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The Metabotropic Glutamate Receptor 4 Positive Allosteric Modulator ADX88178 Inhibits Inflammatory Responses in Primary Microglia

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Abstract

While the specific trigger of Parkinson Disease (PD) in most patients is unknown, considerable evidence suggests that the neuroinflammatory response makes an essential contribution to the neurodegenerative process. Drugs targeting metabotropic glutamate receptors (mGlu receptors), 7 Transmembrane (7TM) spanning/G protein coupled receptors that bind glutamate, are emerging as therapeutic targets for PD and may have anti-inflammatory properties. ADX88178 is novel potent, selective, and brain-penetrant positive allosteric modulator of the mGlu4 which is under evaluation for treatment of PD and other neurological disorders. We used microglia cultured from mouse brain to determine if ADX88178 had direct effects on the inflammatory responses of these cells. We studied both microglia from wild type and *Grm4* knock out mice. We found that activation of mGlu₄ with ADX88178 attenuated LPS-induced inflammation in primary microglia, leading to a decrease in the expression of TNF α , MHCII, and iNOS, markers of pro-inflammatory responses. These effects were absent in microglia from mice lacking mGlu₄. These results demonstrate a cell-autonomous anti-inflammatory effect of ADX88178 mediated mGlu₄ activation on microglia, and suggest that this drug or similar activators or potentiators of mGlu₄ may have disease-modifying as well as symptomatic effects in PD and other brain disorders with an inflammatory component.

Keywords

mGluR4; Parkinson disease; Metabotropic receptor; Microglia; Inflammation

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Compliance with Ethical Standards All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conflict of Interest PJC and CMN received research support from AstraZeneca and are inventors on patents protecting multiple mGlu₄ receptors PAMs.

Introduction

Parkinson disease (PD) is a common neurodegenerative movement disorder. PD is associated with accumulation of aggregated forms of the protein alpha-synuclein (α -syn) in dopaminergic neurons in the substantia nigra pars compacta (SNpc) and in other parts of the brain. One consequence of α -syn accumulation is an increase in microglial activation, which leads to the production of inflammatory cytokines and chemokines and T-cell infiltration (Lang and Lozano 1998). While the specific trigger of PD is in most patients unknown, considerable evidence suggests that neuroinflammatory responses, similar to those observed upon microglial activation, make an essential contribution to the neurodegenerative process.

Inflammation in the CNS can be initiated by microglia, the resident immune cells of the central nervous system, in response to neuronal damage and other molecular triggers (Harms et al. 2013). A characteristic early response of microglia is production of the inflammatory mediator tumor necrosis factor alpha (TNF α). This leads to downstream effects including enhanced expression of induced nitric oxide synthase (iNOS) and major histocompatibility complex II (MHCII) expression. Increased MHCII expression is an important step in α -syn mediated neurodegeneration, and decreased MHCII expression reduces the toxicity of α -syn (Harms et al. 2013).

Metabotropic glutamate receptors (mGlu) are 7 transmembrane spanning/G protein coupled receptors that bind glutamate, and drugs targeting mGlu receptors are emerging as therapeutic targets for PD. mGlu₄ is a heteroreceptor found on the terminals of GABAergic striatopallidal axons, and activation or potentiation of mGlu₄ using small molecule positive allosteric modulators (PAMs) reduces excessive GABA release from these terminals and induces anti-Parkinsonian effects in a range of model systems (Valenti et al. 2003; Marino et al. 2003; Lopez et al. 2007; Bennouar et al. 2013; Beurrier et al. 2009; Engers et al. 2011; Jones et al. 2012; Jones et al. 2011; Niswender et al. 2008; Le Poul et al. 2012; East et al. 2010). An advantage of PAMs over glutamate-site agonists is that these small molecules often have greater selectivity and improved pharmacokinetics compared to conventional orthosteric agonists; for example, ADX88178, the PAM used in this study, has high selectivity for mGlu₄ over other molecular targets (Le Poul et al. 2012).

