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Graduate Program in Physiology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
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THE ROLE OF α5GABA$_A$ RECEPTORS IN BRAIN INFLAMMATION

(Thesis format: Monograph)

by

Jason K. Wong

Graduate Program in Physiology & Pharmacology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
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Abstract

$GABA_A$ receptors that contain the α5-subunit (α5$GABA_A$) exhibit high sensitivity to GABA and confer tonic activity. Moreover, α5$GABA_A$ receptors have been associated with brain inflammation, in which microglia are activated. This study investigates the role of α5$GABA_A$ receptors in microglial activation. Immunohistochemistry revealed that in response to intraperitoneal lipopolysaccharide (LPS), α5-subunit null mice exhibited significantly higher expression of IL-1β in hippocampal microglia. Neuronal-glial co-cultures treated with the α5$GABA_A$ receptor inverse agonist L655,708 drastically increased microglial IL-1β expression. Surprisingly, ELISA of media from L655,708-treated co-cultures showed a considerably lower concentration of IL-1β. Treating cultured primary astrocytes with LPS increased IL-1β secretion, which was reduced by co-treatment with L655,708. Cultured primary microglia did not respond to LPS/L655,708. When grown in LPS-primed astrocytic conditioned media, primary microglia secreted IL-1β, which increased in the presence of L655,708. These data suggest that α5$GABA_A$ receptors in astrocytes indirectly modulate microglial activation.

Keywords

γ-aminobutyric acid, microglia, interleukin-1β, α5$GABA_A$ receptor, CD11b, LPS

Acknowledgments

I would like to thank my supervisor Dr. Wei-Yang Lu for his support, time and teaching for the past two years. Having the opportunity to pursue graduate studies under Dr. Lu has been both exciting and rewarding. I would like to also express my gratitude towards Dr. Yun-Yan Xiang for her teaching, patience and insight in my studies. Other members of the lab, past and present have also been helpful in my studies.

The advisory committee, Drs. Gregory Dekaban, Stephen Ferguson, and Douglas Jones has been instrumental in my research progress; their advice has always been highly appreciated and valued.

Special thanks to my family and friends, with your support, success is easier obtained.

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List of Abbreviations

α5GABA$_A$R: α5-subunit-containing subtype of γ-aminobutyric acid type-A receptor

AA: arachidonic acid

ACM: astrocyte condition medium

Ala: alanine

ANOVA: analysis of variance

ASC: apoptosis-associated speck-like protein

Asp: Aspartic acid

ATP: adenosine triphosphate

BBB: blood brain barrier

Best-1: bestrophin-1

Ca^{2+}: calcium

CA1/3: Cornu Ammonis area

cAMP: cyclic adenosine monophosphate

CARD: caspase recruitment domain

CCL2: chemokine (C-C motif) ligand 2

CCR2: chemokine (C-C motif) receptor type 2

CD: cluster of differentiation

Cl^-: chloride

COX: cyclooxygenase

CNS: central nervous system

CR3: complement component receptor 3

CREB: cAMP response element-binding protein

CVO: circumventricular organ

CX_3CL1: chemokine (C-X3-C motif) ligand 1

DAMP: damage/danger-associated molecular pattern

DMEM: Dulbecco's modified eagle medium

DMSO: dimethyl sulfoxide

ELISA: enzyme-linked immunosorbent assay

eNOS: endothelial nitric oxide synthase

FITC: fluorescein isothiocyanate

GABA: γ-aminobutyric acid

$GABA_A$: γ-aminobutyric acid type A

$GABA_B$: γ-aminobutyric acid type B

Gabra5$^{-/-}$: α5-subunit null mutant

GAD: glutamic acid decarboxylase

GFAP: glial fibrillary acidic protein

GM-CSF: granulocyte/macrophage colony-stimulating factor

HEPES: 4-(2-hyroxyethyl)-1-piperazineethanesulfonic acid

ICAM-1: intercellular cell adhesion molecule-1

IgG: immunoglobulin G

IL: interleukin

IL-1Ra: interleukin-1 receptor antagonist

IL-1RI: interleukin-1 receptor type I

IL-1RII: interleukin-1 receptor type II

iNOS: inducible nitric oxide synthase

K^+: potassium

KO: knock out

kDA: kilodalton

LBP: lipopolysaccharide-binding protein

LPS: lipopolysaccharide

M-CSF: macrophage colony-stimulating factor

MAPK: mitogen-activated protein kinase

MAC-1: macrophage-1 anitgen

MCP-1: monocyte chemotactic protein-1

MEM: minimum essential medium

MIP1-α: macrophage inflammatory protein-1α

mRNA: messenger ribonucleic acid

NALP: NACHT, leucine-rich repeat and pyrin domain

NCM: neuron conditioned media

NDS: normal donkey seurm

NeuN: neuronal nuclei

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

NIH: National Institutes of Health

NO: nitric oxide

NOS: nitric oxide synthase

nNOS: neuronal nitric oxide synthase

$P2X_7$: purinergic $2X_7$

PAMP: pathogen-associated molecular pattern

PBS: phosphate-buffered saline

PDL: poly-d-lysine

PFA: paraformaldehyde

PGG_2: prostaglandin G_2

PGH_2: prostaglandin H_2

PSG: penicillin-streptomycin-glutamine

SEM: standard error of the mean

TGF-β: transforming growth factor β

TLR: Toll-like receptor

TTX: tetrodotoxin

TNF-α: tumour necrosis factor α

WT: wild type

Chapter 1: Introduction

Microglial cells (microglia) play critical roles in the processes of neuronal network formation, maintaining CNS homeostasis, innate immunity and neurodegeneration at different stages of life. As innate immune cells, microglia can be activated by diverse infection-related molecules such as the Gram-negative bacterial endotoxin lipopolysaccharide (LPS). The activity and function of microglia are also effectively regulated by various soluble factors including cytokines and neurotransmitters. Gamma-aminobutyric acid (GABA) is a primary neurotransmitter in the central nervous system (CNS). Recent studies have reported that type-A GABA ($GABA_A$) receptors are expressed on monocytes, macrophages and possibly on microglia. Moreover, a new study indicates that the activity of the α5-subunit-containing $GABA_A$ ($\alpha 5GABA_A$) receptor is associated with brain inflammation (Wang et al., 2012). However, the mechanism by which $\alpha 5GABA_A$ receptors regulate the innate immunity of the CNS and the process of neuroinflammation remains largely unclear. My thesis aims to investigate the role of $\alpha 5GABA_A$ receptors in the regulation of microglial activation by LPS, as well as related cellular and molecular mechanisms. In this chapter, I will provide a brief overview of the current literature regarding microglia activation in response to inflammatory stimulants, with a focus on the role of GABA signaling in the regulation of inflammation.

1.1 Microglia and Neuroinflammation

Microglia are the resident macrophages of the CNS, playing a pivotal role in CNS innate immunity. Microglia make up ~15-20% of brain cells in the healthy adult brain (Polazzi and Monti, 2010; Rojo et al., 2010). Originally from mesodermal origin, myeloid progenitors

migrate into the brain during embryonic development where they settle and further differentiate into microglia (Chan et al., 2007). Since the blood-brain barrier (BBB) largely prevents immune cell infiltration, microglia serve as the major immune cells in the brain although other cell types such as astrocytes and neurons have immunomodulatory functions (Dong and Benveniste, 2001; Veerhuis et al., 2011). However, breakdown of the blood brain barrier can occur (e.g. multiple sclerosis), coinciding with peripheral immune cell infiltration (Engelhardt, 2006; Raine et al., 1990).

1.1.1 Microglial Activity

Microglia constantly survey their surrounding area and readily react to damage in the brain. In the healthy brain, microglia remain in a “resting” state, while they become “activated” when disruptions of brain homeostasis occur (Kettenmann et al., 2011). Although termed “resting”, microglia in this state are still functioning in an important role. Resting microglia are active in the sense that they are always surveying the brain parenchyma. Resting microglia are characterized by having extended, branched processes from a small cell body; this is also known as a ramified morphology (Kettenmann et al., 2011). In the absence of stimulatory signals, microglia retain a ramified, resting state and have minimal phagocytic ability (abd-el-Basset and Fedoroff, 1995; Ribes et al., 2009; Sivagnanam et al., 2010). Regular monitoring of the brain involves the motile microglial processes, which are used to constantly scrutinize a specific, non-overlapping territory monitoring for changes in homeostasis such as injury signals known as pathogen-associated molecular patterns and damage-associated molecular patterns (PAMPs/DAMPs) (Bianchi, 2007; Ohsawa and Kohsaka, 2011). Increased microglia process movement has been linked to microglia activation (Fontainhas et al., 2011; Nimmerjahn et al., 2005).

The morphology and activity of microglia is subject to regulation by other cell types in the brain such as astrocytes and neurons. Studies have indicated that astrocytes can secrete cytokines such as macrophage colony-stimulating factor (M-CSF), transforming growth factor-β (TGF-β), and granulocyte/macrophage colony-stimulating factor (GM-CSF), which keep microglia in a resting state showing ramified processes (Schilling et al., 2001). Neurons also play a role in the control of microglial activity. For example, neurons constitutively express CD200, an immunoglobin superfamily molecule that is on the membrane surface. CD200 interacts with the CD200 receptor expressed on microglia, resulting in an inhibitory effect (Hoek et al., 2000). Another prominent factor secreted by neurons is the chemokine CX_3CL1/fractalkine, which can exist as a membrane bound or a soluble chemokine that recognizes its receptor CX_3CR on microglia protecting against microglial neurotoxicity (Cardona et al., 2006; Cook et al., 2001). In addition, microglia express neurotransmitter receptors. For instance, it is reported that activating $GABA_B$ receptors expressed on microglia attenuates their activity (Kuhn et al., 2004).

Microglia can become activated from a resting state in response to various stimulating signals (Fig. 1.1). These signals include neuronal injuries, infection, inflammation or other homeostatic disruptions. Upon activation, microglia undergo morphological and functional changes. The morphological changes include the retraction of their ramified processes back to the cell body. Fully activated microglia have been described as amoeboid, which allows for increased mobility and promotes phagocytic activity (Dheen et al., 2007; Kettenmann et al., 2011). On the other hand, the functional changes of activated microglia include the increased production and secretion of cytotoxic factors and pro-inflammatory cytokines (Smith et al., 2012). It has been well documented that activated microglia produce cytokines, chemokines, prostaglandins, excitatory amino acids, quinolinic acid, complement proteins,

reactive oxygen intermediates and nitric oxide (Nakajima and Kohsaka, 2001). Prolonged microglial activation has been linked to various neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease, Parkinson's disease, gliomas and others (Chao et al., 1992; Smith et al., 2012).

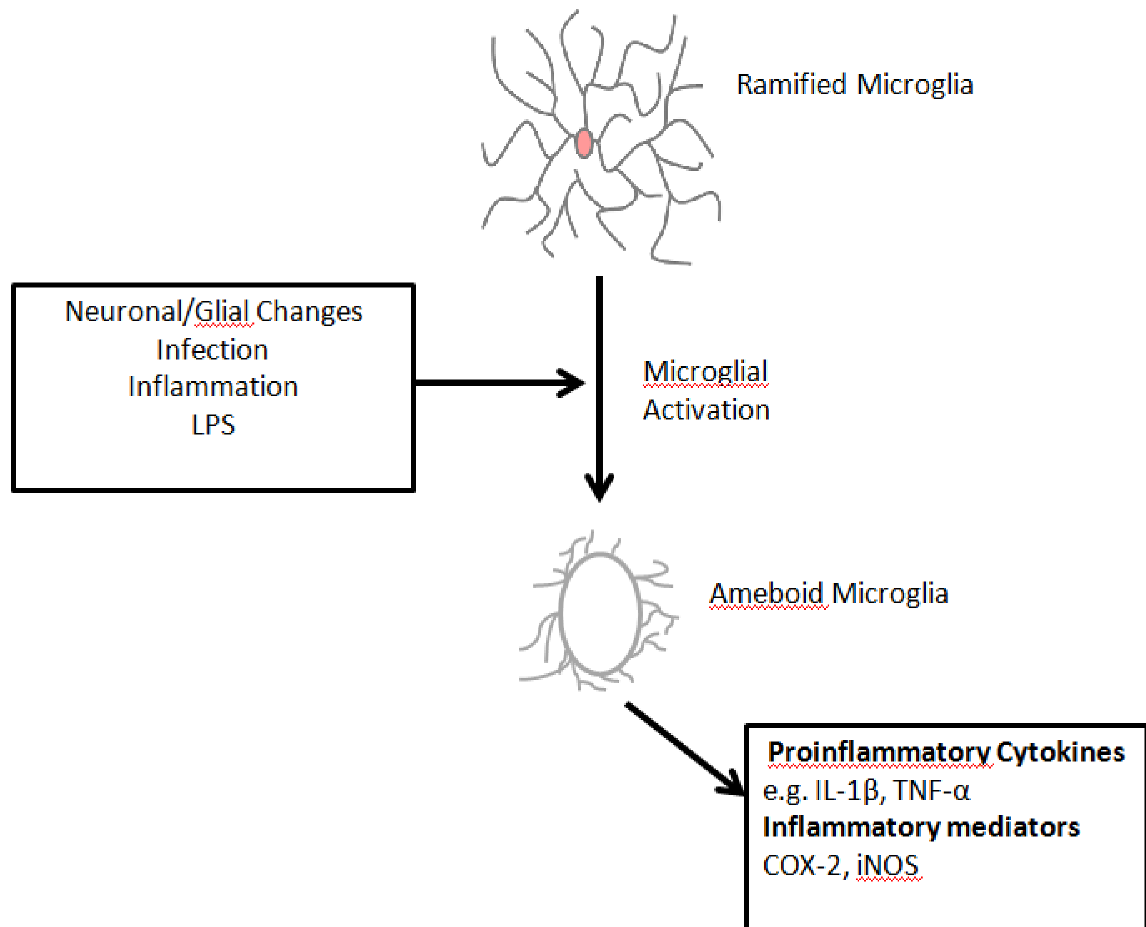


Figure 1.1 Resting, ramified microglia become activated in response to various "injury" signals in/to the brain. This leads to phenotypic (e.g. production of cytokines) and morphological changes (e.g. retraction of processes).

Like other phagocytes, microglia also express phagocyte-specific proteins, such as cluster of differentiation-11b (CD11b), a member of the β2-integrin family expressed on the surface of

phagocytes. CD11b plays a role in microglia activity. Together with CD18, CD11b forms the MAC-1 receptor also known as the complement component receptor 3 (CR3). This receptor is able to bind complement C3bi and intercellular cell adhesion molecule-1 (ICAM-1) (Reichert and Rotshenker, 2003; Ross and Vetvicka, 1993; Ueda et al., 1994), playing a fundamental role in microglial phagocytosis. Microglia have also been reported to utilize CD11b for developmental neuronal apoptosis. (Wakselman et al., 2008). The surface expression of CD11b is up-regulated in activated microglia (Le Cabec et al., 2002; Roy et al., 2006) and reported stimuli that increase CD11b expression include LPS and nitric oxide (NO) (Fenton and Golenbock, 1998; Roy et al., 2006).

1.1.2 Activation of microglia by LPS

LPS consists of a polysaccharide (sugar) chain and a lipid moiety, known as lipid A, which is in the outer membrane of Gram-negative bacteria. Although LPS cannot pass the blood brain barrier in most circumstances, a small amount of LPS may enter the brain (Lacroix et al., 1998). Like macrophages, microglia are highly sensitive to LPS and exhibit robust activation (Singh and Jiang, 2004). Thus, LPS is used as the model stimulant to study activated microglia *in vitro* (Hanisch and Kettenmann, 2007). LPS binds to soluble LPS binding protein (LBP) and is recognized by CD14+ immune cells. The bound LPS is then recognized by the toll-like receptor 4 (TLR4), which in turn activates inflammatory signaling pathways including NF-κB and MAPK pathways (Guha and Mackman, 2001). These signaling pathways are able to induce gene transcription of pro-inflammatory cytokines (e.g. IL-1β, TNF-α) in CD14+/TLR4+ expressing cell types, especially in macrophages and microglia (Guha and Mackman, 2001; Woodroofe et al., 1991).

As a large molecule, LPS does not readily pass through the intact blood brain barrier and is unlikely able to directly activate microglia in most areas of the CNS (Banks and Robinson, 2010; Rothwell and Hopkins, 1995). However, following systemic administration of LPS, vigorous inflammatory reactions occur in the brain showing activated microglia (Dantzer et al., 2008; Yirmiya and Goshen, 2011). There are several ways that systemic inflammation triggered by LPS can translate into CNS inflammation. LPS administration results in increased production of cytokines by peripheral immune cells and the elevated cytokines (including IL-1β) in the circulation can pass through the blood brain barrier, although these transport mechanisms may be inefficient (Banks et al., 1995; Quan et al., 1998). The circumventricular organs (CVOs), choroid plexus, and leptomeninges are regions in the brain that are termed "leaky" as they do not have a blood brain barrier. Moreover in response to systemic inflammation, an upregulated expression of the LPS receptor CD14 occurs in these structures, and in microglia throughout the brain. This suggests that microglia are capable of responding indirectly to LPS in systemic inflammation (Hashimoto et al., 1991; Laflamme and Rivest, 2001; Rivest, 2003). The vagus nerve also provides a means of transducing a negative regulatory response to systemic inflammation as subdiaphragmatic vagotomy reduced the immune response in the CNS in response to LPS or IL-1β (Bluthe et al., 1994; Konsman et al., 2000). However, this mechanism largely depended on the dose of the stimulus (Hansen et al., 2000; Hansen et al., 2001). Another way that systemic inflammation can induce an inflammatory signal in the brain is through the activation of endothelial cells on the blood-brain barrier. In response to LPS or circulating cytokines in the periphery, cerebral vascular cells produce prostaglandin E_2, a small lipophilic eicosanoid, which readily diffuses into the brain parenchyma to mediate inflammation in the brain. (Ek et al., 2001; Lacroix and Rivest, 1998).

1.1.3 Inflammatory mediators secreted by activated microglia

As stated above, LPS activates microglia via NFκB and/or MAP kinase pathways (Kim et al., 2004; Simi et al., 2002), which in turn initiate the production of various inflammatory factors, such as interleukin-1β, cyclooxygenase-2, inducible nitric oxide synthase, and monocyte chemoattractant-1.

1.1.3.1 Interleukin-1β

The IL-1 gene family consists of three products: IL-1α, IL-1β and IL-1 receptor antagonist (IL-1Ra) (Dinarello, 1996). IL-1α is an autocrine growth factor that is constitutively produced but not induced in stimulated blood monocytes. (Dinarello, 2009; Lonnemann et al., 1989; Schindler et al., 1990). IL-1β serves as a widespread proinflammatory cytokine that plays a major role in mediating immune responses to infection, injury, and local and systemic inflammation (Dinarello, 1998). The soluble IL-1Ra binds to IL-1 receptors, competing against IL-1β. Therefore, IL-1Ra serves as an attenuating signal for IL-1β and is a mechanism of controlling IL-1β's effects.

Both IL-1α and IL-1β are recognized by several IL-1 receptors, although the two main receptors are IL-1 type I receptor (IL-1RI) and IL-1 type II receptor (IL-1RII). IL-1RI is expressed ubiquitously on multiple cell types and its cytosolic domain closely resembles the domains of Toll-like receptors (TLR), a receptor for innate immunity. Therefore, activation of this receptor initiates inflammatory signaling pathways similar to TLR activation, such as the complement pathway (Dinarello, 2009). IL-1RII is termed a "decoy" receptor as binding of IL-1 to the type II receptor does not mediate any physiological effects. The type II

receptor is expressed ubiquitously on cells as well as being soluble. IL-1RII serves as another way of tightly regulating IL-1 signaling (Dinarello, 1996).

Via NFκB and/or MAP kinase pathways, activation of TLR4 through LPS can induce IL-1β gene transcription (Kim et al., 2004; Simi et al., 2002). Other documented stimuli for IL-1β production in the brain are β-amyloid, hypoxia, ischemia, and seizures (Simi et al., 2007). Unlike other secreted cytokines, IL-1β does not contain a leading/signaling sequence for endoplasmic reticulum/golgi complex processing. Therefore, IL-1β is not likely to be secreted through lysosomes like other common secreted cytokines (Andrei et al., 1999; March et al., 1985; Singer et al., 1988) . Instead, IL-1β is first transcribed as a 31 kDA immature form termed pro-IL-1β. This immature form is unstable and has no biological activity (Beuscher et al., 1990; March et al., 1985). The biologically active, mature form (13 kDA), has to be cleaved from pro-IL-1β. This process as detailed in (Fig. 1.2), can be catalyzed by the NALP3 inflammasome (Martinon et al., 2002). The inflammasome is a multi-protein complex consisting of various adaptor proteins. In the CNS, an inflammasome initiating event can be the activation of purinergic receptors, including the $P2X_7$ receptor in microglia. Activation of this receptor via ATP leads to K^+ efflux. Although unclear, the decrease in potassium has been implicated in the assembly of the NALP3 inflammasome (Brough et al., 2003; Perregaux and Gabel, 1994). This includes key proteins procaspase-1, NALP3 (cryopyrin), and apoptosis-associated speck-like protein (ASC) (Agostini et al., 2004). Via the caspase activating recruiting domain (CARD) on the ASC protein, the inactive procaspase-1 is cleaved into its mature, active form, caspase-1 (Mariathasan et al., 2006; Srinivasula et al., 2002). This enzyme, a cysteine protease, can then cleave pro- IL-1β between its Asp 116 and Ala 117 yielding mature IL-1β (Howard et al., 1991; Sleath et al., 1990).

