms: chemokinesis, which is the increased random cell migration in response to a chemical stimulus, and chemotaxis, which is the directed migration towards an increasing chemical gradient (Wilkinson, 1998; Miller and Stella, 2008). We tested whether the prototypical AI analogue ST-11 alters basal and/or ATP-stimulated migration to determine if AI-sensitive receptors control these functions. Specifically, we measured cell migration by quantifying the number of cells labelled with a near-infrared (NIR) emitting dye that had migrated to the bottom surface of a Boyden chamber filter (Figure 2a) (Miller and Stella, 2009). ATP was chosen as a stimulus a because it is released by injured cells and promotes both chemokinesis and chemotaxis in microglia (Haynes et al., 2006). We found that low levels of basal cell migration were significantly inhibited by ST-11 ($34 \pm 10\%$, $n = 8$, $P < 0.001$ compared with vehicle-treated cells) (Figure 2a). ATP increased migration by 2.9 fold over basal migration (Figure 2b), and ST-11 dose-dependently inhibited this response ($IC_{50} = 868$ pM, 46% maximal inhibition of ATP response) (Figure 2c). To exclude the possibility that this inhibitory response was due to acute cellular toxicity of ST-11, cell viability was assessed using the tetrazolium salt WST-1 (an index of mitochondrial activity) (Berridge et al., 2005). We found that ST-11 did not affect cell viability at concentrations up to 3 μ M (Figure 2d). These

results suggest that AI receptors negatively modulate both basal and stimulated microglial cell migration.

In addition to cell migration, cell proliferation is either positively or negatively affected in response to mediators acting through GPCRs, depending on the receptor subtype (Derocq et al., 1995; Carrier et al., 2004). To determine if AI-sensitive receptors regulate basal cell proliferation, we incubated mouse microglia in primary culture with [³H]thymidine and ST-11 for 72 hrs and measured the amount of [³H]thymidine incorporated into the cells by liquid scintillation. We found that ST-11 had no significant effect on basal microglial cell proliferation (Figure 3a). To determine if AI-sensitive receptors regulate stimulated cell proliferation, we incubated microglia with L929-conditioned media containing M-CSF, an agonist at CSF1R known to trigger microglia cell proliferation and to be produced in response to certain brain injuries (Théry and Mallat, 1993; Imai and Kohsaka, 2002). M-CSF increased microglial cell proliferation by $69 \pm 10\%$ (Figure 3a, b), and ST-11 decreased this response (IC_{50} of 23 nM, 53% maximal inhibition of L929 response) (Figure 3a). We verified that the decrease in proliferation was not due to cellular toxicity by concomitantly measuring cell viability with WST-1 and found no significant effect on cell viability (Figure 3c). These data suggest that AI-sensitive receptors do not affect the basal rate of cell proliferation yet inhibit cell proliferation stimulated by M-CSF.

AI-sensitive receptors do not control microglial cell M1 and M2 phenotypes

An M1 phenotype is typified by the production of pro-inflammatory cytokines and free radicals and can be triggered by various stimuli, including lipopolysaccharide (LPS) and interferon γ ($IFN\gamma$) with tumor necrosis factor α ($TNF\alpha$). The M2 phenotype can be induced by IL-4 and is characterized by the up-regulation of Arginase 1, Chitinase-3-like protein 3 (Ym1), and Found in inflammatory zone 1 (FIZZ1) expression, which participate in tissue repair. Several subtypes of GPCRs control microglial cell activation by modulating the effector proteins that are characteristic of the M1 and M2 phenotypes (Colton, 2009). To determine if AI-sensitive receptors control the M1 phenotype, we incubated microglia with $TNF\alpha$ and $IFN\gamma$ and measured nitrite accumulation as an index of iNOS induction (Mosser and Edwards, 2008) and IP-10 accumulation as index of cytokine production (Vanguri and Farber, 1994; Hua and Lee, 2000). We found that $TNF\alpha$ and $IFN\gamma$ stimulated microglia nitrite production by 4.2-fold and IP-10 production by 4.3-fold (Figure 4a-c). Remarkably, ST-11 did not affect either of these responses (Figure 4d-g). We next measured the effect of ST-11 on the M2 phenotype by incubating microglia with IL-4 and quantifying Ym1 and FIZZ1 mRNA expression by qRT-PCR (Raes et al., 2002) (Figure 5a, b). We found that IL-4 induced a strong increase in Ym1 and FIZZ1 mRNA expression and that ST-11 did not affect either basal or IL-4-induced Ym1 and FIZZ1 expression (Figure 5c). Together, these results suggest that AI-sensitive receptors do not control the induction of M1 or M2 phenotypes.