Recent work has revealed that mGlu₄ orthosteric agonists and PAMs may also have anti-inflammatory effects. Microglia express mGlu₄ receptor protein, and treatment with of microglial/neuronal cultures in vitro with the orthosteric mGlu₄ activators (*L*)-2-amino-4-phosphono-butyrac acid (*L*-AP-4) or (*R,S*)-phosphonophenylglycine (*RSPPG*) reduces neuronal apoptosis resulting from microglial activation (Taylor et al. 2003). *In vivo*, activation of mGlu₄ with orthosteric agents in neurotoxin-based rat models of PD preserves motor function and reduces dopamine neuron loss (Zhou et al. 2003; Betts et al. 2012). Additionally, targeting mGlu₄ with a PAM in a 6-hydroxydopamine rat model of PD results in decreased levels of inflammatory markers, in addition to providing significant protection against dopamine neuron loss and preservation of motor function (Betts et al. 2012). Together, these data suggest that mGlu₄ PAMs could have important effects on both the short-term symptoms and the long-term progression of PD.

The present study sought to examine several key issues with respect to the anti-inflammatory effects of the mGlu₄ PAM ADX88178 in PD model systems: 1) does ADX88178 have cell-autonomous effects on the inflammatory responses of brain microglia, and 2) are the anti-inflammatory effects of ADX88178 the result of specific interaction with the mGlu₄ receptor? Results of our study indicate that treatment of primary microglial cells with ADX88178 attenuates lipopolysaccharide (LPS)-induced inflammation, characterized by reduced TNF α levels and decreased MHCII and iNOS expression. Anti-inflammatory effects of ADX88178 were not observed in microglia with genetic deletion of the mouse *Grm4* gene. These data support a cell-autonomous effect of ADX88178 on brain microglia, mediated by interaction with the mGlu₄ receptor. The ability of ADX88178 to reduce inflammation as well as improve circuit function in PD models suggests that it along with other similar mGlu₄ PAMs may be valuable symptomatic and disease-modifying agents for human PD.

Materials and Methods

Mouse Model and Reagents

Wild-type (*mGlu4*^{+/+}), and knockout mice lacking the *Grm4* gene (*mGlu4*^{-/-}) in C57Bl/6 background were purchased from the Jackson Laboratories. (Strain 8815915) Animals were maintained in a UAB Animal Care Facility. All animal studies were performed following approved guidelines of the Institutional Animal Care and Use Committee. Breeding pairs were used to obtain neonates from both WT and mGlu₄ KO mouse colonies. Homozygous knockout status for the *Grm4* gene was confirmed by genomic PCR. ADX88178 was synthesized at Vanderbilt as described in (Le Poul et al. 2012; Yin et al. 2013). Primary antibodies used in the study were rat anti-mouse MHCII and iNOS from eBioscience. Biotinylated secondary antibody (anti-rat) and Alexa Fluor 488- Streptavidin were purchased from Jackson Immuno Laboratories. An enzyme-linked immunosorbent assay kit for mouse TNF α was purchased from R&D Systems (CA).

Microglia Extraction

Primary microglia were isolated from WT and *mGlu4* KO mice on postnatal days 0–2 as described by previously published protocols (Harms et al. 2013). The brains were isolated, the meninges were removed, and the cells were allowed to dissociate at 37 °C for 10 min with periodic agitation. Mixed glial populations were plated and cultured for 11 days or until confluent. Upon confluency, microglia were isolated from the astrocyte bed by a mechanical shaking method in which culture flasks are placed in a cell shaker for 45 min at 195 rpm at 37 °C. Prior to treatment, microglia from WT and mGlu₄ KO mice were plated in chamber slides at a density of 100,000 cells/well and allowed to adhere for 1 h in serum-free and glutamate-free DMEM/F12 nutrient mixture (Sigma).