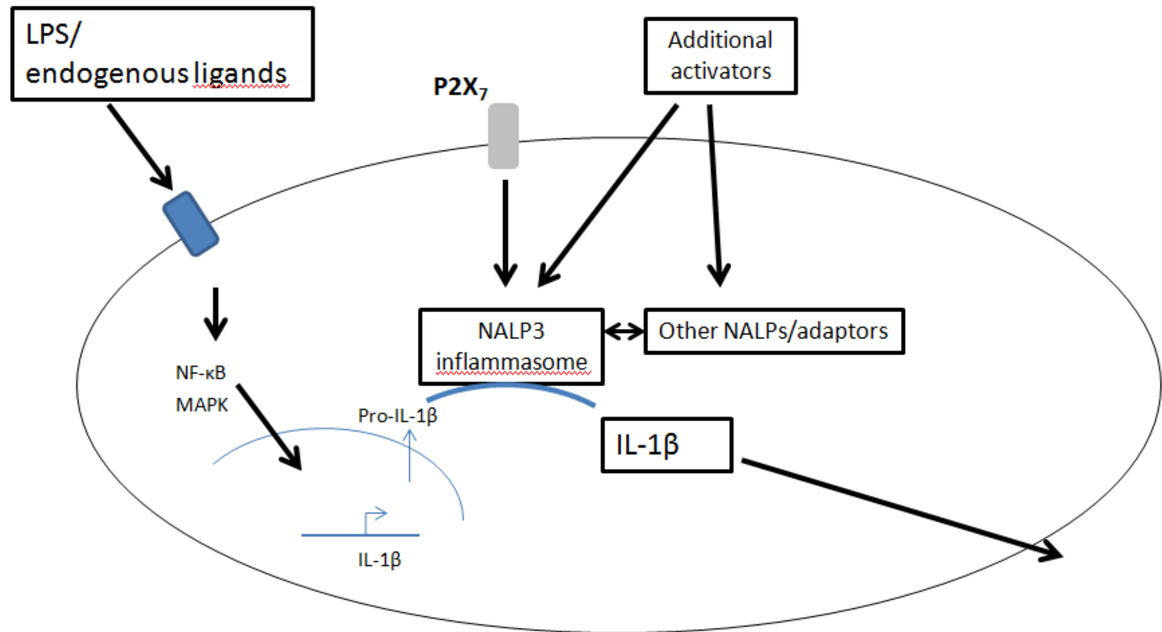


Figure 1.2 The NALP3 inflammasome processes IL-1β utilizing the ATP receptor $P2X_7$

The key molecular signal for the NALP3 inflammasome is extracellular ATP to activate $P2X_7$ receptors (Solle et al., 2001). ATP can act as an inflammatory mediator and many cell types have purinergic receptors that are activated by ATP. The matter of which cell types express the $P2X_7$ receptor in the brain is controversial. Studies have cited that neurons, microglia and astrocytes express them (Lee et al., 2008; Surprenant et al., 1996), while others have indicated microglia are the only cell type to express the $P2X_7$ receptor (Sim et al., 2004). In microglia, activation of the $P2X_7$ receptor plays a critical role in NALP3 inflammasome activation and ultimately mature IL-1β production (Solle et al., 2001). Sources of extracellular ATP in the brain include neurons, astrocytes, microglia, and endothelial cells. In many cases, elevated ATP release from these cells occurs during CNS inflammation. Neurons can release ATP in synapses (Gonzalez-Sistal et al., 2007; Sperlagh et al., 1998a; Sperlagh et al., 1998b), while ATP is also released as a consequence of

neuronal damage (Xia et al., 2012). Astrocytes also have been documented to release extracellular ATP, especially during propagated calcium waves (Coco et al., 2003; Cotrina et al., 1998; Guan et al., 1997). ATP can also be released by endothelial cells during inflammation (Bodin and Burnstock, 1998).

In terms of how IL-1β is released from the cell, it is still not definitively determined. Various hypotheses include the following: cell lysis (Le Feuvre et al., 2002), lysosomes (Andrei et al., 1999; Andrei et al., 2004), microvesicle shedding (Bianco et al., 2005b; MacKenzie et al., 2001), and direct passing through the cell membrane (Brough and Rothwell, 2007; Singer et al., 1995). Nevertheless, given that the expression and secretion of IL-1β by microglia increases significantly following activation, I will use the expression level of IL-1β as a major index of microglia activation.

1.1.3.2 Cyclooxygenase-2

Cyclooxygenase (COX) is the enzyme that catalyzes the rate limiting step in the production of prostaglandins. There are two isoforms, COX-1 and COX-2, which are 60% homologous (Smith et al., 2000). Expressed in most tissues, COX-1 is constitutively active and produces prostaglandins involved in maintaining homeostasis (Kraemer et al., 1992). The COX-2 gene is also constitutively active in the central nervous system but can be induced by nuclear NF-κB and cyclic AMP response element binding protein (CREB) (Appleby et al., 1994). Both of these molecules are inflammatory signaling molecules that become activated in response to inflammatory stimuli such as growth factors, LPS and inflammatory cytokines (Akarasereenont et al., 1995; Flynn and Hoff, 1995). COX-2 is readily expressed in immune cells such as microglia as well as neurons, glia, and endothelial cells (Bauer et al., 1997; Hirst et al., 1999; Pasinetti and Aisen, 1998).

Prostaglandins are small, lipophilic eicosanoids that have various functions including mediating inflammation (Funk, 2001). The primary step in prostaglandin synthesis is the breakdown of membrane lipids by phospholipase A_2 into arachidonic acid (AA). Regulated by NF-κB, COX has two functional steps that generate prostaglandin precursors. The first is the cyclooxygenase step in which COX converts AA into prostaglandin G_2 (PGG_2) and then the perioxidase activity of COX converts PGG_2 into prostaglandin H_2 (PGH_2). This last step also produces free radicals. PGH_2 can be modified further by tissue specific prostaglandin synthases to various prostaglandins (Consilvio et al., 2004).

1.1.3.3 Inducible nitric oxide synthase

Nitric oxide is an important intercellular signaling molecule that underlies several physiological processes. As a free radical, NO can act as an inflammatory mediator that is produced by immune cells. There are three NO synthases, vascular endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). In macrophages and microglia, iNOS is highly inducible in response to inflammatory stimuli such as LPS and cytokines (Bal-Price and Brown, 2001). In the brain, iNOS is expressed in astrocytes and microglia, where they produce and release NO upon activation (Brown et al., 1995; Chao et al., 1992).

1.1.3.4 Monocyte chemoattractant-1

Monocyte chemoattractant-1 or chemokine (C-C motif) ligand 2 (MCP-1/CCL2), is a member of the β-chemokine subfamily. MCP-1 regulates the migration of inflammatory cells such as microglia and lymphocytes to the site of inflammation (Babcock et al., 2003; Zhou et al., 2007). This chemokine acts on the MCP-1 receptor (CCR2), which is expressed by many cells including endothelial cells. Activation of the MCP-1 receptor in endothelial

cells results in upregulation of certain adhesion molecules (e.g. ICAM-1) that are involved in recruitment of immune cells such as microglia.

In the brain, MCP-1 secretion is mostly from astrocytes and microglia (Hayashi et al., 1995; Zhou et al., 2007), although it has been reported that neurons also release MCP-1 as a mechanism to recruit microglia (Hayashi et al., 1995; Yang et al., 2011). MCP-1 production and secretion by microglia is dependent on NF-κB activation and microglia use MCP-1 to migrate to the site of inflammation (Deng et al., 2009).

1.2 GABA signaling in the regulation of inflammation

GABA is synthesized in a highly regulated metabolic pathway (Martin and Rimvall, 1993). Specifically, it is synthesized from glutamate via the enzyme glutamic acid decarboxylase (GAD), which uses pyridoxal 5'-phosphate as a cofactor. GABA is used as a signaling molecule or as an energy source (Martin and Rimvall, 1993). In neurons, GABA is packaged in vesicles that are readily released by GABAergic neurons following depolarization in a calcium dependent manner (Farrant and Nusser, 2005). The secreted GABA then diffuses across the synaptic cleft and activates GABA receptors on the postsynaptic neuron. It has been estimated that 20 – 50% of all neuronal synapses in the CNS utilize GABA as a neurotransmitter (Bloom and Iversen, 1971; Young and Chu, 1990). Extracellular GABA is rapidly cleared from the synaptic cleft by GABAergic neurons and surrounding glia, via the activity of GABA transporters (Liu et al., 1993; Roettger and Amara, 1999), while some may diffuse away from the synaptic cleft. GABA concentrations in presynaptic GABAergic terminals range from 50 – 100 mM (Petroff, 2002) while the concentration of GABA in the extracellular space can reach as low as 160 nM (Santhakumar et al., 2006). GABA acts on

two types of receptors: $GABA_A$, and $GABA_B$ receptors. $GABA_A$ receptors are ligand-gated anionic channels while $GABA_B$ receptors are metabotropic (G-protein coupled) receptors. My thesis study will mainly focus on the role of $GABA_A$ receptors in the regulation of microglial activity.

1.2.1 $GABA_A$ receptor signaling in neurons

$GABA_A$ receptors are heteropentamers; i.e. a functional $GABA_A$ receptor consists of 5 subunits. There are 19 $GABA_A$ receptor subunits: α1-6, β1-3, γ1-3, δ, ε, θ, π and ρ1-3. Different subunit compositions confer different pharmacological and physiological properties of the $GABA_A$ receptor (Sieghart, 1995). In neurons, the majority of $GABA_A$ receptors consist of two α subtypes, two β subtypes, and one γ subtype (McKernan and Whiting, 1996; Sieghart et al., 1999). As members of the ligand gated ion channel superfamily, $GABA_A$ receptor subunits share molecular structures similar to that of glycine and nicotinic acetylcholine receptor subunits. Specifically, one subunit has four transmembrane domains, with a large amino terminus domain located extracellularly that can bind GABA and other modulators (Moss and Smart, 2001). $GABA_A$ receptors are pharmacologically important as they recognize drugs such as benzodiazepines, barbiturates, steroids, and convulsants (Sieghart, 1995, 2000). As ligand-gated anionic channels, $GABA_A$ receptors open and allow mainly chloride (Cl^-) ions to pass through its pore upon binding with GABA. In mature CNS neurons, the opening of $GABA_A$ receptors usually leads to an influx of Cl^-, resulting in a hyperpolarization (inhibition) of the membrane potential.

In mature neurons, $GABA_A$ receptors mediate two types of inhibition (or two-types of anionic currents): phasic and tonic inhibition. Phasic inhibition is characterized by a quick onset, short lasting and large amplitude current. This is mediated by postsynaptic $GABA_A$

receptors in response to large-quantity vesicular release of GABA from GABAergic synaptic terminals. Tonic inhibition, on the other hand, is characterized by persistent low-amplitude current, which is due to constitutive activation of *extrasynaptic* $GABA_A$ receptors by ambient extracellular GABA. These extrasynaptic receptors have a high sensitivity to GABA, allowing them to respond to relatively low concentrations (~100 nM) of GABA in the extracellular space (Farrant and Nusser, 2005; Santhakumar et al., 2006). Sources of ambient GABA include spill-over GABA from synaptic release (Mitchell and Silver, 2000; Semyanov et al., 2003), and non-synaptic release of GABA from astrocytes (Semyanov et al., 2004)(also see below). The high sensitivity of extrasynaptic $GABA_A$ receptors to GABA largely depends on subunit composition, specifically receptors that contain the α5 or δ subunits (Caraiscos et al., 2004).

1.2.2 GABA signaling in astrocytes

Astrocytes, the major type of glial cells in the brain (Liu et al., 2011), play an essential role in neuron signalling, survival and development (Ridet et al., 1997). Not only do they provide metabolic support for neurons, but they are also involved in the uptake and clearance of released neurotransmitters (Liu et al., 1993; Roettger and Amara, 1999). Moreover, astrocytes have been described to be GABAergic – that is, they have the cell machinery to produce and release GABA utilizing glutamic acid decarboxylase (Lee et al., 2011). As mentioned above, astrocytes have been implicated as a source for GABA in the extracellular space that is responsible for tonic activation of $GABA_A$ receptors (Lee et al., 2010). However astrocytes release GABA via a non-vesicular mechanism. It is reported that in astrocytes GABA is constitutively released into the extracellular space through bestrophin-1 (Best-1) channels independent of neuronal activity and extracellular calcium. Bestrophin-1 channels

are capable of passing GABA through its pore (Lee et al., 2010). Other mechanisms of release of GABA from astrocytes have been proposed such as reversed transport, stretch activated ion channels and $P2X_7$ receptor-mediated release (Kimelberg et al., 1990; Volknandt, 2002; Wang et al., 2002).

Astrocytes are also GABA receptive. Activation of $GABA_A$ receptors leads to depolarization of the membrane potential (Kettenmann et al., 1984; Serrano et al., 2006) because of an inherently high intracellular Cl^- concentration. This leads to the extrusion of these anions when the $GABA_A$ receptor gated channels open (Kimelberg et al., 1990). As a result of depolarization, intracellular calcium concentration increases and results in astrocyte activation (Bernstein et al., 1996; Fraser et al., 1995; Meier et al., 2008). Elevated intracellular calcium leads to the initiation of various signalling pathways, including the propagation of a calcium wave across astrocytes. Astrocytic release of ATP has been implicated in the propagation of calcium waves as adding antagonists to P_2-purinergic receptors blocked this propagation (Guan et al., 1997; Zanotti and Charles, 1997). Activation of astrocytes has broad implications on the activity of other cells in the CNS including regulating neuronal and microglial function (Liu et al., 2011; Perea et al., 2009). Interestingly, astrocyte progenitor proliferation in the postnatal brain has been found to be modulated by their $GABA_A$ receptors. These receptors are tonically activated by nonsynaptic release of GABA from neuroblasts in the subventricular zone (Liu et al., 2005). Moreover, activated astrocytes dynamically regulate the activity and function of microglial cells (see below).

1.2.3 Regulation of microglia activity by GABA signaling

Microglia express various neurotransmitter receptors that have been documented to modulate their function. These include purinergic (Bianco et al., 2005a; Xiang and Burnstock, 2005), glutamate (Hagino et al., 2004; Noda et al., 2000), cholinergic (De Simone et al., 2005; Shytle et al., 2004) dopamine (Farber et al., 2005), adrenergic (Prinz et al., 2001) and GABA receptors (Kuhn et al., 2004; Lee et al., 2011).

Activation of microglial $GABA_B$ receptors exhibit an outward K^+ current (membrane hyperpolarization) and a transient increase in intracellular calcium (likely through the calcium-release activated calcium channel). In addition, $GABA_B$ receptor activation in LPS-stimulated microglia results in a decrease of the release of proinflammatory cytokines IL-6 and IL-12p40 (Kuhn et al., 2004). On the other hand, the presence of functional $GABA_A$ receptor-mediated signalling in microglia remains controversial. One recent study has shown that adult human microglia express $GABA_A$ receptors and express the mRNA of the α1, α3 and β1 subunits (Lee et al., 2011). Furthermore, the authors indicated that stimulation of $GABA_A$ receptors in activated microglia lead to the attenuation of increases in inflammatory intracellular signalling mediators NF-κB and p38 MAP kinase. This resulted in a decrease in the secretion of proinflammatory cytokines TNF-α and IL-6(Lee et al., 2011). However, several studies have reported that stimulating microglia with GABA or $GABA_A$ receptor agonists does not lead to any measurable current (Cheung et al., 2009; Wu and Zhuo, 2008) or change in process motility (Chen et al., 2010).

Nevertheless, an increasing amount of evidence indicates that $GABA_A$ receptor-mediated signaling in the brain can indirectly affect microglia activity. For example, the $GABA_A$ receptor agonist muscimol has been shown to induce a transient increase in the conductance

of K^+ channels in microglia located on the surface of a mouse brain slice. This resulted in the release of an inflammatory cytokine, macrophage inflammatory protein-1α (MIP-1α). However, microglial $GABA_A$ receptors were not responsible for this effect as isolated microglial cells did not respond to muscimol when removed from the brain slice. (Cheung et al., 2009). In addition, retinal microglial morphology has also been shown to be modulated by neurotransmission. Process motility was increased in response to ionotropic glutamatergic neurotransmission but decreased in response to ionotropic GABAergic neurotransmission. Furthermore, these effects were not directly mediated as direct application of GABA did not affect microglia process motility. Instead, ATP seemed to be a downstream modulator that is released in response to neurotransmission, and enables microglial activation (Fontainhas et al., 2011). ATP has been indicated to play a critical role in microglial activation, especially in the production and secretion of the proinflammatory cytokine interleukin 1-β (Clark et al., 2010; Inoue, 2006). These findings indicate that the activation of $GABA_A$ receptors in other cell types can indirectly regulate microglia activity.

1.2.4 GABA signaling in peripheral cells

Not only does GABA have an important role in neuronal signaling, but a growing amount of evidence supports $GABA_A$ receptor signaling outside the CNS. Specifically, $GABA_A$ receptors have been documented in peripheral tissues such as the pancreas, adrenal, ovary, testis, small intestine (Akinci and Schofield, 1999), lung (Jin et al., 2005) as well as in immune cells including T-lymphocytes (Tian et al., 1999) and macrophages (Reyes-Garcia et al., 2007). A recurring theme in these studies indicates that $GABA_A$ receptors have an anti-inflammatory effect. For example, $GABA_A$ receptors are expressed on islet β-cells and activation of these receptors depolarizes the membrane potential. This leads to an increase in

insulin secretion (Dong et al., 2006) and the promotion of β-cell survival (Soltani et al., 2011). In the lung, $GABA_A$ receptors are expressed in pulmonary epithelial cells on the airway apical membrane (Xiang et al., 2007). In addition, ongoing studies in our lab show that the $\alpha5GABA_A$ receptor is expressed in the type II epithelial cell, and these receptors may play an essential role in pulmonary innate immunity (laboratory communication).

$GABA_A$ receptors have also been shown to modulate immune cell function. Studies have shown that $GABA_A$ receptors are expressed in neutrophils, lymphocytes, and macrophages (Nigam et al., 2010). For example, a study showed that T-lymphocytes express $GABA_A$ receptors that resemble extrasynaptic receptors contained in neurons. These receptors were activated at low concentrations of GABA (100 nM) and contained the α1,4, β2,3, γ1 and δ subunits (Bjurstom et al., 2008). Activation of $GABA_A$ receptor in human T-lymphocytes inhibits proliferation (Dionisio et al., 2011). In addition, activation of $GABA_A$ receptors on peritoneal macrophages leads to reduced production of cytokines IL-6 and IL-12 *in vitro* (Reyes-Garcia et al., 2007). Gabapentin, a GABA analogue, inhibits platelet aggregation (Pan et al., 2007). Moreover, oral administration of GABA reduced antigen presenting cell (APC) function and T cell activation and proliferation in a mouse model of rheumatoid arthritis (Tian et al., 2011). Together these available data imply that GABA signalling is fundamentally associated with negatively regulating innate and adaptive immunity of the body.

1.2.5 $GABA_A$ receptors that contain the α5 subunit

In the mammalian brain, $\alpha5GABA_A$ receptors are predominantly expressed in the hippocampus (Sur et al., 1999). Located in the dendritic fields of the hippocampus, $\alpha5GABA_A$ receptors make up ~20% of all $GABA_A$ receptors in the hippocampus (Sur et al.,

1999; Sur et al., 1998). The α5 subunit is also expressed in other parts of the brain such as the olfactory bulb while its presence is low in the cerebral cortex and not expressed in most other parts of the brain (Fritschy and Mohler, 1995; Gao and Fritschy, 1995; Sur et al., 1999). In the entire brain, α5GABA$_A$ receptors make up less than 5% of all GABA$_A$ receptors (Sur et al., 1999). Specifically, α5GABA$_A$ receptors are expressed extrasynaptically in pyramidal neurons in the CA1 and CA3 regions and generate a tonic inhibitory conductance (Caraiscos et al., 2004). This tonic conductance occurs because α5GABA$_A$ receptors have two properties that are important for tonic signaling. First, α5GABA$_A$ receptors have a relatively high affinity for GABA compared to other subtypes and therefore α5GABA$_A$ receptors are able to be activated at low, ambient concentrations of GABA (~100 nM) in the extracellular space (Caraiscos et al., 2004; Santhakumar et al., 2006). It has been documented that α5GABA$_A$ receptors containing the α5β3 subunits have 2-fold higher sensitivity to GABA compared to GABA$_A$ receptors consisting of the subunits α1β2, thought to be the most common GABA$_A$ receptor located in brain synapses (Caraiscos et al., 2004; McKernan and Whiting, 1996). Secondly, α5GABA$_A$ receptors desensitize more slowly than synaptic receptors, therefore the conductance is persistent and long lasting, known as tonic conductance (Yeung et al., 2003). α5GABA$_A$ receptors regulate the intrinsic excitability of pyramidal neurons in the hippocampus as activation of these receptors result in hyperpolarization (Bonin et al., 2007; Brickley et al., 1996; Hamann et al., 2002). The roles of α5GABA$_A$ receptors in the regulation of brain functions have been studied extensively using genetic knockout of the α5 subunit (*Gabra5-/-*) or pharmacological inhibition such as utilizing the selective α5GABA$_A$ receptor inverse agonist L655,708. Among others, several important studies have shown that *Gabra5*$^{-/-}$ mice have “improved” hippocampus dependent spatial learning (Collinson et al., 2002), likely due to altered synaptic plasticity (Martin et al.,

2010). Notably, a recent study has indicated that α5GABA$_A$ receptor activity increases during systemic inflammation, and such an alteration is important for inflammation-induced memory deficits (Wang et al., 2012).