M1 and M2 microglia cell activation differentially affects AI-sensitive receptor signaling

The results obtained so far have described the role of AI-sensitive receptors on non-activated microglia. We next considered whether the microglial response to ST-11 varies if cells assumed either an M1 (induced by $TNF\alpha/IFN\gamma$) or M2 (induced by IL-4) phenotype. We

pretreated mouse microglia in culture with these cytokines and measured changes in cAMP levels, cell migration and cell proliferation in response to ST-11. When measuring cAMP levels, we found that none of the cytokine treatments significantly changed basal cAMP levels yet these treatments significantly potentiated the isoproterenol-stimulated increase in cAMP (approximately 3-fold increase in the isoproterenol response in TNF α /IFN γ - and IL-4-treated cells compared with the response in non-treated cells) (Figure 6a, b). With regard to the ST-11 response, we found that the 3-fold ST-11-stimulated increase in cAMP was preserved in TNF α /IFN γ -treated cells and that this response was lost in IL-4 treated cells (Figure 6a, b). These results suggest that AI-sensitive receptor signaling might be selectively reduced in microglia adopting a M2 phenotype.

To determine whether the M1 and M2 phenotypes affect the inhibitory response produced by ST-11 on microglial cell migration, we treated cells with the respective cytokines and measured their migratory in response to ST-11 and the positive control ATP. We found that both TNF α /IFN γ - and IL-4 treated cells exhibited a reduced basal migration (by $39 \pm 6\%$ and $52 \pm 6\%$ compared with non-treated cells, respectively) (Figure 6c, d). ST-11 further reduced migration in TNF α /IFN γ treated cells, and did not further affect IL-4 treated cells (Figure 6c, d). Importantly, the ATP-stimulated migration was unaffected in M1 microglia and reduced in M2 microglia (Figure 6c, d). To determine whether the inhibitory effect of ST-11 on microglia cell proliferation was affected by M1 and M2 activation, cells were pretreated with the respective cytokines and their proliferation response to both ST-11 and the positive control M-CSF measured. We found that both cytokine treatments significantly reduced basal microglia cell proliferation (by 21 ± 3 and $16 \pm 3\%$ compared with non-treated cells, respectively) (Figure 6e, f). ST-11 did not further reduce the level of cell proliferation nor did M-CSF increase it (Figure 6e, f). Together, these data suggest that M1 and M2 polarization of the microglia phenotype differentially affects the microglia cell migration and proliferation response to ST-11.

Discussion

We found that microglial cells express functional AI-sensitive receptors that couple to G $_s$ proteins. Agonists acting at these receptors reduce both basal and stimulated microglial cell migration, suggesting that this subtype of membrane receptor controls both the basal and directed movement of microglia. Agonists acting at AI-sensitive receptors do not affect basal cell proliferation but significantly reduce MCSF-stimulated cell proliferation, suggesting that AI-sensitive receptors do not broadly control the proliferation machinery but likely cross-talk with the signaling pathways triggered by M-CSF. Agonists acting at AI-sensitive receptors do not modulate the expression level of select effector proteins of M1 and M2 phenotypes, indicating that this membrane receptor subtype only controls specific components (migration and proliferation) of their activation process. Finally, we found that M2 activation of microglia reduces the functionality of AI-sensitive receptors (cAMP), whereas M1 and M2 activation inhibit overall microglia cell migration and proliferation, altering the sensitivity of these cells to modulation by membrane receptors.

Multiple studies suggest that microglia express novel cannabinoid-like receptors (Facchinetti et al., 2003; Franklin et al., 2003; Kreutz et al., 2009). Whether these receptors were