Microglia Treatment with mGlu₄ Activators

After adhesion to the chamber slides, microglia from WT and *mGlu4* KO microglia pre-treated with 1 nM, 10 nM, 100 nM ADX88178, or 100 nM L-AP4. Each treatment was performed in quadruplicate. Thirty minutes after treatment with ADX88178 or L-AP4, 100 ng/mL LPS was added to the cultures and the cells were incubated at 37 °C for an additional

24 h. At the end of the 24 h treatment period, media was collected and analyzed for TNF α , and the cells were for iNOS and MHC II expression by immunocytochemistry.

Enzyme-Linked Immunosorbent Assay for Measurement of TNF α

To measure the amount of soluble TNF α produced by microglia following treatments, supernatants were collected from cultured cells and assayed using a TNF α DuoSet ELISA kit according to the guidelines of the manufacturer (R&D Systems). Culture supernatants were diluted with reagent diluent in a 1:5 ratio in accordance with kit instructions. Fluorescence was quantified using a microplate reader at 450 nm and analyzed using GraphPad software.

Immunocytochemistry Analysis for iNOS and MHCII Expression

After harvesting media for the TNF α measurement, the treated microglia were fixed with 2 % PFA and stained for iNOS and MHCII using the primary and secondary antibodies mentioned previously (Harms et al. 2012). The MHCII staining was identified using a biotinylated goat-anti-rat IgG secondary antibody at 1:500 dilution. Alexa Fluor 488 conjugated streptavidin was used at 1:500 dilution for detection.

Imaging and Quantification

A Leica TCS-SP5 laser-scanning confocal microscope was used to capture images as previously described (Harms et al. 2013). Images were exported and processed using ImageJ. For antigen processing and presentation, immunofluorescence from four representative confocal images was quantified using ImageJ software. The corrected total cell fluorescence was quantified using the following equation: Integrated density (area of selected cell $\times$ mean fluorescence of background readings).

Results

LPS-induced TNF α is attenuated by ADX88178 treatment

To characterize the TNF α response in our system, microglial cells from C57BL/6 mice were either treated with LPS for 24 h or untreated and cultured in serum- and glutamate-free medium in which glutamate concentrations were undetectable. Replicates treated with DMSO, the vehicle for subsequent studies with ADX88178, were also included. TNF α levels were measured from culture supernatants using an ELISA assay. We observed a marked enhancement of TNF α after LPS treatment of microglial cells. There was also no effect observed of DMSO vehicle on LPS-induced TNF α production (Fig. 1).

Next, we determined if pretreatment of microglia with ADX88178 would attenuate inflammatory responses induced by 100 ng/mL LPS treatment. Microglia were pretreated with 1, 10 or 100 nM ADX88178 or 100 nM LAP4 for 30 min followed by LPS treatment for 24 h prior to collecting culture supernatant for ELISA measurement of TNF α levels. We found that that pre-treatment with ADX88178 and LAP4 both significantly attenuated LPS-induced TNF α levels (Fig. 1A). As little as 1 nM of ADX88178 was sufficient to inhibit TNF α , and was as effective at concentrations of 10 and 100 nM ($p < 0.01$, one-way ANOVA with a Tukey's *post hoc* test).

mGlu₄ Specificity of the Anti-Inflammatory Effect of ADX88178

We evaluated the specificity of the anti-inflammatory effect of ADX88178 using microglia from animals with genetic deletion of the *Grm4* gene. We cultured microglial cells from mGlu₄ KO mice and treated them with LPS as described above. This produced marked enhancement of TNF α production, but neither ADX88178 nor LPS inhibited the TNF α response in the KO microglia (Fig. 1B).