1.3 Rationale and Hypothesis

1.3.1 Rationale

An accumulating amount of evidence has implicated an immunomodulatory role for GABA$_A$ receptor mediated signaling in immune cells such as macrophages and microglia (Lee et al., 2011; Reyes-Garcia et al., 2007). Microglia critically regulate inflammation in the brain where they are activated by various stimuli. The activity and function of microglia are also tightly regulated by neurotransmitters. Since microglia are not subject to GABAergic innervations, I propose that microglial activity is regulated, directly or indirectly, by ambient GABA in the extracellular space through the high affinity, constitutively active α5GABA$_A$ receptor. In this regard, a recent study indicates that α5GABA$_A$ receptor activity is associated with brain inflammation (Wang et al., 2012). However, the mechanism by which α5GABA$_A$ receptors regulate the innate immunity of the CNS and the process of neuroinflammation remains largely unclear. This thesis investigates the role of α5GABA$_A$ receptors in the regulation of microglial activation, as well as the related cellular and molecular mechanisms. To this end, I proposed 1) to investigate the effect of intraperitoneal LPS on the expression level of IL-1β in microglia of *Gabra5*$^{-/-}$ mice, in comparison with their wild type littermates; and 2) to study how selectively blocking the α5GABA$_A$ receptor affects the expression and secretion of IL-1β by cultured primary microglia primed with LPS.

1.3.2 Hypothesis

On the basis of available knowledge, I hypothesize that interrupting α5GABA$_A$ receptor signaling intensifies the LPS-induced microglia activation and the expression of IL-1β in microglia.

Chapter 2: Materials and Methods

To study the role of α5GABA$_A$ receptors in the regulation of microglial responses to systemic inflammatory challenges, I examined the expression levels of CD11b and IL-1β in the microglia of mice that lack the α5-subunit of GABA$_A$ receptor (*Gabra5*$^{-/-}$) and their wild type (WT) littermates. The *Gabra5*$^{-/-}$ mice were generated from the C57/BL6 mouse strain (Collinson et al., 2002). To further elucidate possible molecular mechanisms by which α5GABA$_A$ receptor regulates microglia activity, I prepared various cultures of primary brain cells and tested the effects of an inverse agonist selective for α5GABA$_A$ receptors (L655,708). Microglial activation was examined primarily through microglial morphology and IL-1β expression/secretion, by means of immunochemistry and enzyme-linked immunosorbent assays (ELISA).

2.1 *Gabra5*$^{-/-}$ mice

In collaboration with Dr. Beverley Orser in the Department of Physiology at the University of Toronto, *Gabra5*$^{-/-}$ mice (Collinson et al., 2002) and their WT littermates were housed in the University of Toronto animal care facility. The *in vivo* experiments in *Gabra5*$^{-/-}$ mice were approved by the University of Toronto Animal Use Committee, in line with the regulations of Canadian Council on Animal Care.

Male *Gabra5*$^{-/-}$ mice and their WT littermates aged 7–8 weeks and weighing 22–25 g were divided into four groups (n = 4 in each group): WT control, WT-LPS, *Gabra5*$^{-/-}$ control, and *Gabra5*$^{-/-}$-LPS. Male mice were purposely chosen to avoid the reported effect of the female hormonal cycle on GABA$_A$Rs subunit expression (Maguire et al., 2005).

Mice in WT control and Gabra5$^{-/-}$ control groups were injected intraperitoneally (i.p.) with 0.2 ml phosphate-buffered saline (PBS), while mice in WT-LPS and Gabra5$^{-/-}$-LPS groups were injected i.p. with LPS (serotype 026:B6, Sigma, 4.0 mg/kg in 0.2 ml PBS). Six hours after LPS administration, mice were perfused transcardiacally with 20-25 ml PBS and then with 10 mL 4% paraformaldehyde (PFA). Mouse brains were fixed in 4% PFA for 24 hours and then stored in 30% sucrose solution at 4°C for 2-5 days. Brains were glued on a metal block and coronal sections (40 µm thick) were cut using a vibratome (PELCO). Brain slices were stored in cryoprotectant solution at -20°C until further use for immunohistochemistry. To ensure consistent experimental conditions, a mouse brain from each group was sectioned at the same day and stored in cryoprotectant. Brain sections cut from the same day were used for immunohistochemistry analysis at the same time. Sections containing the same plane of the hippocampus were used for immunohistochemistry.

2.2 Cell cultures

2.2.1 Primary rat hippocampal co-cultures

Cell culture: Primary *mixed* neuronal-glial cultures were prepared from the hippocampi of embryonic day 18 (E18) rats, following a previously described protocol (Dinkel et al., 2004). Briefly, pregnant (18 days) Sprague-Dawley rats were euthanized by cervical dislocation under isoflurane anesthesia. Embryonic brains were removed and placed in ice-cold dissection buffer containing Hanks balanced salt solution – Ca^{2+}/Mg^{2+} free (Life Technologies), 15 mM HEPES (Sigma), 0.5% glucose (Sigma), 2% sucrose (Sigma), 1 mM sodium pyruvate (Sigma). Fetal hippocampi were isolated from each embryo and cells were dissociated using mechanical trituration. Single cell suspensions were plated on poly-d-lysine

(MW 70,000-150,000; P 6407, Sigma) coated 18-mm–diameter round glass cover slips (Deckglaser) in 12-well plates (Nunc), at a density of 1 x 10^6 cells/well. Cultures were maintained in a humidified atmosphere of 5% CO_2 and 95% air in minimum essential medium (MEM; 10370-021, Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies), 10% heat-inactivated horse serum (HS; Life Technologies), 1.0 g/L glucose (Sigma), 1 mM sodium pyruvate (Life Technologies), and 50 U/mL penicillin, and 50 μg/mL streptomycin, and 2 mM L-glutamine (PSG) (Life Technologies).

Cell Treatment: Cells on the fifth day in culture were switched to serum-free MEM supplemented with 1 mM sodium pyruvate and PSG. To determine the role of $\alpha5GABA_A$ receptor on microglia activity, the $\alpha5GABA_A$ receptor inverse agonist L655,708 (1327, Tocris), dissolved in dimethyl sulfoxide (DMSO; D5879, Sigma), was added to the culture media at concentrations of 0.5 and 5.0 μM. It is reported that L655,708 has an affinity for the $GABA_A$ receptor containing the α5 subunit 50-fold greater than $GABA_A$ receptors containing other α subunits (Quirk et al., 1996). Experimental concentrations of L655,708 (0.5 and 5.0 μM) was used consistent with previous studies (Glykys et al., 2008; Khom et al., 2006; Numata et al., 2012; Wang et al., 2012). Twenty-four hours after L655,708 pre-treatment, the cultured cells were treated with both L655,708 and LPS serotype 026:B6 (L2654, Sigma, 50 ng/mL) for another 24 hours. The control cells were subject to the same concentration of DMSO used in L655,708 treatment. To confirm the role of $GABA_A$ receptors in microglial activation, the $GABA_A$ receptor antagonist bicuculline (Tocris; 50 μM) was added to the culture media in combination with LPS.

As α5$GABA_A$ receptors inhibit the intrinsic excitability of neurons, I explored whether neuronal activity affects the L655,708 induced IL-1β expression. Neuronal-glial cultures were pre-treated for 24 hours with tetrodotoxin (TTX), which blocks voltage-gated, Na^+ channels. L655,708 was then treated 24 hours after TTX pretreatment. The media from the control and treated cells were collected and centrifuged at 3000 rpm for 5 minutes. The supernatant was collected and aliquots were stored at -80°C for future ELISA.

2.2.2 Primary rat microglia

***Microglial culture**:* Primary microglia were prepared from cortices of E18 Sprague-Dawley rats as previously described (Liu et al., 2001). Briefly, isolated cortical neuronal-glial cells were first cultured in 80 cm^2 flasks (Nunc) maintained in a humidified atmosphere of 5% CO_2 and 95% air in DMEM supplemented with 10% FBS and PSG. Upon reaching confluency (10–14 days), microglia were separated from astrocytes by shaking flasks on a benchtop shaking incubator (VWR) at 200 rpm, for 4 hours at 37°C. Suspended microglia in the media were centrifuged at 1000 rpm for 5 minutes at 4°C. The supernatant was discarded and single cell suspensions were plated on poly-d-lysine (MW 70,000–150,000; P 6407, Sigma) coated 18-mm–diameter round glass cover slips in 12-well plates, at a density of 2.5 x 10^5 cells/well. Microglial cultures were maintained in a humidified atmosphere of 5% CO_2 and 95% air in DMEM supplemented with 10% FBS and PSG.

Microglial treatment: Twenty-four hours after plating, microglial cultures were switched to serum free MEM supplemented with 1 mM sodium pyruvate and PSG. Microglial cultures were treated the following day with LPS and L655,708 using the same procedure as the neuronal-glial co-cultures. The media of microglial cultures were collected and centrifuged at

3000 rpm for 5 minutes. The supernatant was collected and aliquots were stored at -80°C for future ELISA.

2.2.3 Primary rat neurons

Neuronal culture: Following a previously described protocol (Dong et al., 2004), suspension of dissociated hippocampal cells obtained from E-18 rats were plated on poly-d-lysine (MW 30,000–70,000; P7280, Sigma) coated 18-mm–diameter round glass cover slips in 12-well plates, at a density of 1 x 10^5 cells/well. Cultures were initially plated in a humidified atmosphere of 5% CO_2 and 95% air. The plating medium consisted of Neurobasal medium (Life Technologies) supplemented with 0.5 mM L-glutamine, 1x B-27 supplement (Life Technologies), 0.5% FBS, 25 µM glutamic acid (Sigma), and 0.5 mM sodium pyruvate. Two-thirds of the medium was replaced every two days with maintenance media which consisted of Neurobasal medium supplemented with 0.5 mM L-glutamine and 1X B-27 supplement.

Neuronal treatment: Five days after plating, the neuronal cultures were switched to serum free MEM supplemented with 1 mM sodium pyruvate and PSG. Neuronal cultures were treated the following day with LPS and L655,708 using the same procedure as the neuronal-glial co-cultures. The media from the cultured neurons were collected and centrifuged at 3000 rpm for 5 minutes. The supernatant was collected and aliquots were stored at -80°C for future ELISA.

2.2.4 Primary rat astrocytes

Astrocytic cultures: Similar to the procedure used for primary microglial cultures, cell suspensions of mixed neuronal-glial cells obtained from E18 rat cortices were plated on 100

mm culture dishes at a density of 1 x 10^5 cells/mL. Cells were incubated in a humidified atmosphere of 5% CO_2 and 95% air in DMEM supplemented with 10% FBS and PSG. Upon reaching confluence, cells were split by trypsinization and re-seeded in 100 mm culture dishes for the generation of a new passage. After three passages, cells were seeded on 18-mm–diameter round glass cover slips in 12-well plates, at a density of 3 x 10^6 cells/well.

Astrocytic treatments: Upon reaching 80% confluence, astrocytic cultures were switched to serum free MEM supplemented with 1 mM sodium pyruvate and PSG. Astrocytic cultures were treated the following day with LPS and L655,708 using the same procedure as the mixed neuronal-glial co-cultures. A438079 (100 μM), a $P2X_7$ receptor antagonist was pretreated for 24 hours and astrocytes were subsequently treated with both A438079 and LPS for 24 hours. The media of the treated astrocytes were collected and centrifuged at 3000 rpm for 5 minutes. The supernatant was collected and aliquots were stored at -80°C for future ELISA.

2.2.5 Preparation of neuronal- and astrocytic-conditioned media

In the intact brain, microglia activity is subject to soluble factors from neurons and astrocytes (Farber et al., 2005; Kuhn et al., 2004; Schilling et al., 2001). To study whether the $\alpha5GABA_A$ receptor regulates secreted soluble factors from these cells, primary microglial cultures were treated with astrocytic or neuronal conditioned medium (ACM or NCM). Specifically, primary astrocytes or neurons were treated using the same procedure as mentioned above; cells were treated with the vehicle (control), LPS (50 ng/mL), LPS + L655,708 (5 μM) or with LPS + A438079 (100 μM). After treatment, ACM or NCM were collected and centrifuged at 1000 RPM for 5 minutes. The supernatant was then immediately applied to primary microglia cultures. After 24 hours, the media were collected and

centrifuged at 3000 rpm for 5 minutes. The supernatant was collected and aliquots were stored at -80°C for ELISA.

2.3 Immunochemistry and confocal microscopy

Immunohistochemistry of brain sections: Brain sections of *Gabra5*$^{-/-}$ and WT mice were washed three times with PBS and then permeablized with 0.25% Triton-PBS for 5 minutes. Normal donkey serum (NDS; 10% in 0.25% Triton-PBS) was used to block sections for 2 hours at room temperature. Sections were then washed once for 5 minutes. Primary antibody was applied in 2% NDS/0.25% Triton-PBS for 24 hours at room temperature. After three washes, the secondary antibody was applied in 2% NDS/0.25% Triton-PBS for two hours at room temperature. Double staining was achieved by applying another primary antibody and subsequent secondary antibody. Sections were mounted between microscope slides and cover slips with Fluoromount–G (#17984-25, Electron Microscopy Sciences). Images were taken using a Zeiss confocal microscope (Carl Zeiss, Gottingen, Germany) primarily using a 40x objective lens. Images were taken and saved in a computer via the LSM Image Browser (LSM 510) for analysis.

Primary antibodies used in these studies included Rabbit Anti-Mouse IL-1β (1:200; 500-P51, Peprotech) and Rat Anti-Mouse CD11b (1:150; MCA74G, AbD Serotec). Corresponding secondary antibodies applied were Cy3-Donkey-Anti Rabbit immunoglobulin G (IgG) (1:400; 70295, Jackson ImmunoResearch) and Fluorescein (FITC) Donkey-Anti Rat (1:200; 79415, Jackson ImmunoResearch).

Immunocytochemistry on primary cultures. After specific treatments as described above, cultured cells were rinsed once with PBS and immediately fixed with 4% PFA for 10

minutes. Cells on glass cover slips were then washed for 5 minutes with 0.1 M Glycine-PBS and washed again with PBS 2 X 5 minutes. Cells were permeablized with 0.1% Triton-PBS for 5 minutes and PBS washed 3 X 5 minutes. Blocking consisted of 1% NDS in PBS for 1 hour at room temperature. Primary antibodies were applied in 1% NDS solution overnight at 4 °C. After incubation, cells were washed 3 X 5 minutes in PBS and a corresponding secondary antibody applied in 1% NDS. Negative controls consisted of applying secondary antibodies only. For double staining, another primary antibody and subsequent secondary antibody was applied. Finally, cells were mounted on a coverslip using Fluromount G. The threshold of fluorescent detection was set to a level of ~1.5 times the fluorescent intensity of the negative control. Electronic images of test tissues and cells were taken via the LSM 510 program.

Primary antibodies used in these studies included Rabbit Anti-Rat IL-1β (1:200; 500-P80, Peprotech), Mouse Anti-Rat CD11b (1:150; MCA275FT, AbD Serotec), Mouse Anti-Neuronal Nuclei (NeuN), clone 60, Alexa Fluor®488 conjugated (1:500; MAB377X, Millipore), and Anti-Glial Fibrillary Acidic Protein (GFAP) Clone G-A-5 Cy3 Conjugate (1:1000; C9205, Sigma). Corresponding secondary antibodies applied were Cy3-Donkey-Anti Rabbit IgG (1:400; 70295, Jackson ImmunoResearch) and FITC Donkey-Anti Mouse (1:200; 75450, Jackson ImmunoResearch).

***Image Analysis*:** All confocal microscopy images were analyzed by means of the java based software ImageJ, provided by The National Institute of Health (NIH). For images taken from hippocampal regions, special analyses were focused on the region just below the dentate gyrus, because first, the α5-subunit is highly expressed in the hippocampus (Sur et al., 1999; Sur et al., 1998) and that it is suggested that α5GABA$_A$ receptor expression increases in

inflammation (Clarkson et al., 2010; Wang et al., 2012). Second, I observed a high density of CD11b-positive microglia in this region. As illustrated in Figure 2.1, average intensities of CD11b and IL-1β immunofluorescence were calculated by converting each image to despeckled, black and white images. For each test mouse, a minimum of five images of the hippocampus was analyzed.

For quantification of the immunofluorescent intensity of a test protein (e.g. IL-1β) in primary cell cultures, the cell size and mean fluorescent intensity of single cells were measured (as illustrated in Figure 2.2). In each experiment, a minimum of 70 cells per treatment were analyzed. Specifically, the fluorescent intensities of test proteins were quantified and averaged. The mean values of fluorescence measurements of each treatment group were normalized to the mean measurement obtained from the control (non-treated) group.

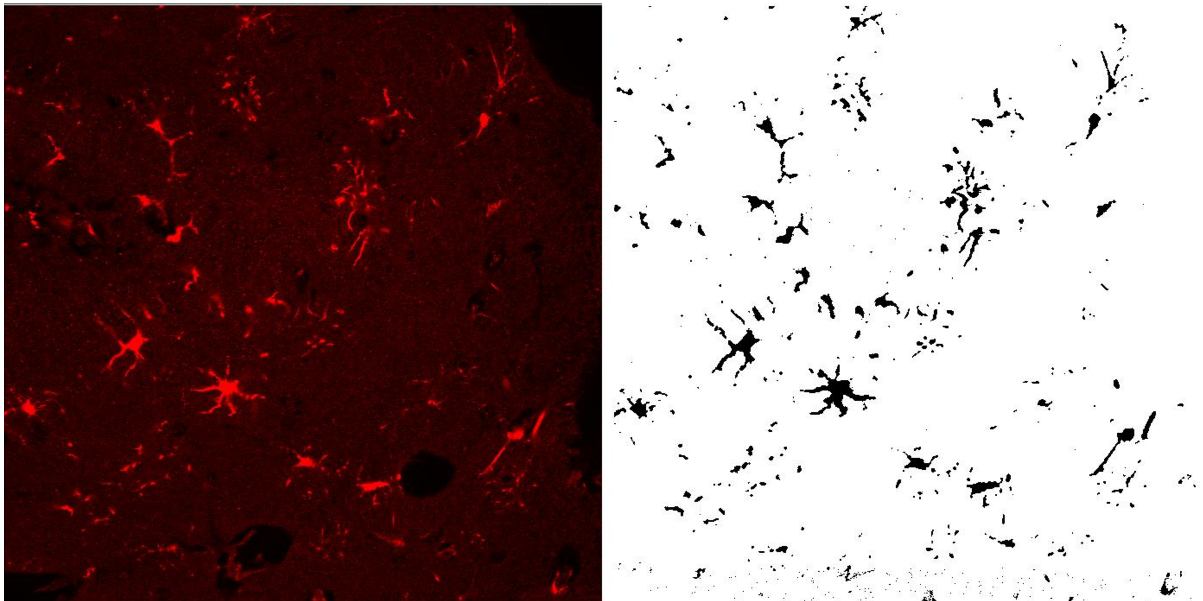


Figure 2.1 Example image illustrating ImageJ analysis of IL-1β in hippocampal tissues. Confocal images (left) are converted to black and white images (right) in ImageJ to calculate expression intensity

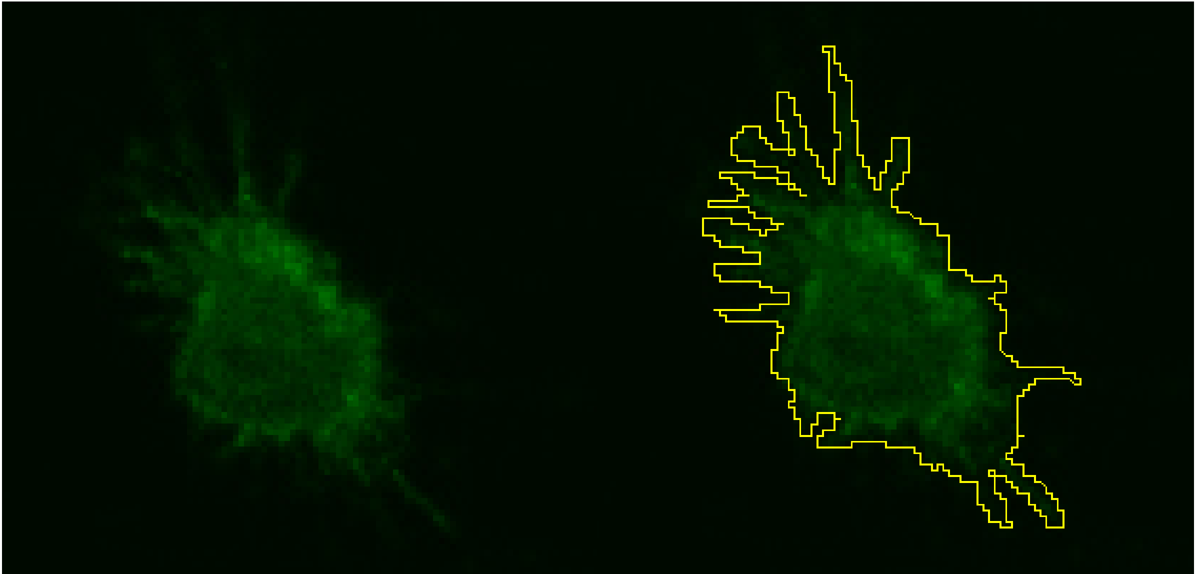


Figure 2.2 Example images illustrating ImageJ analysis of CD11b-immunofluorescence in cultured microglia. Confocal images (left) are traced using ImageJ to calculate intensity of CD11b expression and microglia cell size within the outlined area (right)

2.4 Phagocytosis assay

Phagocytosis is one of the primary functions of microglial cells. To examine the role of $\alpha 5GABA_A$ receptors in the regulation of microglial phagocytosis, hipoocampi neuronal-glial cell cultures were treated for twenty-four hours in the absence (control) or in the presence of L655,708 (0.5 μM). The phagocytotic ability of microglia was analyzed by using the Phagocytosis Assay Kit following the manufacturer's (Cayman Chemical Company) instructions. In this assay, Latex Bead-Rabbit IgG-FITC Complex is added to cultures which can be engulphed by microglia cells. FITC fluorescence in the microglia were taken by confocal microscopy and the intensity of FITC fluorescence in the cells was analyzed using ImageJ.