alkylindole-activated GPCRs (AI-sensitive receptors) or other unknown protein targets activated by cannabinoid-like ligands, including phytocannabinoids and other synthetic cannabinoids, is unclear. Specifically, conclusions regarding the involvement of a specific subtype of cannabinoid-like receptor were difficult to resolve because these studies used micromolar concentrations of cannabinoid agonists known to activate multiple targets, including CB₁ and CB₂ receptors (with nanomolar potency) and additional unknown off-targets (with micromolar potency). For example, micromolar concentrations of CP55940 and the AI analogue JWH-015 are required to decrease microglial NO production induced by IFN γ and LPS (Waksman et al., 1999; Ehrhart et al., 2005), cytokine production induced by LPS (Puffenberger et al., 2000; Facchinetti et al., 2003) and cell migration stimulated by ADP (Romero-Sandoval et al., 2009), which raises the question of whether these effects were mediated through CB₁ receptors, CB₂ receptors, AI-sensitive receptors or other unknown targets. The novel AI analogues used in our study exhibit nanomolar potencies at AI-sensitive receptors, reduced affinity at CB₁/CB₂ receptors and they allowed us to identify the specific components of the microglia activation process (*i.e.* migration and proliferation) that are regulated by this novel GPCR subtype. When measuring cAMP levels, we found that microglia adopting an M2 (but not M1) phenotype do not respond as well to ST-11, whereas microglia adopting either M1 or M2 exhibit a greatly increased response to isoproterenol. These results suggest that the microglial cell activation process is associated with differential modifications of the functionality of specific GPCRs.

Microglial cell migration can be triggered by an array of chemoattractants acting at GPCRs, including peptides and proteins (*e.g.*, chemokines), small hydrophilic molecules (*e.g.*, nucleotides) and bioactive lipids (*e.g.* endocannabinoids) (Thelen, 2001; Inoue, 2002; Miller and Stella, 2008). While stimulated microglial migration has been extensively studied, little data is available detailing the microglial cell migration “stop signal”, a response studied in more detail in other immune cell types, including T cells (Dustin et al., 1997). Such a “stop signal” may serve not only to instruct microglial cells to become less motile but may be used to guide microglial cell migration toward lesions by decreasing migration speed as the gradients of such signals increase nearing the injury site. Examples of GPCR subtypes that reduce cell migration include CB₂ receptors stimulated by the HIV Tat protein (Fraga et al., 2011), mu opioid receptors stimulated by C5a (Chao et al., 1997) and Y1 receptors stimulated by the pro-inflammatory cytokine IL-1 β (Ferreira et al., 2012). Here we show that AI-sensitive receptors reduce both basal and ATP-stimulated microglial cell migration, suggesting that the signaling pathways regulated by these novel GPCRs directly control the cell migration machinery. Interestingly, microglia that adopt M1 or M2 phenotypes migrate less both under basal conditions and in response to ATP. Furthermore, ST-11 further reduced the level of microglial cell migration in M1 microglia. Together, these results suggest that the molecular machinery mediating microglial cell migration is primarily controlled by M1 and M2 phenotype over AI-sensitive and purinergic receptors.

Growth factors, such as M-CSF, increase cell proliferation by activating CSF1R and increasing tyrosine kinase activity within the signaling network controlling cell proliferation (Théry and Mallat, 1993). Endocannabinoids, such as 2-AG, further increase cell proliferation stimulated by M-CSF (or CD40) by acting CB₂ receptors that cross talk with

tyrosine kinase signaling (Derocq et al., 1995; Carrier et al., 2004). Thus, while some studies suggest that some membrane receptors may control the initiation of cell proliferation, others, including ours, indicate that GPCRs modulate the effect of specific growth factors (Derocq et al., 1995; Bouaboula et al., 1996; Carrier et al., 2004). In line with this notion, a study has shown that a cannabinoid-like receptor modulates a microglial cell line that exhibits constitutively increased proliferation (Carrier et al., 2006). We found that the signaling network controlled by AI-sensitive receptors likely cross talk with signaling networks controlled by the tyrosine kinase receptor CSF1R. Furthermore, we demonstrated that this cross talk may be overridden when microglia adopt either M1 or M2 phenotypes. Thus, our study raises the possibility that the cross talk between the signaling pathways controlled by AI-sensitive receptors and CSF1R might overlap with the signaling pathway stimulated by IL-4 that controls cell proliferation.

In summary, we have shown that mouse microglia in culture express functional AI-sensitive receptors that control cell migration and stimulated proliferation. Accordingly, agonists at this novel target are likely to reduce the number of microglia migrating toward brain lesion sites, while not affecting their M1 or M2 phenotypes. Such compounds may help elucidate how reducing the number of microglia cells at lesion sites without affecting their phenotype might influence the course of brain diseases associated with abundant accumulation of microglia, such as malignant brain tumors and ischemic injuries (Turner et al., 2013; Fung and Stella, 2014). Several important questions remain unanswered with regard to AI-sensitive receptors expressed by microglia. Among these unknowns are the molecular identity of these novel GPCRs and their endogenous ligand(s), and whether other cell types in the brain and peripheral tissues express this target. Additional unknowns include the mechanism by which AI-sensitive receptors control the signaling network modulating microglia cell migration and proliferation, and how it is differentially controlled by M1 and M2 phenotypes. Based on the therapeutic potential of novel drugs that selectively control discrete components of the microglial cell activation process, our study clarifies the presence and role of AI-sensitive receptors in microglia, and provides a foundation for further study of the therapeutic potential of targeting this novel subtype of GPCR to treat brain diseases and injuries.