Effects of ADX88178 on MHCII in LPS-Stimulated Microglia

TNF α signaling is an important component of the inflammatory response, but another key function of microglial cells is antigen presentation, mediated by MHCII. To determine if activation of mGlu₄ on microglial cells also leads to alterations in MHCII expression, microglia from WT and mGlu₄ KO mice were pretreated for 45 min with ADX88178 and then stimulated with LPS for 24 h. Based on the potency of the ADX88178 compound in the TNF assay, this experiment was performed with 1 nM, 10 nM, and 100 nM ADX88178. The cells were then fixed and stained for immunocytochemical detection. MHCII expression was determined using confocal microscopy and quantified with ImageJ software. As shown in Fig. 2A, we found that LPS led to a marked enhancement of MHCII staining in WT microglia. There was a significant decrease ($p < 0.001$, one-way ANOVA with a Tukey's *post hoc* test) in MHCII expression when microglia from WT mice were pretreated with ADX88178 prior to LPS stimulation. Similar pretreatment of microglia from *mGlu₄* KO mice showed that ADX88178 treatment did not alter MHCII expression in the KO cells (Fig. 2B), confirming the specificity of attenuation by ADX88178 through mGlu₄.

Pre-Treatment of Microglia with ADX88178 Decreases iNOS Levels

An additional function of activated microglia is upregulation of pro-oxidant enzymes, including inducible nitric oxide synthase (iNOS). In particular, iNOS production is associated with elevated levels of reactive oxygen species (ROS) and nitric oxide (NO) (Zhang et al. 2005; Li et al. 2010). To determine if modulation of mGlu₄ activity by a PAM affects iNOS level, microglia from WT and mGlu₄ KO mice were treated with ADX88178 following LPS activation. iNOS measurement after 24 h in culture demonstrated a significant decrease in microglia from WT mice (Fig. 2D, E) but not from mGlu₄ KO mice (Fig. 2F).

Discussion

In this study, we found that the mGlu₄ PAM ADX88178 is anti-inflammatory, decreases MHC II and iNOS expression, and decreases TNF α release in microglia from WT mice, while none of these effects are observed using microglia from mGlu₄ KO mice. This suggests that the ADX88178 has broad anti-inflammatory effects on microglia that are mediated by the mGlu₄ receptor protein.

One goal of this study was to determine whether the effect of mGlu₄ agonists on microglia were cell-autonomous. Our data demonstrate clearly that activation of mGlu₄ has a direct anti-inflammatory effect on brain-derived microglial cells. Of course, this does not preclude the existence of additional anti-inflammatory effects mediated indirectly. It will be important

to determine whether anti-inflammatory effects of mGlu₄ agonists, such as those observed in 6-OHDA treated rats, are due solely to effects on microglia (Betts et al. 2012), or whether there are also effects of mGlu₄ activation on other components of the immune system.

To activate the immune response in microglia, we have used LPS. This is a specific activator of toll-like receptor 4 (TLR4), which produces rapid activation of microglia via nuclear factor kappa B-induced cytokine and chemokine release. LPS is useful as a probe for TLR4 signaling, but there is also some evidence which connects the actions of LPS directly to the pathophysiology of PD. A large body of experimental work shows that LPS can induce prominent degeneration of dopaminergic neurons when administered directly into the brain (Fleegler 1987). There is also evidence that mutations in the gene for leucine-rich repeat kinase 2 (LRRK2), the most common monogenic cause of human PD, strongly modulate the response of microglia to LPS (Moore et al. 1986). It will be important to study the effects of mGlu activation with ADX88178 and other PAMs on other triggers for activation of microglia.

Anti-inflammatory properties of mGlu receptor other than mGlu₄ have also been reported. Activation of mGlu₅ plays a role in modulating neuronal cell death, significantly reduces microglial activation in response to LPS as measured by TNF α and iNOS levels, and decreases neuroinflammation. (Byrnes et al. 2009) Group 1 mGlu receptors have also been shown to mediate inflammation-induced sensitization to neuronal excitotoxicity in neonatal and adult neurons across species, suggesting a promising direction for the field using mGlu receptors as therapeutic targets for neurodegenerative disorders (Degos et al. 2013).