2.5 ELISA

Media from co-cultures, primary neurons, astrocytes, and microglia were stored at -80°C until they were used for ELISA. To measure secreted IL-1β levels in the media of primary cultures, the rat IL-1β Quantikine ELISA kit (RLB00) was used according to the manufacturer's (R&D) instructions. In each individual experiment, samples were measured in triplicate; three wells of cultured primary cells were treated with the same treatment and IL-1β was measured from each of the three wells. Measurements were averaged and the mean value would constitute a measurement for one treatment in one experiment.

2.6 Statistical analysis

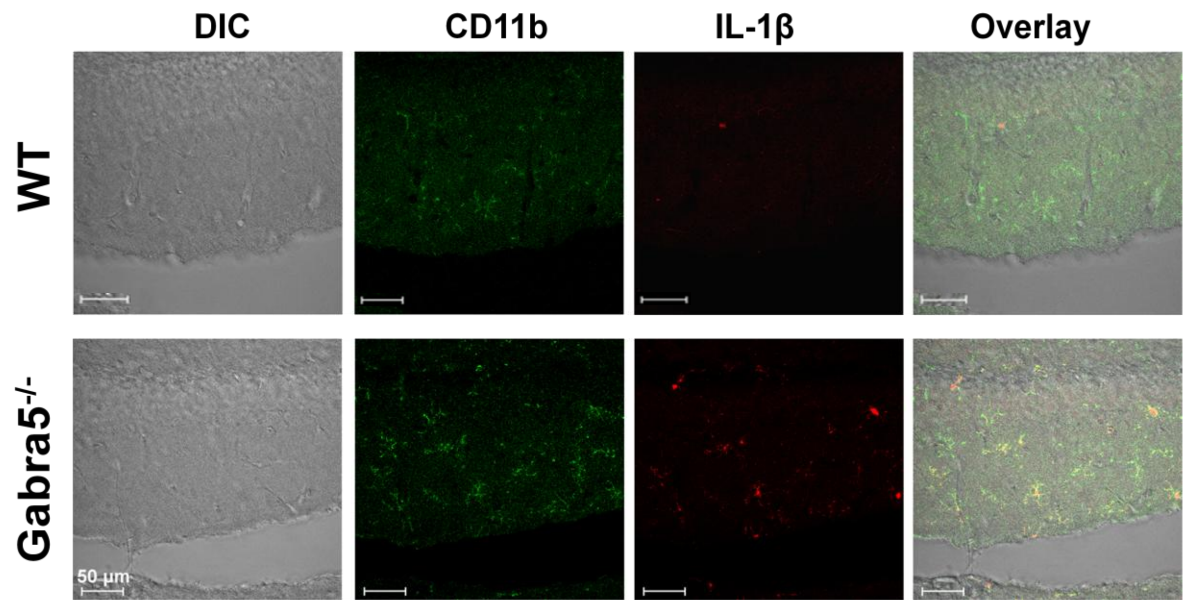
Statistical analysis was conducted using the program GraphPad Prism 5 (San Diego, California, USA). All data are presented as mean ± standard error of the mean (SEM) and was examined using one way analysis of variance (ANOVA) followed by Tukey's *t*-test. Statistical significance was considered at the $p < 0.05$ level unless otherwise stated.

Chapter 3: Results

3.1 Deletion of the $GABA_A$ receptor–α5 subunit increases the microglial expression of IL-1β in mice challenged by systemic administration of LPS

By immunohistochemical analysis of hippocampal sections of *Gabra5*$^{-/-}$ mice and their WT littermates, I observed that under control conditions, CD11b-positive microglial cells in the hippocampus (as well as other brain regions) displayed ramified processes. In addition, no immunoreactivity of IL-1β was detected in hippocampal cells of *Gabra5*$^{-/-}$ mice and WT mice. Moreover, in the region under the dentate gyrus and other regions of the hippocampus, the immunofluorescent intensity of CD11b was significantly higher in the microglial cells of control *Gabra5*$^{-/-}$ compared to their WT littermates ($p < 0.01$; Fig. 3.1B). Six hours after i.p. administration of LPS, the immunoflouorescence of IL-1β was detected in few CD11b-positive microglial cells of WT mice. Notably, microglia in the hippocampus of *Gabra5*$^{-/-}$ mice exhibited a significantly higher expression level of IL-1β compared to that of WT mice ($p < 0.01$; Fig. 3.1C). I focused on the level of IL-1β in microglia as it is an important proinflammatory cytokine that is critically involved in various inflammatory reactions (Simi et al., 2007). The significant difference in the expression levels of both CD11b and IL-1β between the *Gabra5*$^{-/-}$ and WT mice was noticeable only in hippocampal microglia. This result implies that α5$GABA_A$ receptor mediated signaling is critically involved in the regulation of microglial response to systemic inflammation.

A



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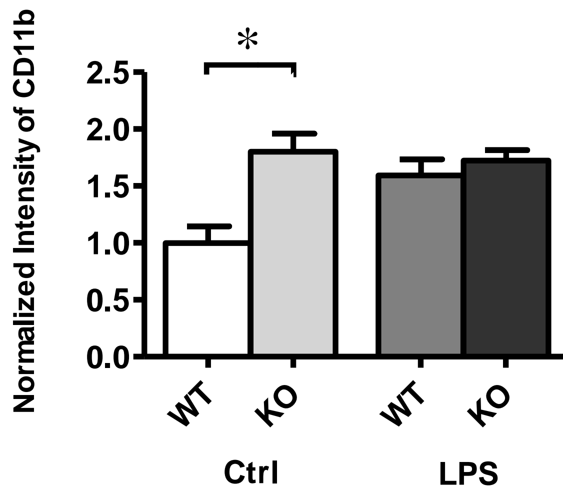

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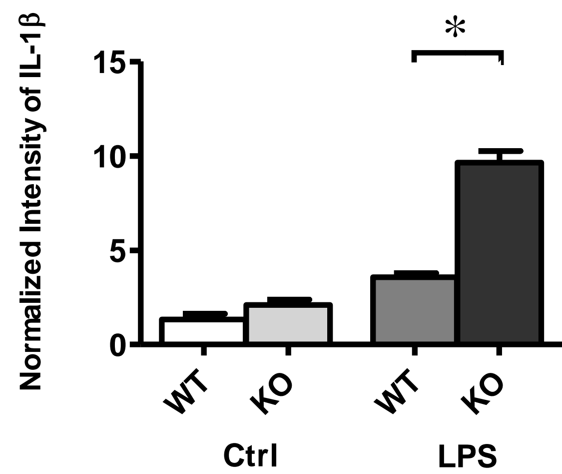


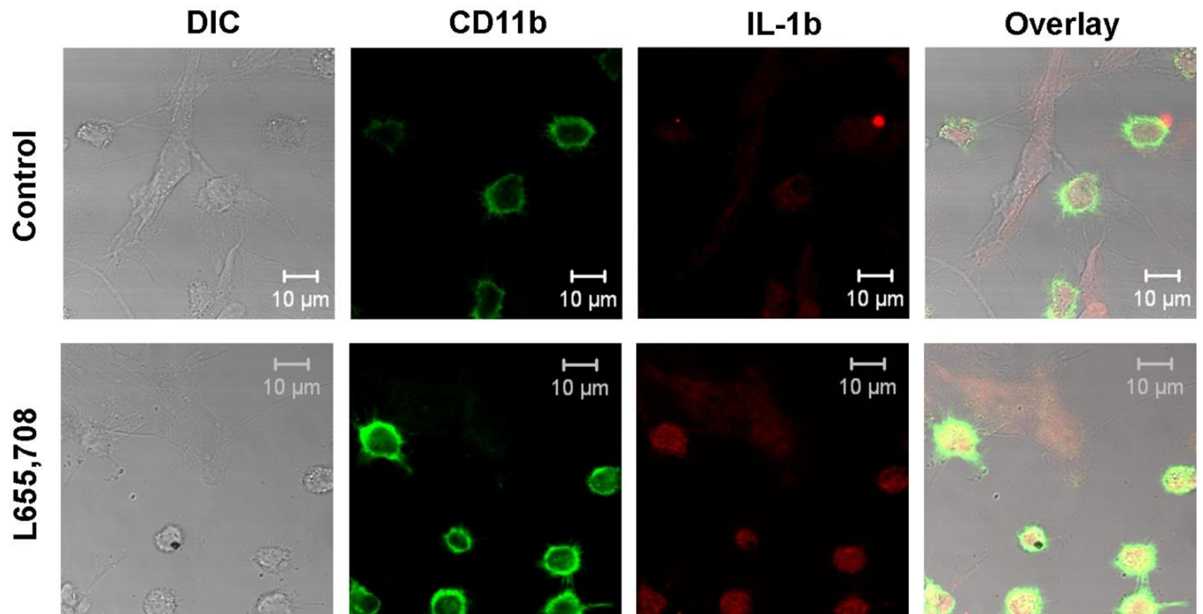
Figure 3.1 Higher expression of CD11b and IL-1β in microglia of *Gabra5*$^{-/-}$ mice compared to WT mice *(see previous page.)* **A)** Representative images of CD11b and IL-1β staining in the area under the dentate gyrus of the hippocampus in mice injected i.p. with LPS. *(See next page)* **B)** Normalized immunofluorescent intensity of CD11b in the hippocampus. *Gabra5*$^{-/-}$ (KO) mice displayed significantly higher expression in saline injected (Ctrl) mice (* $p < 0.01$, n = 4). **C)** Normalized intensity of IL-1β in the hippocampus.

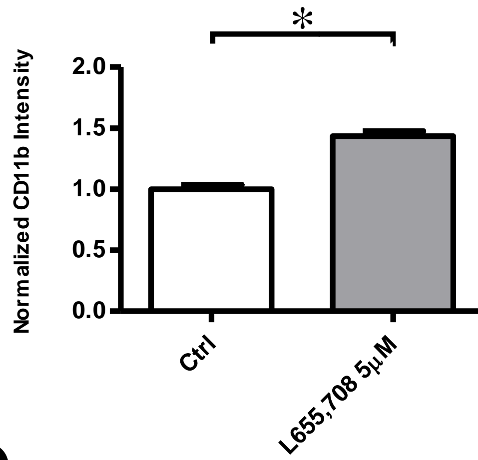
3.2 L655,708 increases microglial activation in mixed neuronal-glial cultures

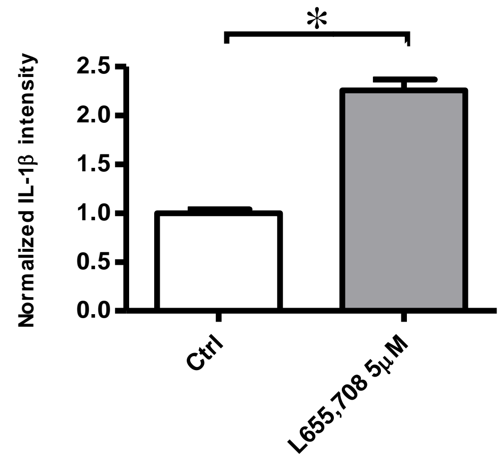
Next I investigated the potential cellular and molecular mechanisms by which $\alpha5GABA_A$ receptor-mediated signaling regulates microglial activation in LPS-induced systemic inflammation. To this aim, I treated mixed neuronal-glial co-cultures with LPS to evoke immune reactions in brain cells, and used L655,708 to selectively block $\alpha5GABA_A$ receptor signaling. Mixed neuronal-glial cultures consisted primarily of neurons, astrocytes and microglia. The effects of LPS and L655,708 were analyzed by examining the morphology of microglial cells and the expression and secretion of IL-1β. I had the following findings.

First, CD11b-positive microglia in mixed neuronal-glial co-cultures exhibited an oval shape with short ramified processes. In control cells, a basal level of IL-1β immunofluorescence was observed in almost all CD11b-positve microglia (Fig. 3.2A). Treating the mixed co-cultures with L655,708 (5 μM) significantly increased the expression levels (measured by immunofluorescent intensity) of CD11b and IL-1β in microglia (Fig. 3.2; $p < 0.01$).

Second, selective blockade of $\alpha5GABA_A$ receptor signaling [L655,708 (0.5 – 5 μM)] increased the phagocytotic activity of microglia, displaying higher fluorescent intensity of FITC in mixed neuronal-glial co-cultures in comparison to control cultures (Fig. 3.3; $p < 0.01$) .


A
DIC
CD11b
IL-1b
Overlay
Control
L655,708
10 µm


B
*
Normalized CD11b Intensity
2.0
1.5
1.0
0.5
0.0
Ctrl
L655,708 5µM


C
*
Normalized IL-1β intensity
2.5
2.0
1.5
1.0
0.5
0.0
Ctrl
L655,708 5µM

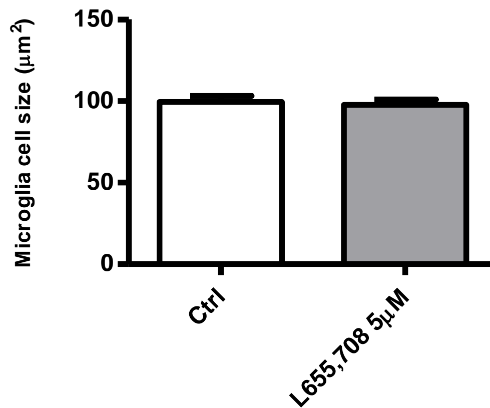

D
Microglia cell size (µm²)
150
100
50
0
Ctrl
L655,708 5µM

Figure 3.2 Microglia exhibit higher expression of CD11b and IL-1β in mixed hippocampal co-cultures treated with α5GABA$_A$ receptor inverse agonist L655,708. (*See previous page)* **A)** Representative images of co-cultures with CD11b and IL-1β. **B)** Normalized CD11b-immunofluorescent intensity (* $p < 0.01$, n = 6 separate experiments). **C)** Normalized IL-1β intensity (* $p < 0.01$, n = 6). **D)** The average size of microglia cells (* $p < 0.01$, n = 6).

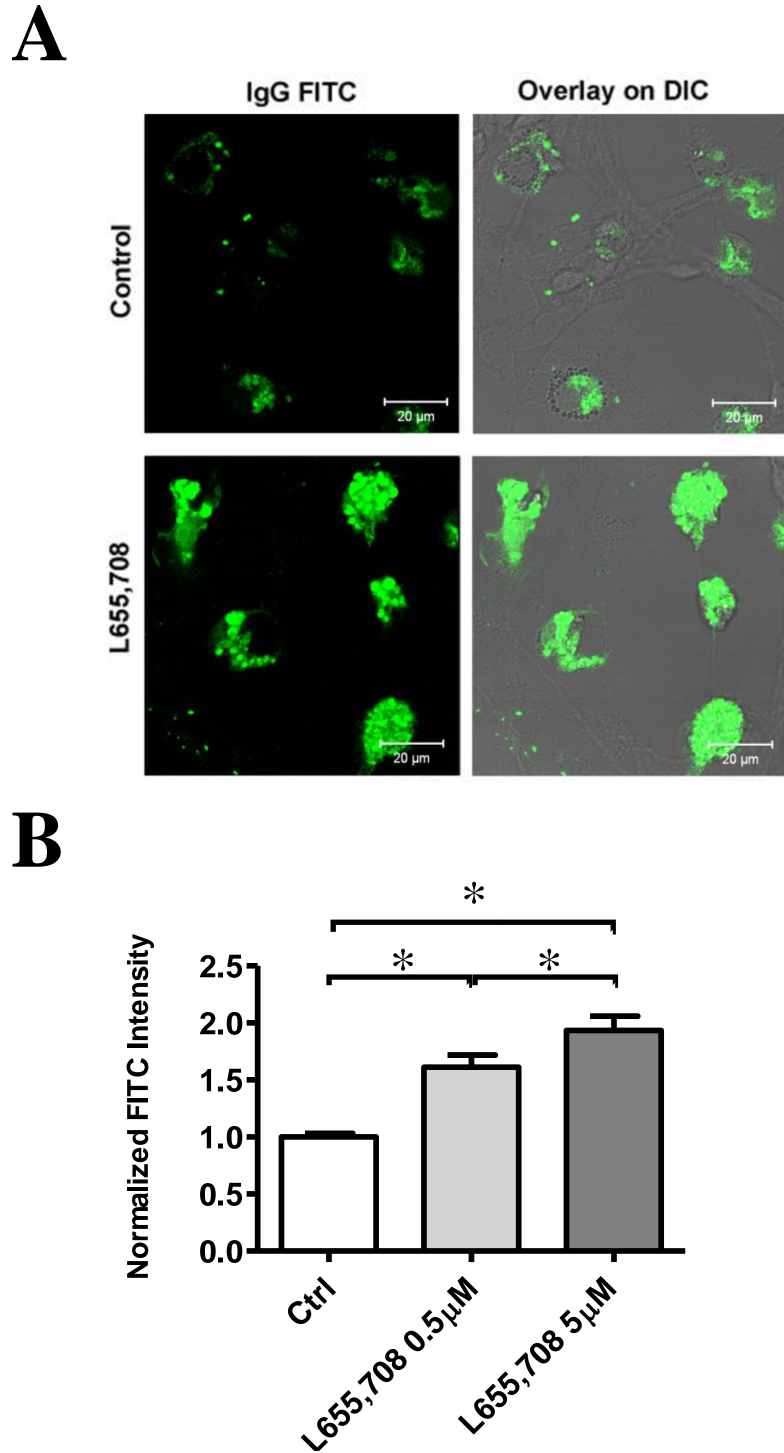


Figure 3.3 Microglia exhibit higher phagocytotic activity in mixed hippocampal co-cultures treated with L655,708. A) Representative images of FITC-IgG in co-cultured cells that were treated with L655,708 (5 μM) and without (control) L655,708. **B)** Normalized FITC intensity. (* $p < 0.01$, in each group n = 6-8 images of cultured cells in 2 separate experiments).