Acknowledgments

The authors would like to thank Drs. Gwenn Garden for helpful discussions and Thomas Möller for helpful discussions and help with IP-10 measurements, as well as Brian Haas, Eric Horne and Alipi Naydenov from the Stella laboratory for help editing this manuscript. Dr. Stella holds both patent and stock ownership linked to the compounds disclosed in this study, all of which is managed by the Division on Compliance and Regulatory Affairs of the University of Washington. This work was funded by NIH (DA014486 to NS) and NIDA (DA007278 to SF).

References

- Abood, ME.; Sorensen, RG.; Stella, N., editors. EndoCANNABINOIDS: Actions at non CB1/CB2 cannabinoid receptors. Springer; 2012.
- Berridge MV, Herst PM, Tan AS. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnol Annu Rev* [Internet]. 2005; 11:127–52. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/16216776>.
- Bouaboula M, Poinot-Chazel C, Marchand J, Canat X, Bourrié B, Rinaldi-Carmona M, Calandra B, Le Fur G, Casellas P. Signaling pathway associated with stimulation of CB2 peripheral cannabinoid

receptor. Involvement of both mitogen-activated protein kinase and induction of Krox-24 expression. *Eur J Biochem* [Internet]. 1996; 237:704–11. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/8647116>.

Breivogel CS, Griffin G, Di Marzo V, Martin BR. Evidence for a new G protein-coupled cannabinoid receptor in mouse brain. *Mol Pharmacol* [Internet]. 2001; 60:155–63. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/11408610>.

Carlisle SJ, Marciano-Cabral F, Staab a, Ludwick C, Cabral G a. Differential expression of the CB2 cannabinoid receptor by rodent macrophages and macrophage-like cells in relation to cell activation. *Int Immunopharmacol* [Internet]. 2002; 2:69–82.. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/11789671>.

Carrier EJ, Auchampach J a, Hillard CJ. Inhibition of an equilibrative nucleoside transporter by cannabidiol: a mechanism of cannabinoid immunosuppression. *Proc Natl Acad Sci U S A* [Internet]. 2006; 103:7895–900. Available from: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1472541&tool=pmcentrez&rendertype=abstract>.

Carrier EJ, Kearn CS, Barkmeier AJ, Breese NM, Yang W, Nithipatikom K, Pfister SL, Campbell WB, Hillard CJ. Cultured rat microglial cells synthesize the endocannabinoid 2-arachidonylglycerol, which increases proliferation via a CB2 receptor-dependent mechanism. *Mol Pharmacol* [Internet]. 2004; 65:999–1007. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/15044630>.

Chao CC, Hu S, Shark KB, Sheng WS, Gekker G, Peterson PK. Activation of mu opioid receptors inhibits microglial cell chemotaxis. *J Pharmacol Exp Ther* [Internet]. 1997; 281:998–1004. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/9152411>.

Colton, C a. Heterogeneity of microglial activation in the innate immune response in the brain. *J neuroimmune Pharmacol* [Internet]. 2009; 4:399–418. Available from: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2773116&tool=pmcentrez&rendertype=abstract>.

Correa F, Docagne F, Mestre L, Clemente D, Hernangómez M, Loria F, Guaza C. A role for CB2 receptors in anandamide signalling pathways involved in the regulation of IL-12 and IL-23 in microglial cells. *Biochem Pharmacol* [Internet]. 2009; 77:86–100. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/18848818>.

Derocq JM, Ségué M, Marchand J, Le Fur G, Casellas P. Cannabinoids enhance human B-cell growth at low nanomolar concentrations. *FEBS Lett*. 1995; 369:177–182. [PubMed: 7544292]

Dustin ML, Bromley SK, Kan Z, Peterson D a, Unanue ER. Antigen receptor engagement delivers a stop signal to migrating T lymphocytes. *Proc Natl Acad Sci U S A* [Internet]. 1997; 94:3909–13. Available from: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=20541&tool=pmcentrez&rendertype=abstract>.