Activators of mGlu₄ are of special interest in PD because they can modulate the circuit abnormalities produced by dopamine deficiency, and have direct therapeutic benefit on motor function. The basis for this action is the localization of mGlu₄ receptors on the presynaptic terminals of neurons arising in the striatum and projecting to the globus pallidus (Bradley et al. 1999). This projection constitutes the indirect pathway of striatal outflow, and in this location mGlu₄ receptors act as heteroreceptors, modulating the release of GABA from striatopallidal terminals (Valenti et al. 2003). The effect of mGlu₄ activation is to reduce GABA release from these synapses, and since overactivity of the striatopallidal pathway is believed to contribute to parkinsonism, activation of the mGlu₄ terminals was predicted to have therapeutic benefit. Indeed, mGlu₄ agonists were shown to have marked antiparkinson benefit in reserpinized rodents (Marino et al. 2003) and to directly modulate firing of globus pallidus neurons in non-human primates (Bogenpohl et al. 2013). As a result of these findings, there are active programs seeking to bring mGlu₄ activators in to clinical trials in human PD (Mueller et al. 2012; Johnson et al. 2009).

In this study, we have focused on ADX88178, a novel mGlu₄ PAM. Unlike direct agonists of glutamate receptors, most of which act as glutamate analogs and bind directly to the ligand-binding site, mGlu₄ PAMs bind to a distinct allosteric site, allowing more specificity. ADX88178 is a next-generation mGlu₄ PAM which is potent, selective, and orally bioavailable (Kalinichev et al. 2014). It is effective in rodent models of parkinsonism (Le Poul et al. 2012), and is considered a prototype for compounds that will advance to clinical testing.

Microglia have been shown to play a role in a range of neurodegenerative disorders other than PD. Since mGlu₄ activation is a novel mechanism that targets microglia to achieve anti-inflammatory effects through a novel signaling pathway, mGlu₄ may also be of utility in other disorders where microglial activation is a significant contributor to the disease state.

Collectively, the results of the present study provide evidence that ADX88178, and likely other PAMs targeting mGlu₄, can act directly on microglial mGlu₄ receptors to suppress inflammatory responses. Drugs of this class may be particularly useful in human PD because they may offer both symptomatic effects through circuit modulation as well as additive neuroprotective effects by attenuating pro-inflammatory immune mechanisms associated with PD progression.

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Fig. 1.

a WT TNF ELISA following 1 nM, 10 nM, and 100 nM ADX and 100nM L-AP4 pretreatment and 100 ng/mL LPS treatment shows decreased levels of TNF output in response to LPS and PAM treatment, $p < 0.01$, one-way ANOVA with a Tukey's post hoc test. **b** mGlu4 KO TNF ELISA shows no significant response to LPS and ADX treatment $p =$ not significant, one-way ANOVA with a Tukey's post hoc test

Fig. 2.

a Images of MHCII stained WT microglia following 100ng/mL LPS and 1 nM, 10 nM, and 100 nM ADX treatment taken with confocal microscopy at 63x with 2x zoom. **b** Immunofluorescence from four representative confocal images of each sample was quantified using ImageJ software. The corrected total cell fluorescence was quantified using the following equation: Integrated density (area of selected cell $\times$ mean fluorescence of background readings) showing decreased WT MHCII expression in response to 100ng/mL LPS and 1 nM, 10 nM, and 100 nM ADX treatment, $p < 0.01$, one-way ANOVA with a Tukey's post hoc test and **c** no significant decrease in mGluR4 KO MHCII expression, $p =$ no significance, one-way ANOVA with a Tukey's post hoc test. **d** Images of iNOS stained WT microglia following 100ng/mL LPS and 1 nM, 10 nM, and 100 nM ADX treatment taken with confocal microscopy at 63x with 2x zoom. **e** Immunofluorescence from four

representative confocal images of each sample was quantified using ImageJ software. The corrected total cell fluorescence was quantified using the following equation: Integrated density (area of selected cell $\times$ mean fluorescence of background readings) showing decreased WT iNOS expression in response to 1 nM, 10 nM, and 100 nM ADX pretreatment and 100 ng/mL LPS treatment, $p < 0.01$, one-way ANOVA with a Tukey's post hoc test and **f** no significant change in mGluR4 KO iNOS expression, $p =$ no significance, one-way ANOVA with a Tukey's post hoc test