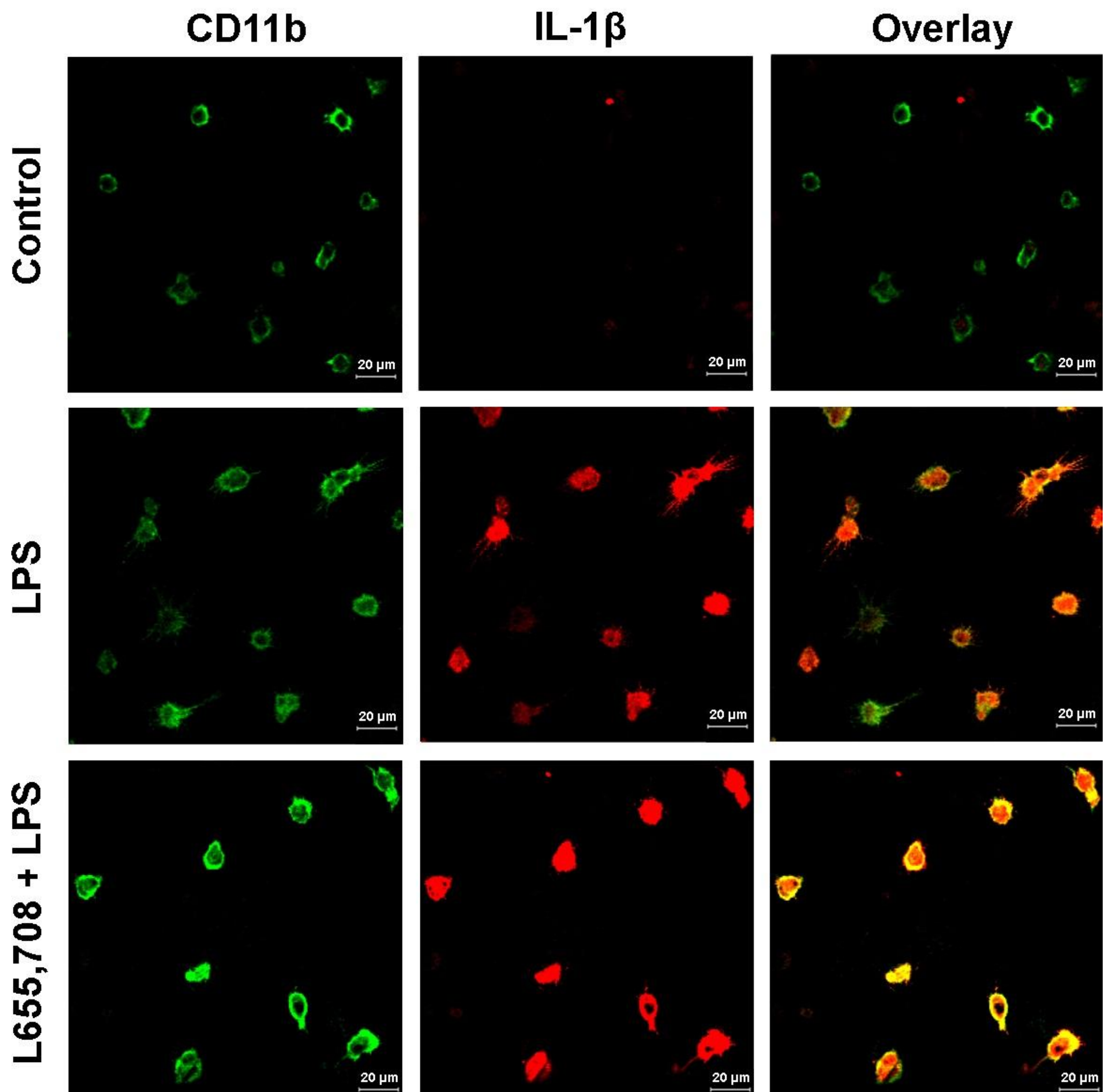
Treating the co-cultures with LPS (50 ng/mL) also significantly increased the expression levels of CD11b and IL-1β. Notably, when co-cultures were treated with both L655,708 (5 μM) and LPS (50 ng/mL), microglia exhibited significantly higher expression levels of CD11b and IL-1β, in comparison to co-cultures treated with LPS alone (Fig. 3.4; $p < 0.01$).

Another important finding was that microglial morphology changed from a larger oval shape with extended processes to a smaller round shape without processes when co-cultures were treated with both L655,708 and LPS, in comparison to co-cultures that were treated with LPS alone (Fig. 3.4A). This was evident by measuring the size of CD11b-positve microglia (Fig. 3.4D; $p < 0.01$). Such a change in microglial morphology may imply increased activation of microglia.

Treating the mixed co-cultured cells with bicuculline (50 μM), a $GABA_A$ receptor antagonist, mimicked the effect of L655,708, resulting in a higher expression of IL-1β in microglia. Bicuculline also increased the LPS-induced IL-1β expression in microglia when applied together with LPS (Fig. 3.5; $p < 0.01$;).

Finally, ELISA of the culture media from neuronal-glial cultures showed that treating the co-cultures with LPS significantly increased the concentration of IL-1β in the culture medium, although no IL-1β was detectable in the medium of control cells. Surprisingly, the concentration of secreted IL-1β from co-cultures treated with both LPS and L655, 708 (0.5-5.0 μM) was significantly lower than that of co-cultures treated with LPS alone (Fig. 3.6; $p < 0.01$). IL-1β was not detected from co-cultures treated with L655,708.

A



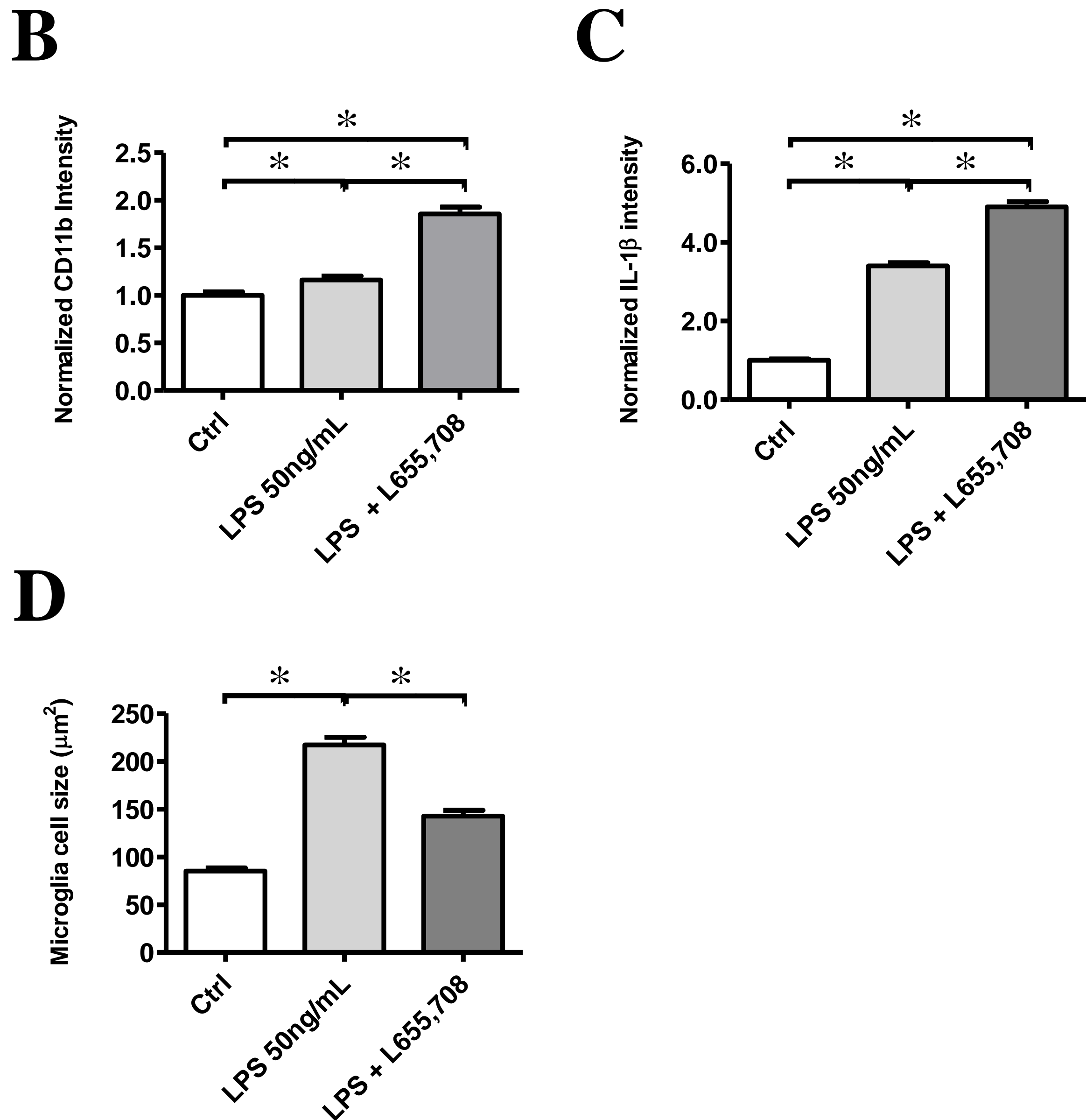
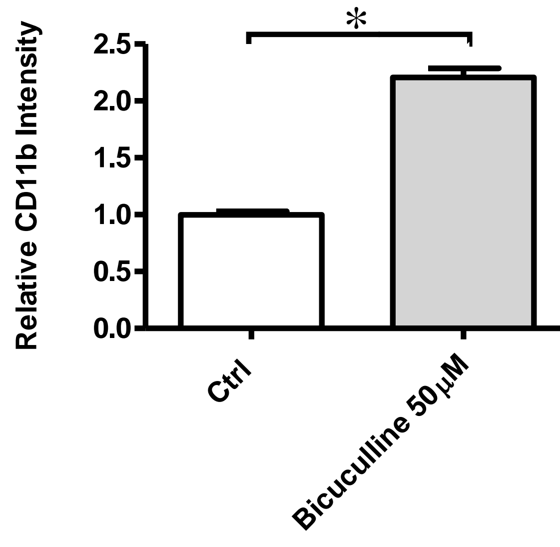


Figure 3.4 Microglia in mixed hippocampal co-cultures treated with L655,708 and LPS exhibit higher expression of CD11b and IL-1β compared to co-cultures treated with LPS alone. (*See previous page*) **A)** Representative images of co-cultures treated with LPS (50 ng/mL) and/or L655,708 (5 μM). **B)** Normalized CD11b-immunofluorescent intensity. (* $p < 0.01$, n = 6 separate experiments) **C)** Normalized IL-1β-immunofluorescent intensity (* $p < 0.01$, n = 6). **D)** Microglia cell size (* $p < 0.01$, n = 6).

A



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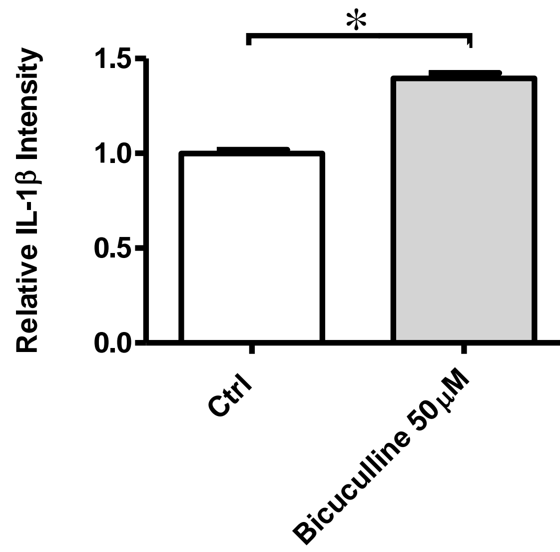

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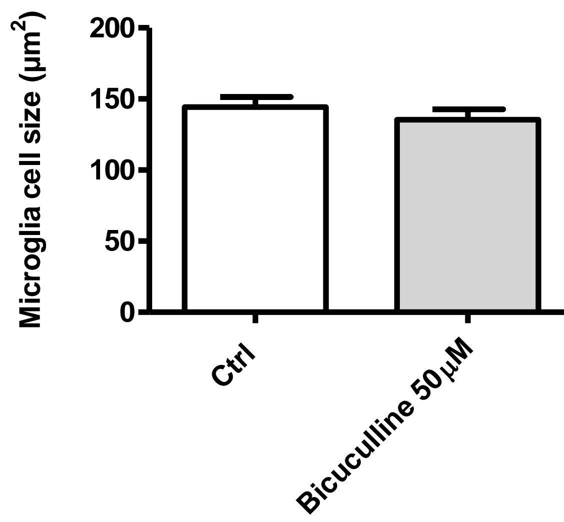
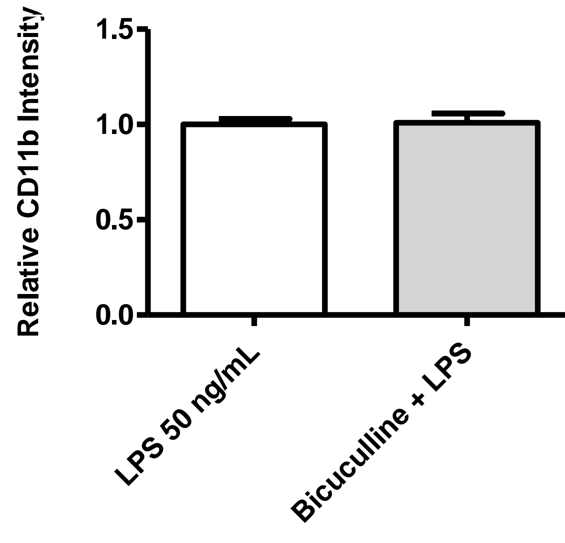


D



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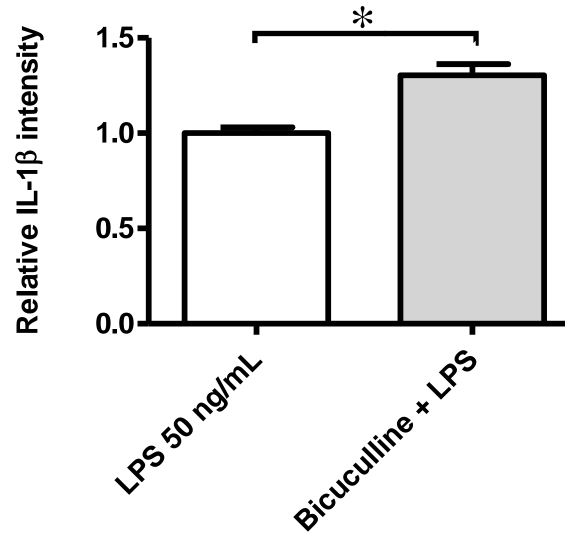


F

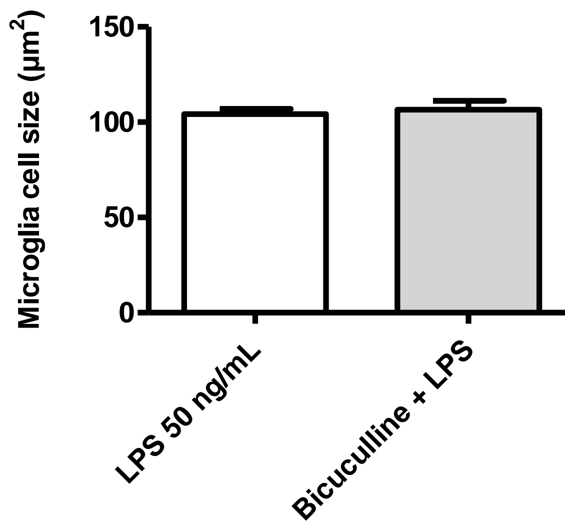


Figure 3.5 Blockade of $GABA_A$ receptors increases the expression of CD11b and IL-1β. *(See previous two pages)* **A-C)** Microglia in mixed hippocampal co-cultures treated with bicuculline exhibit higher expression of CD11b and IL-1β compared to control. **A)** Normalized CD11b intensity. (* $p < 0.01$, n = 3 separate experiments) **B)** Normalized IL-1β intensity (* $p < 0.01$, n = 3) **C)** Microglia cell size (n = 3) **D-F)** Co-cultures treated with both bicuculline and LPS compared to co-cultures treated with LPS alone. **D)** Normalized CD11b intensity (n = 3) **E)** Normalized IL-1β (* $p < 0.01$, n =3) **F)** Microglia cell size (n = 3)

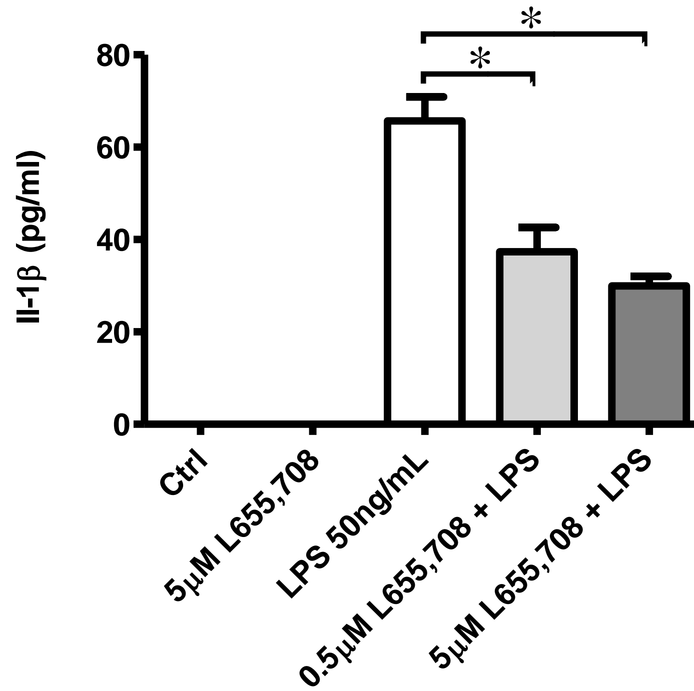


Figure 3.6 Treatment of LPS-primed hippocampal co-cultures with L655,708 decreases the extracellular concentration of IL-1β. ELISA of IL-1β in the medium of co-cultures treated without (Ctrl) or with LPS and/or LPS + L655,708. IL-1β concentrations in the culture media: LPS (50 ng/mL) treatment = 66 ± 5 pg/mL; LPS + L655,708 (0.5 μM) = 37 ± 5 pg/mL; LPS + L655,708 (5.0 μM) = 30 ± 2 pg/mL; * $p < 0.01$, n = 3.

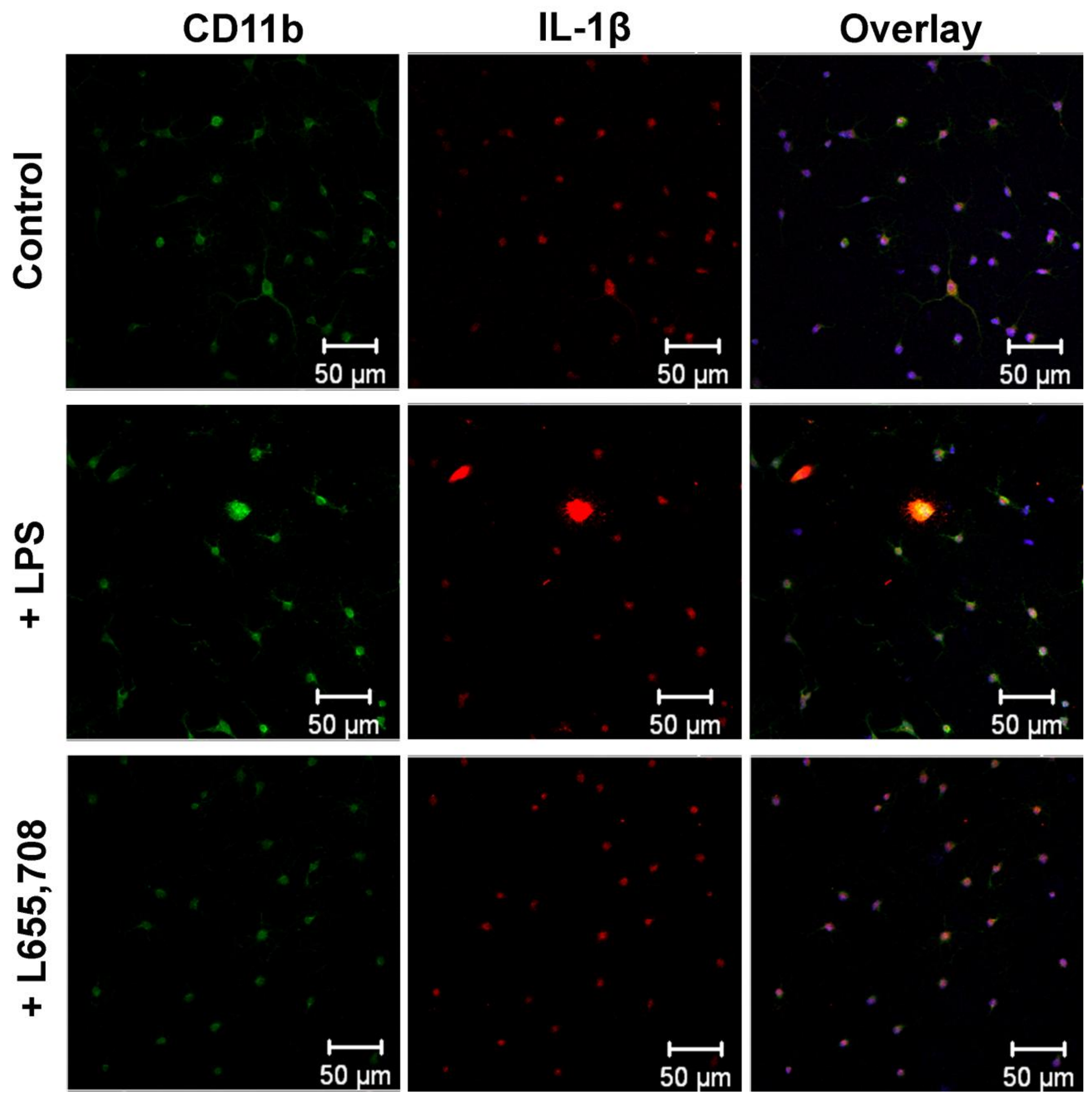
Taken together, these *in vitro* results indicate that 1) consistent with the finding in *Gabra5*$^{-/-}$ mice, interrupting α5GABA$_A$ receptor signaling increased the activity of microglial cells to inflammatory stimulants such as the bacterial endotoxin LPS; and 2) these changes in microglial activities are due to alterations in α5GABA$_A$ receptor mediated signaling among brain cells, but not associated with peripheral cells.