Ehrhart J, Obregon D, Mori T, Hou H, Sun N, Bai Y, Klein T, Fernandez F, Tan J, Shytle RD. Stimulation of cannabinoid receptor 2 (CB2) suppresses microglial activation. *J Neuroinflammation* [Internet]. 2005; 2:29. Available from: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1352348&tool=pmcentrez&rendertype=abstract>.

Facchinetti F, Del Giudice E, Furegato S, Passarotto M, Leon A. Cannabinoids ablate release of TNFalpha in rat microglial cells stimulated with lypopolysaccharide. *Glia* [Internet]. 2003; 41:161–8. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/12509806>.

Falkenburger BH, Dickson EJ, Hille B. Quantitative properties and receptor reserve of the DAG and PKC branch of G(q)-coupled receptor signaling. *J Gen Physiol* [Internet]. 2013; 141:537–55. Available from: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3639584&tool=pmcentrez&rendertype=abstract>.

Ferreira R, Santos T, Cortes L, Cochaud S, Agasse F, Silva AP, Xapelli S, Malva JO. Neuropeptide Y inhibits interleukin-1 beta (IL-1β)-induced microglia motility. *J Neurochem* [Internet]. 2012; 120:93–105. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/22007767>.

Fraga D, Raborn ES, Ferreira G a, Cabral G a. Cannabinoids inhibit migration of microglial-like cells to the HIV protein Tat. *J neuroimmune Pharmacol* [Internet]. 2011; 6:566–77. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/21735070>.

- Franklin A, Parmentier-Batteur S, Walter L, Greenberg D a, Stella N. Palmitoylethanolamide increases after focal cerebral ischemia and potentiates microglial cell motility. *J Neurosci* [Internet]. 2003; 23:7767–75. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/12944505>.
- Fung S, Stella N. Targeting endocannabinoid signaling in tumor-associated macrophages as treatment for glioblastoma multiforme. *WIREs Membr Transp Signal*. 2014; 3:39–51.
- Hanisch U- K, Kettenmann H. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat Neurosci* [Internet]. 2007; 10:1387–94. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/17965659>.
- Haynes SE, Hollopeter G, Yang G, Kurpius D, Dailey ME, Gan W- B, Julius D. The P2Y₁₂ receptor regulates microglial activation by extracellular nucleotides. *Nat Neurosci* [Internet]. 2006; 9:1512–9. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/17115040>.
- Hua LL, Lee SC. Distinct patterns of stimulus-inducible chemokine mRNA accumulation in human fetal astrocytes and microglia. *Glia* [Internet]. 2000; 30:74–81. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/10696146>.
- Huffman JW, Padgett LW. Recent Developments in the Medicinal Chemistry of Cannabimimetic Indoles, Pyrroles and Indenes. *Curr Med Chem*. 2005; 12:1395–1411.. [PubMed: 15974991]
- Imai Y, Kohsaka S. Intracellular signaling in M-CSF-induced microglia activation: role of Iba1. *Glia* [Internet]. 2002; 40:164–74. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/12379904>.
- Inoue K. Microglial activation by purines and pyrimidines. *Glia* [Internet]. 2002; 40:156–63. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/12379903>.
- Kallendrusch S, Kremzow S, Nowicki M, Grabiec U, Winkelmann R, Benz A, Kraft R, Bechmann I, Dehghani F, Koch M. The G protein-coupled receptor 55 ligand l- α -lysophosphatidylinositol exerts microglia-dependent neuroprotection after excitotoxic lesion. *Glia* [Internet]. 2013; 61:1822–31. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/24038453>.
- Kettenmann H, Hanisch UK, Noda M, Verkhratsky A. Physiology of microglia. *Physiol Rev* [Internet]. 2011; 91:461–553. Available from: <http://physrev.physiology.org/content/91/2/461.short>.
- Kohno M, Hasegawa H, Inoue A, Muraoka M, Miyazaki T, Oka K, Yasukawa M. Identification of N-arachidonylglycine as the endogenous ligand for orphan G-protein-coupled receptor GPR18. *Biochem Biophys Res Commun* [Internet]. 2006; 347:827–32. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/16844083>.
- Kozela E, Juknat A, Kaushansky N, Rimmerman N, Ben-Nun A, Vogel Z. Cannabinoids decrease the Th17 inflammatory autoimmune phenotype. *J Neuroimmune Pharmacol* [Internet]. 2013; 8:1265–76. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/23892791>.
- Kozela E, Pietr M, Juknat A, Rimmerman N, Levy R, Vogel Z. Cannabinoids Delta(9)-tetrahydrocannabinol and cannabidiol differentially inhibit the lipopolysaccharide-activated NF-kappaB and interferon-beta/STAT proinflammatory pathways in BV-2 microglial cells. *J Biol Chem* [Internet]. 2010; 285:1616–26. Available from: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2804319&tool=pmcentrez&rendertype=abstract>.
- Kreutz S, Koch M, Böttger C, Ghadban C, Korf H-W, Dehghani F. 2-Arachidonoylglycerol elicits neuroprotective effects on excitotoxically lesioned dentate gyrus granule cells via abnormal-cannabidiol-sensitive receptors on microglial cells. *Glia* [Internet]. 2009; 57:286–94. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/18837048>.
- Lambert C, Ase AR, Séguéla P, Antel JP. Distinct migratory and cytokine responses of human microglia and macrophages to ATP. *Brain Behav Immun* [Internet]. 2010; 24:1241–8. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/20206681>.
- Luongo L, Palazzo E, Tambaro S, Giordano C, Gatta L, Scafuro M a, Rossi FS, Lazzari P, Pani L, de Novellis V, Malcangio M, Maione S. 1-(2',4'-dichlorophenyl)-6-methyl-N-cyclohexylamine-1,4-dihydroindeno[1,2-c]pyrazole-3-carboxamide, a novel CB₂ agonist, alleviates neuropathic pain through functional microglial changes in mice. *Neurobiol Dis* [Internet]. 2010; 37:177–85. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/19804829>.
- McHugh D, Hu SSJ, Rimmerman N, Juknat A, Vogel Z, Walker JM, Bradshaw HB. N-arachidonoyl glycine, an abundant endogenous lipid, potently drives directed cellular migration through GPR18, the putative abnormal cannabidiol receptor. *BMC Neurosci* [Internet]. 2010; 11:44. Available