3.3 α5GABA$_A$ receptors indirectly affect microglial IL-1β expression through modulating astrocytes and neurons

In neuronal-glial cultures, the effects of L655,708 on microglial morphology and expression of IL-1β could be due to its direct action on microglial cells and/or because of its indirect action on neurons or astrocytes. Given the experiments performed by other members in my laboratory showing that 1) GABA failed to evoke detectable current in primary rat microglia or in mouse microglial line of BV2 cells; and 2) no mRNA of α5-subunit was detectable in BV2 cells by polymerase chain reaction (PCR) assays (*acknowledged* by laboratory communications), I tested whether α5GABA$_A$ receptors in neurons or astrocytes indirectly regulate microglial activation through the following experiments.

First I tested the effects of L655,708 and LPS on the expression of IL-1β in isolated primary microglia cultures. Immunocytochemical analyses showed that after described procedures of microglia isolation, ~95% of cells in the cultures exhibited immunofluorescence for CD11b. As shown in Fig. 3.7, these isolated CD11b-positive microglia displayed a ‘resting’, ramified morphology – branched, extended processes from a small cell body. Interestingly, treating isolated microglia with LPS (50 ng/mL) increased CD11b and IL-1β expressions only in a few (less than 0.1%) cells. In addition, treating the microglia with L655,708 (5 μM) had no

A



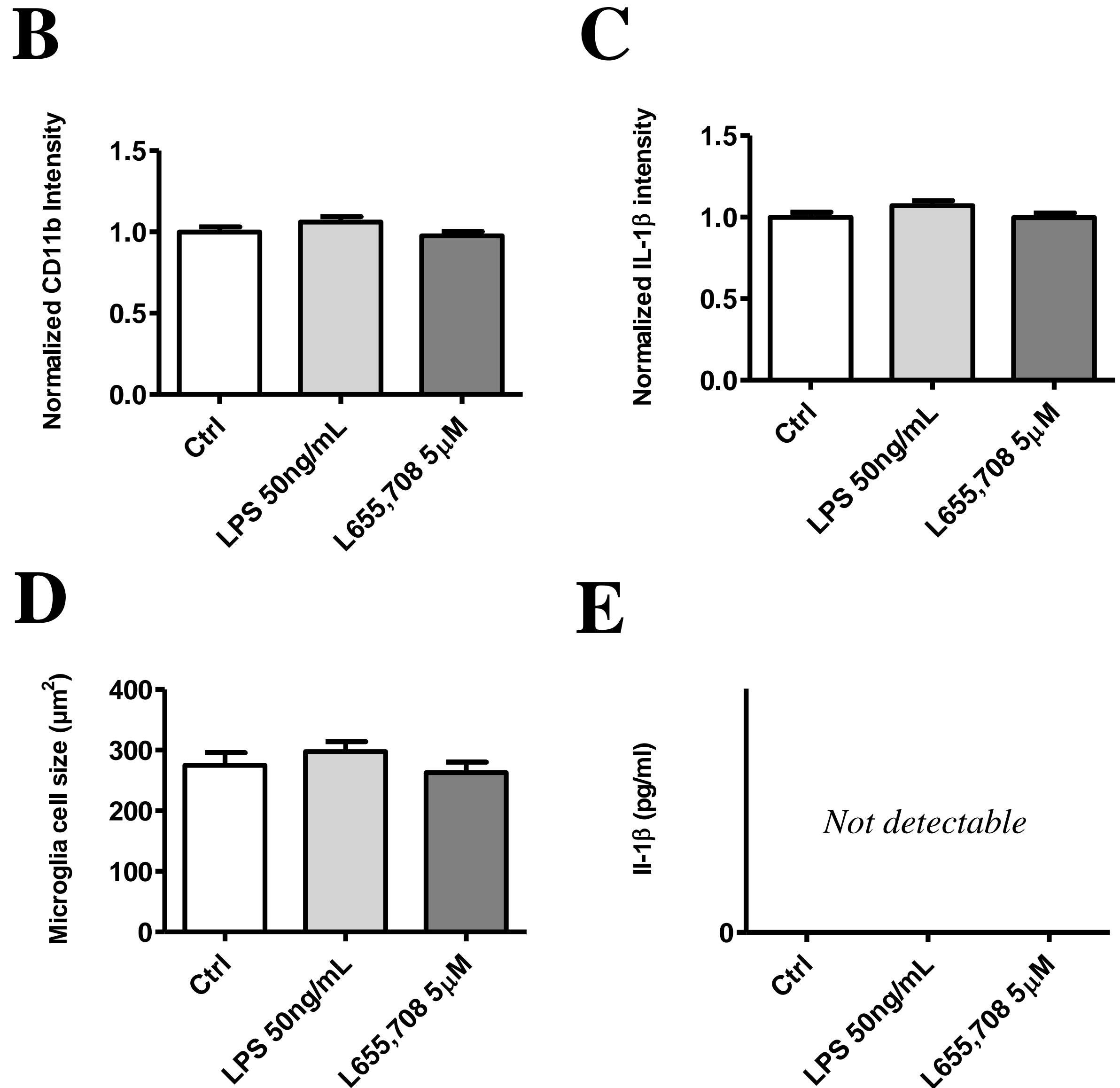


Figure 3.7 Treatment of isolated cortical microglia with L655,708 and/or LPS does not significantly affect the expression level of CD11b and IL-1β. (*See previous page*) **A)** Representative images of primary microglia treated with L655,708 (5 μM) and LPS (50 ng/mL). **B)** Normalized CD11b-immunofluorescent intensity in microglia (n = 2 separate experiments) **C)** Normalized IL-1β intensity in microglia (n = 2) **D)** Microglia cell size (n =2) **E)** ELISA did not detect IL-1β in the media of primary microglia cultures that were treated without (Ctrl), or with L655,708, or with LPS.

effect on the expression levels of CD11b and IL-1β (Fig. 3.7B-C). Moreover, IL-1β in the medium of microglial cultures was not detectable (Fig. 3.7E). The absence of IL-1β expression and secretion suggest that 1) LPS-induced microglial activation requires co-factor(s) from astrocytes or neurons; and 2) the α5GABA$_A$ receptor regulates microglial activation possibly through modulating the activity of astrocytes or neurons.

I also determined the effects of L655,708 and LPS on CD11b and IL-1β expressions in isolated primary neurons. Treating the cultured neurons with L655,708 (5 μM) and LPS (50 ng/mL) had no effect on the expression of CD11b and IL-1β (Fig. 3.8). IL-1β was not detectable in the medium of isolated primary neurons treated with L655,708 or with LPS.

Next, the effects of L655,708 and LPS on the expression of IL-1β in isolated primary astrocytes were examined. Immunocytochemical analysis showed that under my experimental conditions, astrocyte cultures were ~98% pure, with ~2% of cells consisting of microglia (Fig. 3.9). Treating the isolated astrocytes with LPS (50 ng/mL) increased the immunofluorescent intensity of IL-1β in astrocytes, in comparison to control conditions. However, L655,708 (5 μM) did not significantly affect the LPS-induced increase of IL-1β expression (Fig. 3.9A). The concentration of IL-1β in the medium of astrocytes significantly increased following LPS treatment. Remarkably, the LPS-induced increase of IL-1β in the astrocytic media was significantly reduced by treating astrocyte cultures with L655,708 (Fig. 3.9B; $p < 0.05$). These results imply that α5GABA$_A$ receptors are expressed in primary astrocytes and they down-regulate their response to inflammatory stimulants such as LPS.

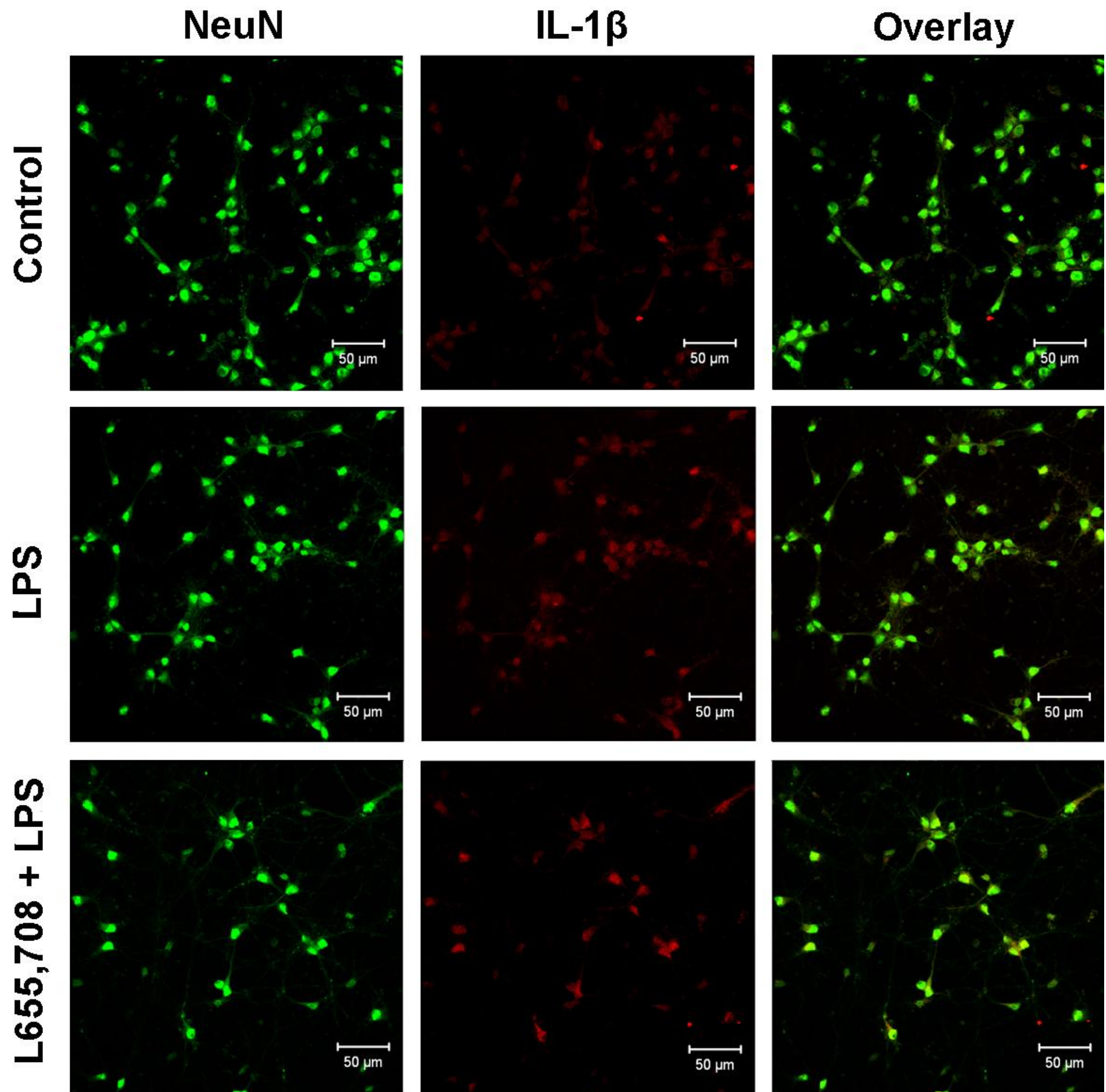
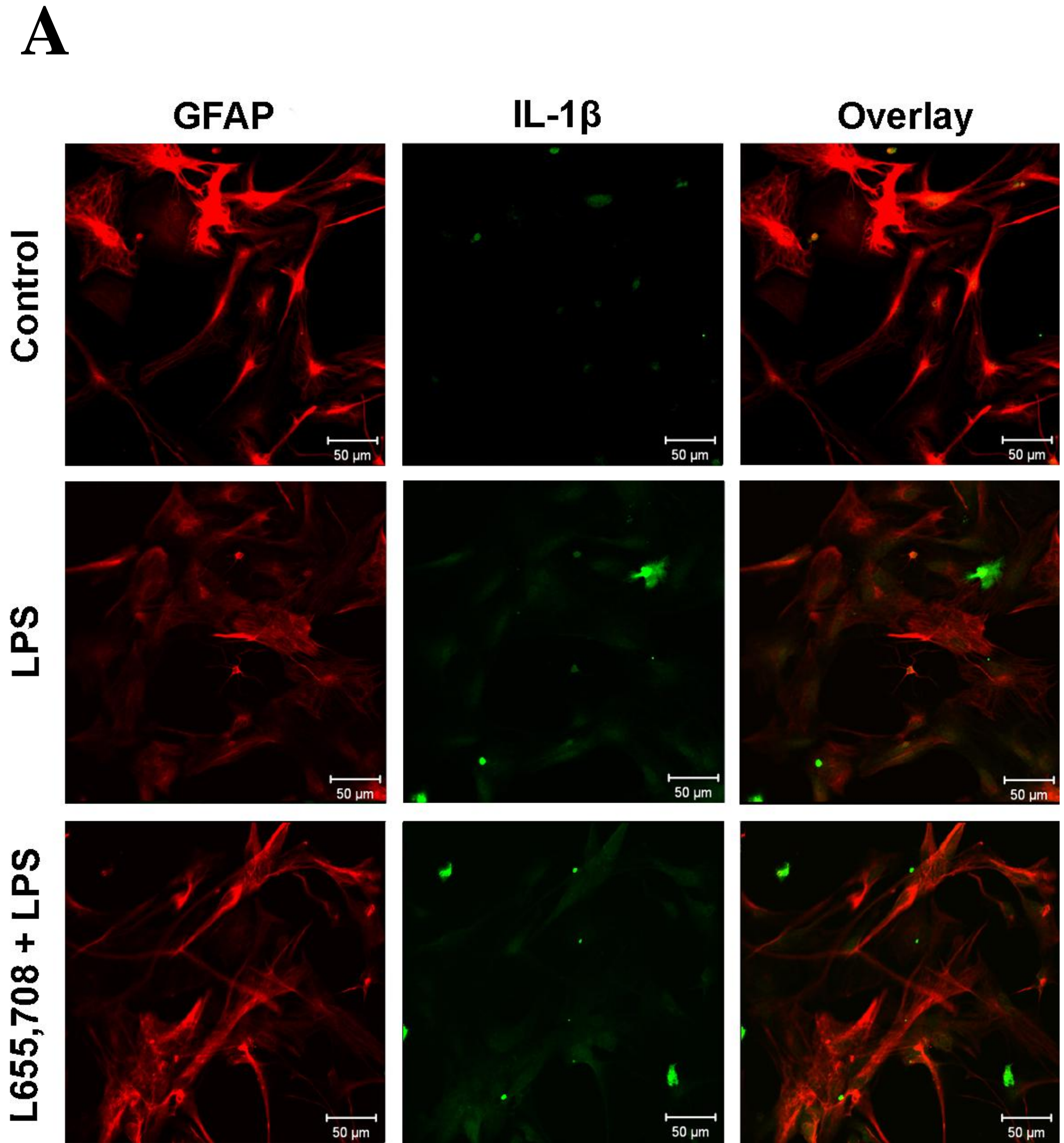


Figure 3.8 Treatment of isolated hippocampal neurons with L655,708 and/or LPS does not affect the expression level of IL-1β. Representative images of immunofluorescence of NeuN and IL-1β in hippocampal neurons treated with L655,708 (5 μM) and LPS (50 ng/mL).


A
GFAP
IL-1β
Overlay
Control
LPS
L655,708 + LPS
50 μm

B

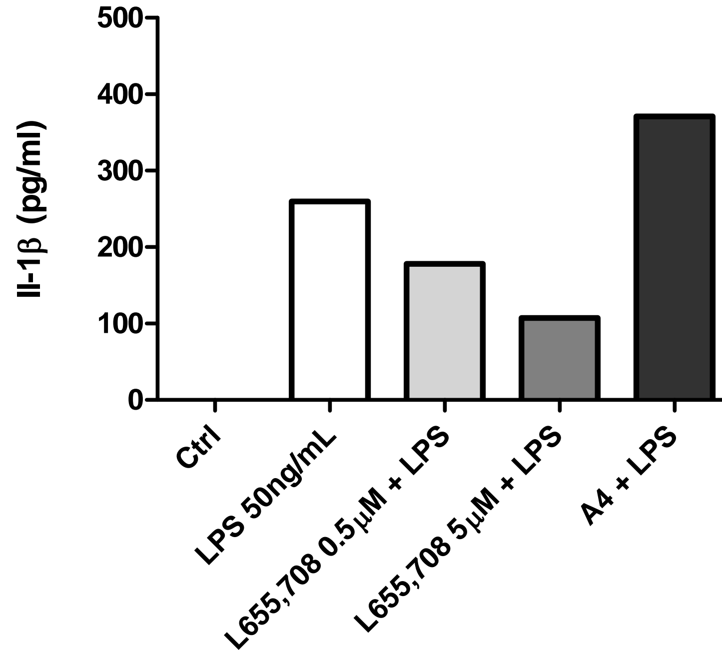


Figure 3.9 Treatment of isolated astrocytes with L655,708 and/or LPS increases IL-1β expression. (*See previous page*) **A)** Representative images of isolated cortical astrocytes treated with LPS (50 ng/mL) alone or with LPS+ L655,708 (5 μM). LPS treatment increased the expression level of IL-1β in the astrocytes. L655,708 treatment also increased the IL-1β immunofluorescent intensity in LPS-primed astrocytes. **B)** ELISA revealed a significant decrease of IL-1β in the media of LPS-primed astrocyte cultures treated with L655,708. IL-1β concentrations in the culture media: LPS (50 ng/mL) treatment = 260 pg/mL; LPS + L655,708 (0.5 μM) = 178 pg/mL; LPS + L655,708 (5 μM) = 107 pg/mL; LPS + A438079 (100 μM) = 370; n = 2 separate experiments.

As mentioned above, evidence indicates that microglia do not express α5GABA$_A$ receptors. Therefore, it is likely that α5GABA$_A$ receptor mediated signaling through other cell types may indirectly modulate microglial activity. To examine the possibility that α5GABA$_A$ receptors regulate microglial activation through astrocytes or neurons, isolated microglia were treated with astrocyte (ACM) or neuron (NCM) conditioned media. Conditioned media was obtained by treating astrocytes and neurons with LPS alone or LPS with L655,708. Microglial cultures were treated with CM for 24 hours, and microglial IL-1β expression and secretion were analyzed by immunocytochemistry and ELISA, respectively.

Microglia exhibited different morphologies in response to CM (Fig. 3.10). Primary microglia displayed a ramified morphology in control media and in non stimulated (control) NCM, indicating that they were in a resting state. Microglia treated with control ACM exhibited larger, oval cell bodies with retracted processes. This suggests that they were partially activated. This result indicates that soluble factor(s) secreted from astrocytes regulates the activity of microglia.

Treating primary microglia with LPS-primed or L655,708+LPS-primed NCM, immunofluorescent intensity of CD11b and IL-1β in microglia increased slightly in comparison to the control (Fig. 3.11). IL-1β concentration was not detected in the media.

Treating isolated microglia with LPS-primed ACM significantly increased the CD11b and IL-1β expression in microglia in comparison to control-ACM. In addition, microglia expressed higher levels of CD11b and IL-1β in L655,708+LPS-primed ACM (Fig. 3.12A). IL-1β concentration also increased in the media of microglia cultured in LPS-primed ACM, while L655,708+LPS-primed ACM exhibited a trend of increasing IL-1β secretion further (Fig. 3.12B).

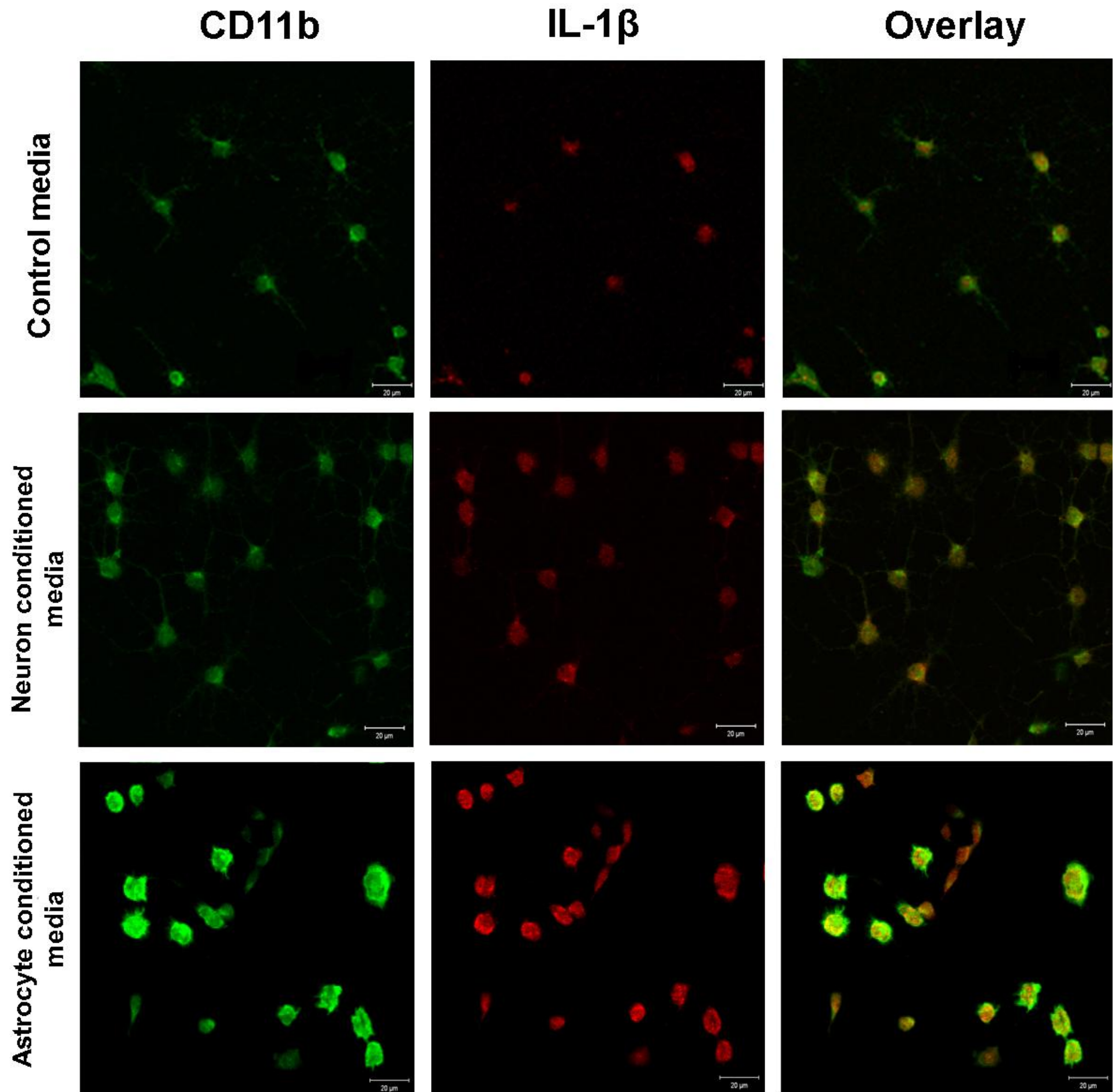


Figure 3.10 Microglia exhibited resting, ramified morphology in nonstimulated conditions and control neuron conditioned media while microglia exhibit an activated morphology when grown in control astrocyte conditioned media. Note that microglia treated with neuronal-conditioned medium show a ramified morphology and lower expression levels of CD11b and IL-1β, which are similar to that of microglia cultured in control condition.

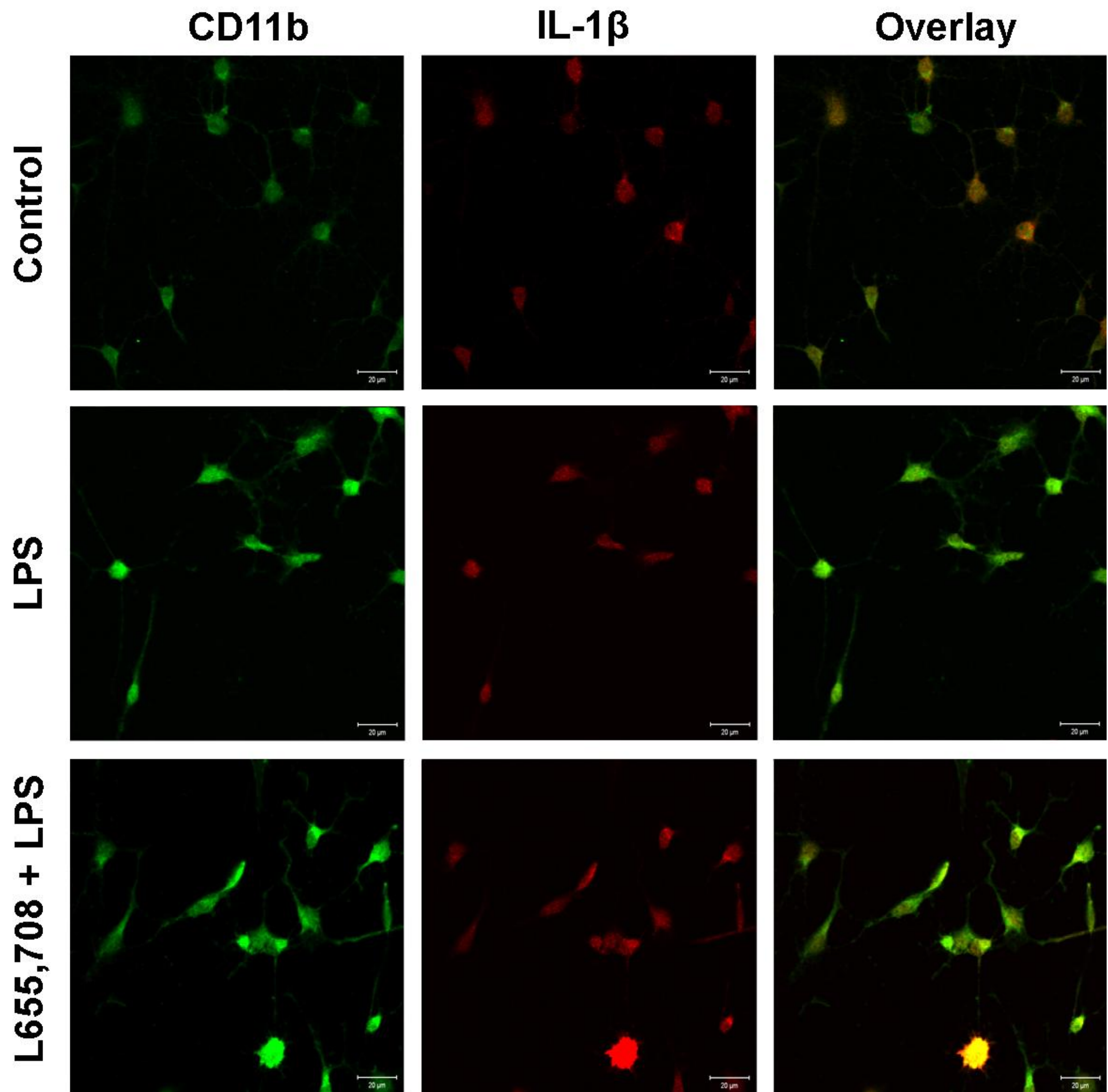
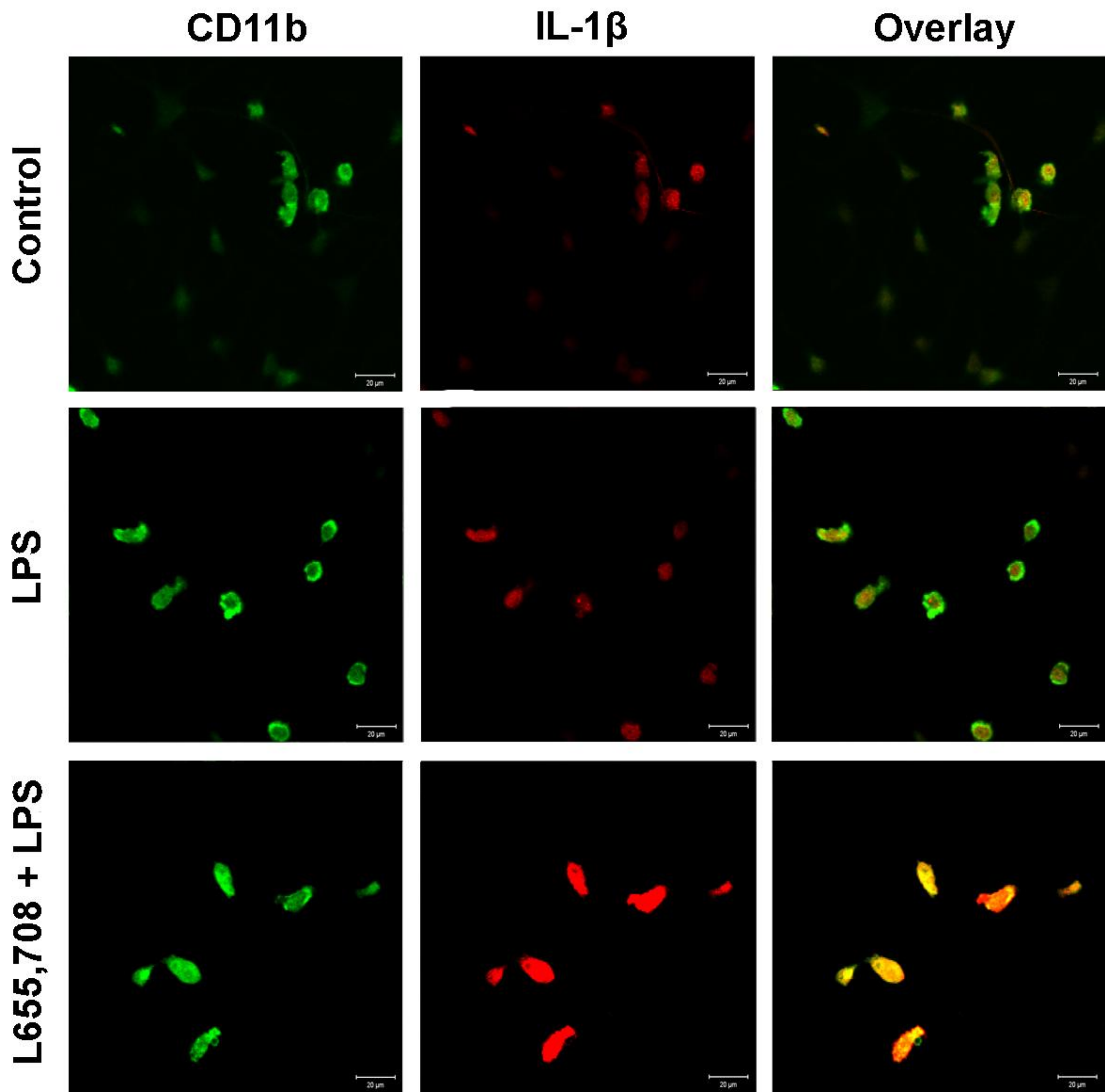


Figure 3.11 Isolated primary microglia exhibit increased IL-1β expression when treated with LPS+L655,708-neuronal conditioned media. Represenative image of microglia treated with L655,708 (5 μM) and LPS (50 ng/mL) conditioned neuronal media.


A
CD11b
IL-1β
Overlay
Control
LPS
L655,708 + LPS

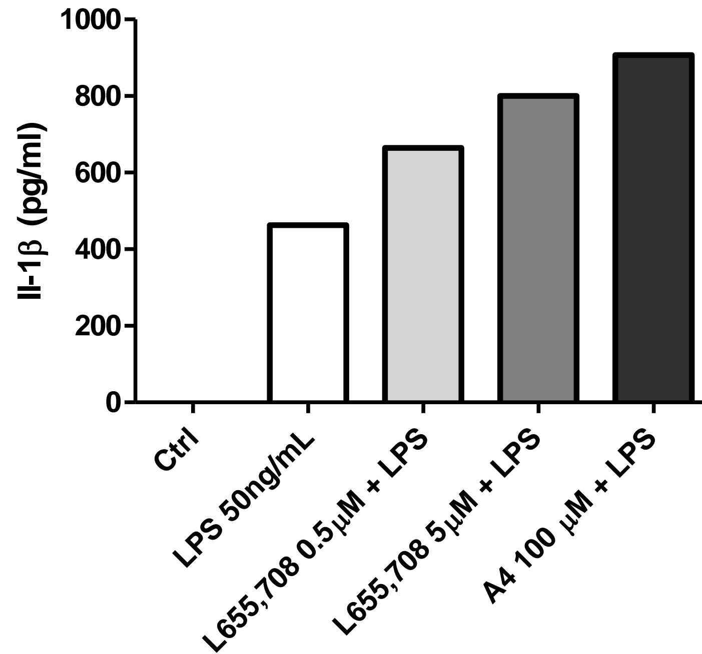


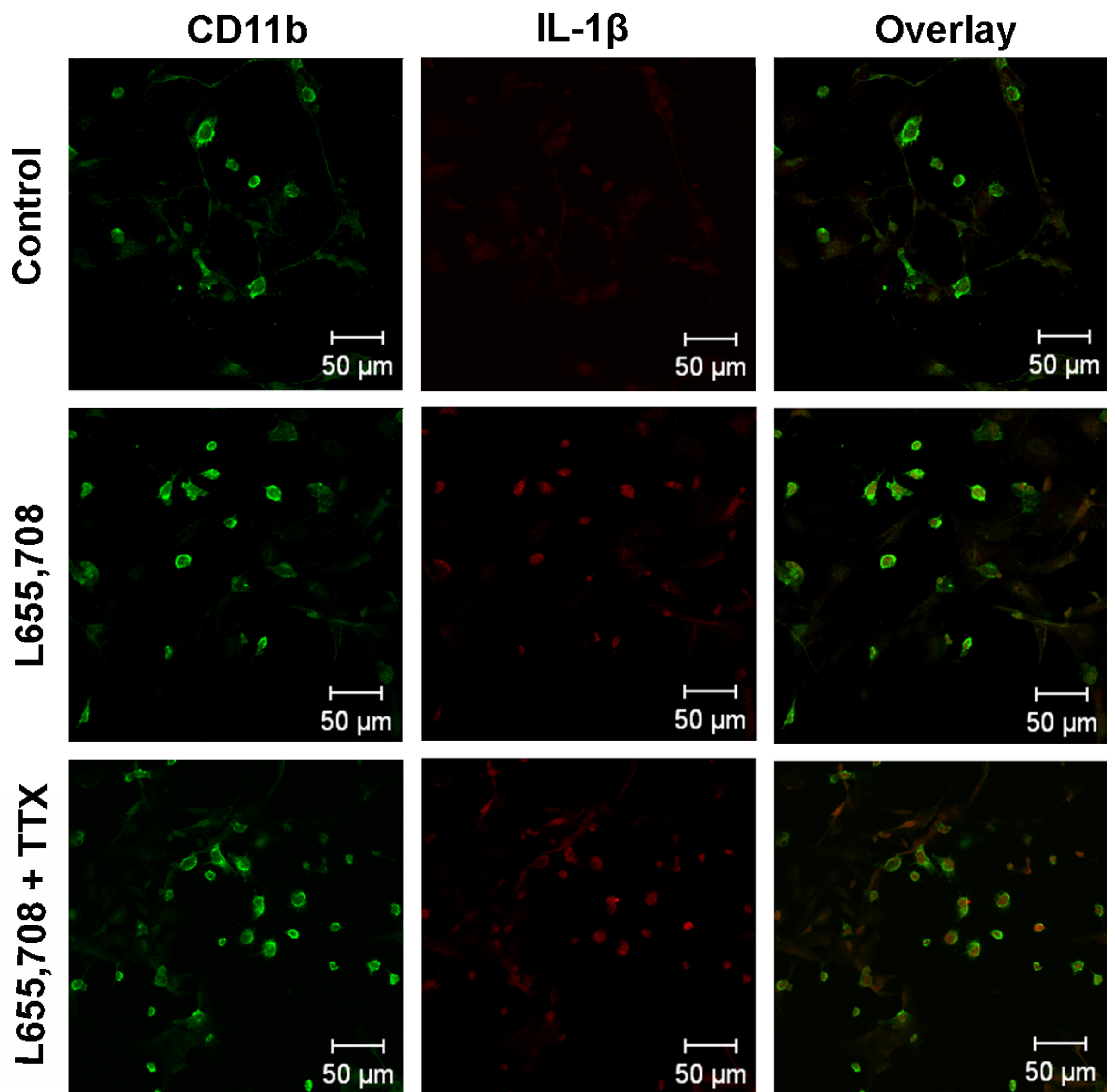
Figure 3.12 Isolated primary microglia exhibit increased IL-1β expression when treated with LPS+L655,708-astrocyte conditioned media. (*See previous page*) **A)** Represenative image of microglia treated with L655,708 (5 μM) and LPS (50 ng/mL) conditioned astrocyte media. Microglia express higher CD11b and IL-1β intensity compared to control. **B)** Secreted IL-1β was higher in microglia treated with L655,708+LPS ACM, relative to LPS-primed ACM. IL-1β concentrations in the culture media: LPS (50 ng/mL) treatment = 463 pg/mL; LPS + L655,708 (0.5 μM) = 664 pg/mL; LPS + L655,708 (5 μM) = 800 pg/mL; LPS+A438079 (100 μM) = 907; n = 2.

3.4 α5GABA$_A$ receptors regulate microglial IL-1β expression possibly through P2X$_7$ receptors

Tetrodotoxin (TTX) decreases neuronal network activities by blocking voltage-gated Na^+-channels and hence eliminating the generation of action potentials by neuronal cells. Treating the mixed cell co-cultures with TTX (100 µM) did not lower the L655,708 induced increase in the immunofluorescent intensities of CD11b and IL-1β in microglial cells (Fig. 3.13). This data suggests that α5GABA$_A$ receptor regulates microglial activation unlikely through regulating the activity of neuronal networks.

As the P2X$_7$ receptor has been implicated in microglial processing of IL-1β, I used a specific antagonist, A438079, to determine if this receptor played a role in IL-1β secretion. A438079 was used in astrocyte cultures as they too have been reported to release ATP and express P2X$_7$ receptors (Coco et al., 2003; Suadicani et al., 2006). In LPS-primed astrocytes, A438079 increased IL-1β secretion (Fig. 3.9B; $p < 0.05$). A438079 had a similar effect in LPS-primed ACM, increasing IL-1β secretion from microglia compared to microglia treated with just LPS-primed ACM (Fig. 3.12B). These data suggest that the P2X$_7$ receptor-mediated signaling in astrocytes and/or microglia modulate IL-1β release from both cell types.

A



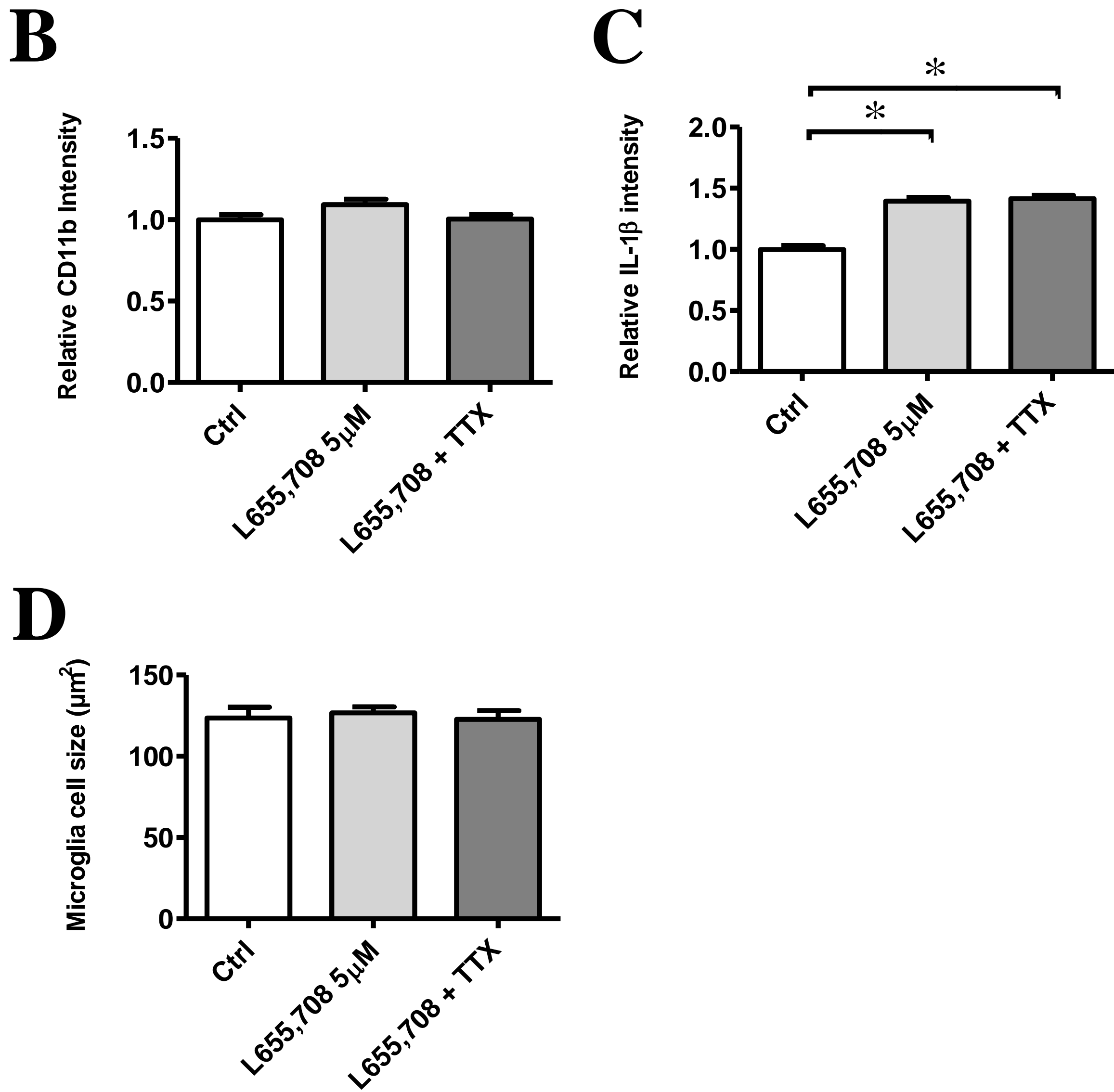


Figure 0.1 Microglia in mixed hippocampal co-cultures treated with L655,708 and tetrodotoxin (TTX) exhibit similar expression of CD11b and IL-1β compared to co-cultures treated with L655,708 alone. *(See previous page)* **A)** Representative images of co-cultures treated with L655,708 (5 μM) and/or TTX (100 μM) **B)** Normalized CD11b intensity. **C)** Normalized IL-1β intensity (* $p < 0.01$, n = 2). **D)** Microglia cell size (n = 2).