from: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2865488&tool=pmcentrez&rendertype=abstract>.

- Miller AM, Stella N. CB2 receptor-mediated migration of immune cells: it can go either way. *Br J Pharmacol* [Internet]. 2008; 153:299–308. Available from: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2219538&tool=pmcentrez&rendertype=abstract>.
- Miller AM, Stella N. Microglial cell migration stimulated by ATP and C5a involve distinct molecular mechanisms: quantification of migration by a novel near-infrared method. *Glia* [Internet]. 2009; 57:875–83. Available from: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2669840&tool=pmcentrez&rendertype=abstract>.
- Monory K, Tzavara ET, Lexime J, Ledent C, Parmentier M, Borsodi A, Hanoune J. Novel, not adenylyl cyclase-coupled cannabinoid binding site in cerebellum of mice. *Biochem Biophys Res Commun* [Internet]. 2002; 292:231–5. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/11890697>.
- Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* [Internet]. 2008; 8:958–69. Available from: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2724991&tool=pmcentrez&rendertype=abstract>.
- Oka S, Nakajima K, Yamashita A, Kishimoto S, Sugiura T. Identification of GPR55 as a lysophosphatidylinositol receptor. *Biochem Biophys Res Commun* [Internet]. 2007; 362:928–34. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/17765871>.
- Ousman SS, Kubes P. Immune surveillance in the central nervous system. *Nat Neurosci* [Internet]. 2012; 15:1096–1101. Available from: <http://www.nature.com/doi/10.1038/nn.3161>.
- Pertwee RG, Howlett AC, Abood ME, Alexander SPH, Di Marzo V, Elphick MR, Greasley PJ, Hansen HS, Kunos G, Mackie K, Mechoulam R, Ross RA. International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid Receptors and Their Ligands : Beyond CB1 and CB2. *Pharmacol Rev*. 2010; 62:588–631. [PubMed: 21079038]
- Puffenbarger, R a.; Boothe, AC.; Cabral, G a. Cannabinoids Inhibit LPS-Inducible Cytokine mRNA Expression in Rat Microglia Cells. *Glia*. 2000; 29:58–69. [PubMed: 10594923]
- Raes G, Noël W, Beschin A, Brys L, de Baetselier P, Hassanzadeh GG. FIZZ1 and Ym as Tools to Discriminate between Differentially Activated Macrophages. *Dev Immunol* [Internet]. 2002; 9:151–159. Available from: <http://www.hindawi.com/journals/cdi/2002/684810/abs/>.
- Romero-Sandoval EA, Horvath R, Landry RP, DeLeo J a. Cannabinoid receptor type 2 activation induces a microglial anti-inflammatory phenotype and reduces migration via MKP induction and ERK dephosphorylation. *Mol Pain* [Internet]. 2009; 5:25. Available from: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2704199&tool=pmcentrez&rendertype=abstract>.
- Ryberg E, Larsson N, Sjögren S, Hjorth S, Hermansson N-O, Leonova J, Elebring T, Nilsson K, Drmota T, Greasley PJ. The orphan receptor GPR55 is a novel cannabinoid receptor. *Br J Pharmacol* [Internet]. 2007; 152:1092–101. Available from: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2095107&tool=pmcentrez&rendertype=abstract>.
- Stella, N.; Kline, T. Composition and methods for treating glioblastoma. 2012. Available from: <https://www.google.com/patents/WO2012024670A3?cl=en&dq=WO+2012/024670&hl=en&sa=X&ei=uq7TVJ2WNNGvyAT314G4BQ&ved=0CB8Q6AEwAA>
- Stella, N.; Kline, T. Composition and methods for treating glioblastoma. 2013. Available from: <https://www.google.com/patents/US20130197026?dq=13/818,026&hl=en&sa=X&ei=5azTVOLoGsaoyATKy4LYDA&ved=0CB0Q6AEwAA>
- Stella N. Cannabinoid and cannabinoid-like receptors in microglia, astrocytes, and astrocytomas. *Glia* [Internet]. 2010; 58:1017–30. Available from: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2919281&tool=pmcentrez&rendertype=abstract>.
- Thelen M. Dancing to the tune of chemokines. *Nat Immunol* [Internet]. 2001; 2:129–34. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/11175805>.
- Théry C, Mallat M. Influence of interleukin-1 and tumor necrosis factor alpha on the growth of microglial cells in primary cultures of mouse cerebral cortex: involvement of colony-stimulating factor 1. *Neurosci Lett* [Internet]. 1993; 150:195–9. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/8469420>.

- Turner RC, Dodson SC, Rosen CL, Huber JD. The science of cerebral ischemia and the quest for neuroprotection: navigating past failure to future success. *J Neurosurg* [Internet]. 2013; 118:1072–85. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/23331000>.
- Vanguri P, Farber JM. IFN and Virus-Inducible Expression of an Immediate Early Gene, crg-2/IP-10, and a Delayed Gene, I-Aa, in Astrocytes and Microglia. *J Immunol*. 1994; 152:1411–1418. [PubMed: 8301141]
- Waksman Y, Olson JM, Carlisle SJ, Cabral G a. The central cannabinoid receptor (CB1) mediates inhibition of nitric oxide production by rat microglial cells. *J Pharmacol Exp Ther* [Internet]. 1999; 288:1357–66. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/10027878>.
- Walter L, Franklin A, Witting A, Wade C. Nonpsychotropic cannabinoid receptors regulate microglial cell migration. *J Neurosci* [Internet]. 2003; 23:1398–1405. Available from: <http://www.jneurosci.org/content/23/4/1398.short>.
- Wilkinson PC. Assays of leukocyte locomotion and chemotaxis. *J Immunol Methods* [Internet]. 1998; 216:139–53. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/9760220>.

Main points

Microglia express membrane receptors, including GPCRs, that control cell function. We show that mouse microglia in culture express alkylindole-sensitive receptors and that novel agonists at these GPCR modulate cell migration and proliferation.

Figure 1.

Microglial AI-sensitive receptors couple to G_s proteins. (A-C) Chemical structures of ST-11 (A), ST-48 (B), and ST-47 (C). (D) WIN55212-2 (WIN), ST-11 and ST-48 dose-dependent competition of [3 H]WIN55212-2 binding in microglial membranes. (E) The inactive compound ST-47 (1 μ M) and cannabinoid ligands, SR2 (300 nM) and O-2050 (100 nM) do not compete for [3 H]WIN55212-2 binding in microglial membranes. (F) ST-11 and ST-48 dose-dependently increase intracellular cAMP levels in primary cultured microglia (** P < 0.01 and *** P < 0.001 compared with vehicle-treated cells). (G) PTX (1 μ g/ml) does not block the ST-11 (300 nM) or ST-48 (1 μ M) stimulated increase of cAMP levels (* P < 0.05, ** P < 0.01, and *** P < 0.001 compared with vehicle-treated cells). Each data point represents at least 3 experiments, each performed in triplicate.

Figure 2.

AI-sensitive receptors inhibit basal and stimulated microglial cell migration. **(A)** Representative images of fluorescent microglia that have migrated through 10 μm pores. Background fluorescence was subtracted from all conditions tested and is represented by the blank condition. This filter shows ST-11 treatment (300 nM) in the presence or absence of ATP (300 μM). **(B)** ATP (300 μM) stimulates primary microglia migration 2.9 fold over basal migration (**P < 0.001 compared with vehicle-treated cells). **(C)** ST-11 dose-dependently inhibits ATP-stimulated migration (*P < 0.05, ***P < 0.001 compared with vehicle-treated cells). **(D)** ST-11 does not cause cellular toxicity to primary microglia during a 3-hr period (***P < 0.001 compared with vehicle-treated cells). Each data point represents at least 3 experiments, each performed in triplicate.

Figure 3.

AI-sensitive receptors inhibit microglial cell proliferation stimulated by M-CSF. (A) ST-11 does not affect basal cell proliferation but dose-dependently inhibits cell proliferation stimulated M-CSF (1% L929 condition media) (* $P < 0.05$ compared with L929 response). (B) 1% L929 added to cell culture media significantly increases cell proliferation (** $P < 0.001$ compared with cells treated with 0.1% DMSO vehicle). (C) ST-11 does not affect microglial cell viability over a 72 hr period. Each data point represents at least 4 experiments, each performed in triplicate.

Figure 4.

AI-sensitive receptors do not modulate the induction of microglial M1 phenotype. (A) Nitrite accumulation measured based of a calibration curve. (B) TNFα/IFNγ significantly increases nitrite levels (**P < 0.01 compared with vehicle-treated cells). Both the basal and TNFα/IFNγ-stimulated increase in nitrate levels are within the linear range of the calibration curve. Nitrite production measured under vehicle or TNFα/IFNγ-stimulated conditions shows no change when treated with ST-11 (300 nM). (C) TNFα/IFNγ treatment significantly increases IP-10 levels (**P < 0.01 compared with vehicle treated cells). IP-10 production measured under basal (vehicle-treated) or when stimulated with TNFα/IFNγ shows no change when treated with ST-11 (1 μM). Each data point represents at least 3 experiments, each performed in triplicate.

Figure 5. AI-sensitive receptors do not modulate the induction of microglial M2 phenotype. **(A, B)** Representative c_t curves of Ym1 (A) and FIZZ1 (B) markers measured by qRT-PCR following treatment with vehicle or IL-4 (10 ng/mL). **(C)** The mRNA expression levels of Ym1 and FIZZ1 in microglia show no change when treated with ST-11 (300 nM). Each data point represents at least 3 experiments, each performed in triplicate.

Figure 6.

M1 and M2 phenotypes differentially regulate the functionality of AI-sensitive receptors. (A, B) Both TNF α /IFN γ (A) and IL-4 (B) do not affect basal cAMP levels. ST-11 (300 nM) induced a significant increase in cAMP levels compared with vehicle in treated cells ($P < 0.05$ compared with vehicle levels in cytokine-treated cells), but did not induce a significant response in IL-4 treated cells. Both cytokine treatments potentiate the 10 μ M isoproterenol-stimulated increase in cAMP levels (+++ $P < 0.001$ compared with isoproterenol response in non-treated cells). (C, D) Both TNF α /IFN γ (C) and IL-4 (D) significantly reduce basal migration (** $P < 0.001$ compared with vehicle level in non-treated cells). ST-11 (300 nM) further reduced migration in both TNF α /IFN γ - and IL-4-treated cells (# $P < 0.05$, ### $P < 0.001$ compared with ST-11 response in non-treated cells). ST-11 further reduced basal migration in TNF α /IFN γ -treated cells (% $P < 0.05$ compared to vehicle levels in cytokine-treated cells) but not in IL-4 treated cells. TNF α /IFN γ treatment did not significantly affect the ATP (300

μM) stimulated migration when compared to the ATP response in non-treated cells, whereas IL-4 treatment significantly reduced the ATP response (++P<0.001 compared with ATP response in non-treated cells). (**E, F**) Both TNFα/IFNγ (E) and IL-4 (F) significantly reduced basal microglial cell proliferation (**P<0.001 compared with vehicle level in non-treated cells). ST-11 (300 nM) did not further reduce proliferation in these cells. Both cytokine treatments blocked the ability of L929 (1%) conditioned media to increase proliferation (+++ P < 0.001 compared with L929 response in non-treated cells). All data points represent at least 3 experiments, each performed in triplicate.