Chapter 4: Discussion

My thesis study explored the role of α5GABA$_A$ receptors in the regulation of microglial activation by *in vivo* and *in vitro* experiments. Three important findings arise from these experiments. First, *Gabra5*$^{-/-}$ mice exhibit increased IL-1β expression in hippocampal microglia in response to systemic inflammation. In addition, selective blockade of α5GABA$_A$ receptor-mediated signaling in mixed neuronal-glial cultures elevated IL-1β expression in microglia. Finally, the regulation of microglial IL-1β expression and secretion by α5GABA$_A$ receptors was mediated indirectly by undetermined soluble factor(s) from neurons and/or astrocytes. These findings established a critical role for α5GABA$_A$ receptor signaling in microglial activation.

4.1 Interrupting α5GABA$_A$ receptor signaling increases microglial activation

Microglia, the resident macrophages of the brain, become “activated” in response to various stimuli and undergo phenotypic and morphological changes to maintain brain homeostasis. These changes include the up-regulation of the production and secretion of proinflammatory cytokines and phagocytic capability. An accumulating amount of evidence indicates that GABA$_A$ receptors regulate the functions of immune cells including peritoneal macrophages and T-lymphocytes (Reyes-Garcia et al., 2007; Tian et al., 1999). Although a previous study reported the expression of GABA$_A$ receptors in microglia (Lee et al., 2011), whether GABA$_A$ receptors regulate microglial activities remains to be examined. Microglial cells lack synaptic innervations because their cellular ultra-structures and their locations change dynamically (Nimmerjahn et al., 2005). Thus I proposed that GABA$_A$ receptors in microglia should be able to sense ambient GABA in the extracellular fluid of brain tissues. In this regard,

previous studies have demonstrated that the α5GABA$_A$ receptor has a high affinity for GABA and thus it is persistently activated by ambient, extracellular GABA (Caraiscos et al., 2004). Importantly, α5GABA$_A$ receptors are critically involved in the process of brain inflammation (Wang et al., 2012). Therefore, I specifically examined the possible role of α5GABA$_A$ receptors in modulating microglial activity.

To address this issue I carried out immunohistochemistry of brain tissues from *Gabra5*$^{-/-}$ mice and their WT littermates, which were challenged by intraperitoneal LPS. Specifically I examined the expression levels of IL-1β in hippocampal microglia that were identified by the immunostaining of CD11b. Intraperitoneal LPS has been widely used as a model of systemic inflammation because it mimics the pathology of systemic bacterial infections. In addition, LPS was used to directly activate microglia *in vitro* as they readily respond to LPS via binding to TLR4 (Olson and Miller, 2004)

In my studies I examined the microglial expression of IL-1β, as it is one of the prototypical proinflammatory cytokines produced by microglia and plays a pivotal role in brain inflammation (Sims and Smith, 2010). CD11b expression reflects microglia acivity as CD11b has been found to be upregulated in microglial activation. (Duan et al., 2006; Frank et al., 2010; Le Cabec et al., 2002; Roy et al., 2006). As such, CD11b was stained as the specific microglial marker in this study. Importantly, CD11b-immunofluorescence is primarily associated with the cell membrane, sketching the morphology of microglia. My analyses were focused in the hippocampus as α5GABA$_A$ receptors are predominantly expressed in this brain region (Sur et al., 1999).

Immunohistochemical results showed that the expression level of CD11b was significantly higher in hippocampal microglia of *Gabra5*$^{-/-}$ mice, relative to their WT littermates (Fig.

3.1B). This implies that without induced inflammation, interrupting $\alpha5GABA_A$ receptor signaling alters microglial activity. As previously reported (Gabellec et al., 1995; Singh and Jiang, 2004), my analyses revealed that intraperitoneal injection of LPS readily induced inflammation in the brain of both *Gabra5*$^{-/-}$ mice and WT mice, displaying increased IL-1β expression in hippocampal microglia 6 hours after LPS injection (Fig. 3.1A). The expression level of IL-1β in hippocampal microglia of *Gabra5*$^{-/-}$ mice was significantly higher than that in WT littermates (Fig. 3.1C). IL-1β positive cells in the hippocampus were also CD11b positive, displaying ramified processes. This indicates that these are microglial cells and likely not parenchymal infiltrated peripheral immune cells. This difference was only observed in the hippocampus and not in other brain regions such as the cortex. This finding not only verifies that the down-regulation of $\alpha5GABA_A$ receptor signaling increases microglial activation in response to systemic inflammation, but also suggests that the increased microglial activity in the hippocampus of *Gabra5*$^{-/-}$ mice is due to alterations in the function of hippocampal cells.

My *in vitro* studies revealed that selectively blocking $\alpha5GABA_A$ receptor signaling in mixed neuronal-glial co-cultures with L655,708 increased the expression level of CD11b and IL-1β significantly in microglial cells in the absence (Fig. 3.2) and presence of LPS (Fig. 3.4). The general $GABA_A$ receptor antagonist bicuculline mimicked the effect of L655,708 (Fig. 3.5). In addition, blocking $\alpha5GABA_A$ receptors in LPS-primed co-cultures with L655,708 changed the morphology of microglia from a larger, oval shape with processes to a rounder, smaller morphology with retracted processes (Fig. 3.4A). This type of amoeboid morphology has been documented to be one of the final stages of microglial activation, where microglia have increased mobility and phagocytic ability (Lynch, 2009; Reichert and Rotshenker, 2003; Stence et al., 2001). Indeed, the phagocytic assay showed that selectively blocking

α5GABA$_A$ receptor signaling in the co-cultures significantly increased the phagocytotic ability of microglia. Specifically treating the mixed neuronal-glial cultures with L655,708 largely increased the fluorescent intensity of IgG-FITC in microglial cells (Fig 3.3). Phagocytosis is a hallmark of innate immune cells such as microglia, and the phagocytotic ability of microglia increases upon activation (abd-el-Basset and Fedoroff, 1995; Lynch, 2009; Reichert and Rotshenker, 2003). Selective blockade of α5GABA$_A$ receptors elevated microglial expression of IL-1β and CD11b and increased microglial phagocytyosis in neuronal-glial co-cultures, which implies that the increased microglial activation in *Gabra5*$^{-/-}$ mice is due to an alteration of α5GABA$_A$ receptor signaling among brain cells. The combined results from my *in vivo* and *in vitro* studies establish that under "normal" conditions, the tonic activity of α5GABA$_A$ receptors restrains the activity of microglia, because down-regulating these receptors leads to the activation of microglia through increased expression of IL-1β and CD11b. Furthermore, inhibiting the tonic activity of α5GABA$_A$ receptors in LPS-primed neuronal-glial cultures increased activated microglia further, exhibiting amoeboid-like morphology and increased expression of IL-1β and CD11b.

Interestingly, blocking α5GABA$_A$ receptors significantly suppressed the LPS-induced increase of IL-1β in the media of mixed neuronal-glial co-cultures (Fig. 3.6). In the brain, IL-1β is expressed and released by various cell types, including neurons (Lechan et al., 1990; Watt and Hobbs, 2000) and astrocytes (Knerlich et al., 1999; Zhang et al., 2000). Thus IL-1β in the media of LPS-treated neuronal-glial cultures could be secreted from multiple types of brain cells in addition to microglia. Furthermore, α5GABA$_A$ receptors may modulate IL-1β secretion from these cells as hippocampal neurons express these receptors (Caraiscos et al., 2004; Collinson et al., 2002). It is undetermined whether astrocytes express the α5GABA$_A$ receptor, but it is well documented that astrocytes contain GABA$_A$ receptors, which activate

astrocytes (Lee et al., 2011; Lee et al., 2010). Therefore, it is possible that blocking α5GABA$_A$ receptor signalling in neuronal-glial cultures by L655,708 increases IL-1β secretion from microglia but decreases IL-1β secretion from other cell types. To explore the possible cellular and molecular mechanisms by which α5GABA$_A$ receptors regulate microglia activity, I decided to dissect the complex actions of GABAergic signaling in the regulation of microglia, astrocytes and neurons under specific conditions. In particular, I investigated the expression profile of IL-1β in microglial cells that were grown in conditioned media from cultured neurons or astrocytes that were treated with or without LPS and the α5GABA$_A$ receptor inverse agonist.

4.2 α5GABA$_A$ receptors regulate microglial activity: an indirect action

GABA$_A$ receptors have been reported to be expressed in microglia (Lee et al., 2011), although a GABA$_A$ receptor-mediated change in membrane potential or in transmembrane current has never been demonstrated (Cheung et al., 2009; Wu and Zhuo, 2008). Nevertheless, it is important for this study to define whether microglial cells express α5-subunit of GABA$_A$ receptors. Reverse Transcriptase Polymerase chain reaction (RT-PCR) analysis conducted in our lab indicates that the murine microglial cell line BV-2 do not express the mRNA of the GABA$_A$ receptor α5-subunit. In addition, patch-clamp recordings failed to detect transmembrane current in primary microglia and/or BV-2 microglia during perfusion with the GABA$_A$ receptor agonist muscimol (by laboratory communications). Indeed, my analyses showed that IL-1β expression (Fig. 3.7A) and secretion (Fig. 3.7E) in isolated microglial cells remained unchanged in response to L655,708. However, it has been reported that GABAergic transmission regulates microglial activity in an indirect manner

(Cheung et al., 2009; Fontainhas et al., 2011). Unlike in neuronal-glial co-cultures, isolated primary microglia remain in a resting status, displaying small cell bodies and long, branched processes even in the presence of LPS and/or L655,708 (Fig. 3.7A). Collectively, available data from the literature and my studies highly suggest that the increased microglial activation in *Gabra5*$^{-/-}$ mice is not due to a direct alteration of $GABA_A$ receptor signalling in microglial cells, but rather through indirectly modulating the secretion of soluble factors from other brain cells which express $\alpha 5GABA_A$ receptors (e.g. neurons and/or astrocytes).

4.2.1 $\alpha 5GABA_A$ receptors regulate microglial activation likely through the modulation of astrocyte activity

Astrocytes have also been reported to secrete proinflammatory cytokines in response to LPS (Carpentier et al., 2008; Guerra et al., 2011). Indeed, my experiments confirmed that treating isolated primary astrocytes with LPS greatly increased the IL-1β expression (Fig. 3.9A) and secretion (Fig. 3.9B) in culture. As mentioned previously, astrocytic $GABA_A$ receptor stimulation leads to elevated intracellular Ca^{2+} and subsequent astrocyte activation. This activation leads to the release of various soluble factors such as ATP. However, whether $GABA_A$ receptors in astrocytes contain α5-subunits remains elusive as it is difficult to purify primary astrocytes for PCR assays and no specific α5-subunit antibody is available for immunocytochemistry. Using the inverse agonist L655,708, I found that selective blockade of $\alpha 5GABA_A$ receptor signalling in primary astrocytes suppressed the LPS-induced increase in IL-1β secretion (Fig. 3.9B). This result implies that astrocytes are endowed with an $\alpha 5GABA_A$ receptor-mediated autocrine signaling mechanism, which constantly stimulates the cells to produce and secrete cytokines such as IL-1β during brain inflammation.

Taking these results into account may help explain how L655,708 treatment in LPS-primed neuronal-glial cultures decreased the IL-1β concentration in the media. That is, in neuronal-glial cultures, both astrocytes and microglia respond to LPS by increased production and secretion of IL-1β. Moreover, blockade of α5GABA$_A$ receptor-mediated excitation in astrocytes largely decreased the astrocytic secretion of IL-1β, but still increased microglial secretion of IL-1β possibly through an astrocytic soluble factor (see below).

To examine whether α5GABA$_A$ receptor indirectly regulates microglia activation via astrocytic secreted soluble factor(s), I tested the effects of astrocyte conditioned media (ACM) on the expression and secretion of IL-1β by isolated primary microglia. Specifically, astrocytes were treated with LPS alone or with L655,708+LPS, and subsequent ACM (*LPS-primed ACM*, which contained LPS; or *L655,708+LPS-primed ACM*, which contained L655,708+LPS) were used to treat primary microglia cultures, respectively. My assays showed that the LPS-primed ACM induced a large amount of IL-1β secretion by the isolated primary microglia (Fig. 3.12B). This result indicates that under my experimental conditions microglial activation by LPS requires undetermined astrocytic soluble factor(s).

Remarkably, L655,708+LPS-primed ACM caused a higher extent of microglial secretion of IL-1β compared to LPS-primed ACM (Fig. 3.12B). On the basis of my results, I propose that the blockade of α5GABA$_A$ receptor mediated autocrine signaling in astrocytes inhibits astrocytic excitation. This inhibition decreases the release of astrocytic soluble factor(s) such as ATP (Davalos et al., 2005), which negatively control microglial production and secretion of IL-1β (see below and illustration in Fig. 4.1). Another possible factor is S100B, a calcium binding protein secreted from astrocytes in response to LPS (Guerra et al., 2011). As a cytokine, S100B from astrocytes has been reported to increase IL-1β release from microglia

in am NF-κB dependent manner (Bianchi et al., 2010). The proposed notion awaits further experiments for validation.

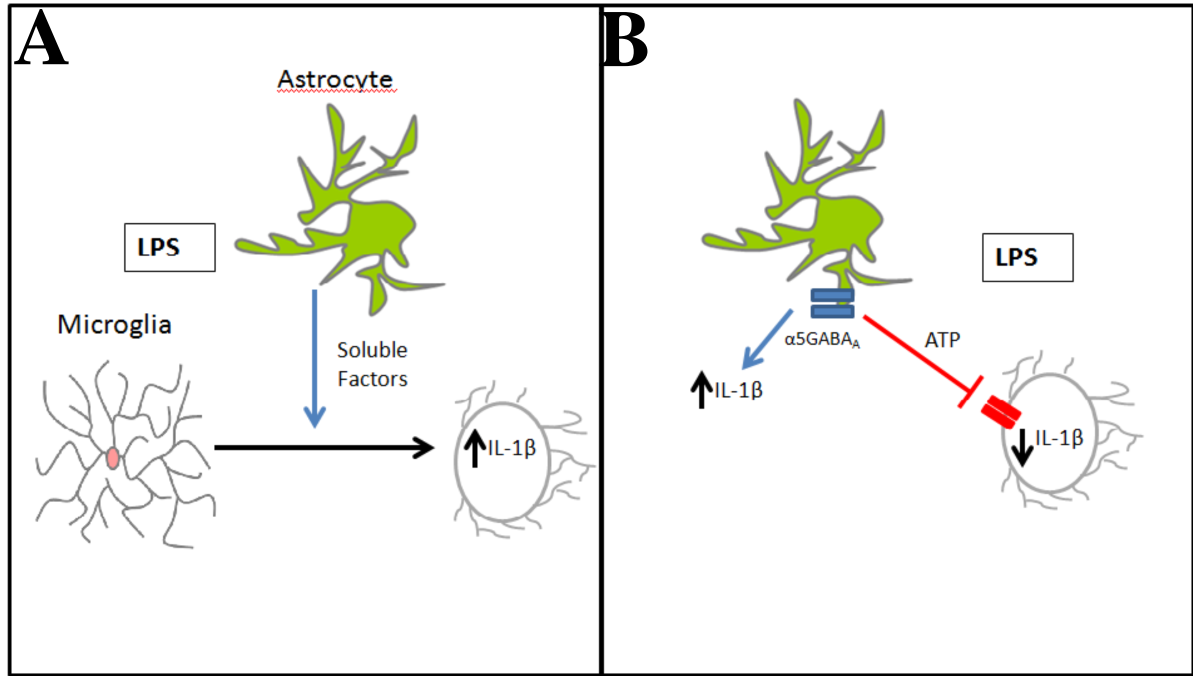


Figure 4.1Proposed mechanism of α5GABA$_A$ receptors on microglial expression of IL-1β. A) In LPS stimulated conditions, astrocytes secrete soluble factors necessary for the production/secretion of IL-1β in microglia. B) α5GABA$_A$ receptors expressed on astrocytes increase IL-1β secretion from astrocytes, however, they release other soluble factors such as ATP that may inhibit microglial IL-1β.

4.2.2 α5GABA$_A$ receptor mediated signaling in neurons play a minor role in regulating the production of IL-1β in the brain

The α5GABA$_A$ receptors generate a tonic conductance in hippocampal neurons and interruption of this signaling leads to increased neuronal excitability (Bonin et al., 2007). Therefore, I also examined whether blockade of α5GABA$_A$ receptors affects neuronal production of IL-1β. Although it has been reported that neurons may secrete IL-1β (Watt and

Hobbs, 2000), isolated hippocampal neurons did not secrete IL-1β in control conditions and in the presence of LPS and/or L655,708 (Fig. 3.11B). Moreover, unlike ACM, the control neuronal conditioned media (NCM), LPS-primed NCM and the L655,708+LPS-primed NCM had minor effects on microglial IL-1β expression. That is, microglial cells cultured in these NCM exhibited ramified morphologies and a low intracellular level of IL-1β (Fig. 3. 11A). Taking into consideration that treating the neuronal-glial co-cultures with tetrodotoxin (TTX, an Na^+ channel blocker that blocks neuronal network activity) did not affect the L655,708-induced increase of IL-1β expression in microglia (Fig. 3.13), I conclude that altered neuronal network activity via α5GABA$_A$ receptors did not play a major role on microglial IL-1β in the brain.

4.2.3 ATP may be an astrocytic soluble factor that mediates the α5GABA$_A$ receptor regulation of microglial activation

Acting as a neurotransmitter and an inflammatory mediator, ATP has many physiological and pathological roles in the brain. During neuroinflammation, ATP can be released from neurons (Bodin and Burnstock, 1998; Gonzalez-Sistal et al., 2007; Xia et al., 2012), activated astrocytes (Coco et al., 2003; Suadicani et al., 2006), and brain vascular endothelial cells (Bodin and Burnstock, 1998), regulating the function of brain cells including microglia.

As introduced before, the processing of IL-1β production and secretion in microglia is largely dependent on the formation of the NALP3 inflammasome, an intracellular protein complex. In microglia, the key molecular signal that regulates the NALP3 inflammasome is extracellular ATP, which regulates the inflammasome functions through purinergic receptors (e.g. $P2X_7$) (Chakfe et al., 2002; Ferrari et al., 1996; Solle et al., 2001). Since one of my focuses is on IL-1β production and secretion by microglia, I sought to determine if astrocytic

α5GABA$_A$ receptors regulate microglial activation through the ATP- P2X$_7$ receptor pathway. As shown in Fig. 3.9B, treating LPS-primed astrocytes with A438079, a specific P2X$_7$ receptor antagonist, increased IL-1β concentration in the media. Notably, treating the isolated microglia with A438079+LPS-primed ACM further increased the IL-1β concentration in the media (Fig. 3.12B). These results imply that P2X$_7$ receptors, contrary to previous literature, have an inhibitory effect on IL-1β secretion. Most importantly, the A438079+LPS-primed ACM increased the secretion of IL-1β by microglia. This result suggests that ATP may be an astrocytic soluble factor that mediates the α5GABA$_A$ receptor regulation of microglial activation. Again, this notion requires more experiments for authentication.

4.3 Future studies

In the present study, the primary readout of the effects of α5GABA$_A$ receptor signalling on the microglial activation is the expression and secretion of IL-1β by microglia. However, in response to systemic inflammation microglia secret a variety of pro- and anti-inflammatory cytokines, which regulate the process of brain inflammation in a complex manner. Studying the changes in a wider range of cytokines and inflammatory regulators such as COX-2 and iNOS in the brain of *Gabra5*$^{-/-}$ mice is critical for understanding the role of GABAergic signalling in the regulation of brain inflammation.

On the other hand, during systemic inflammation, microglia in the brain of the *Gabra5*$^{-/-}$ mice could be activated through a variety of stimuli, such as LPS, proinflammatory cytokines such as TNFα, IL-6 and CCL2/MCP-1, secreted by infiltrating macrophages and inflammatory mediators released from endothelial cells (Banks et al., 1995; Ek et al., 2001; Rivest, 2003). In my *in vitro* study, the neuronal-glial cultures were only challenged by LPS as the sole inflammatory stimulator. It is important to investigate the cellular and molecular

mechanism by which α5GABA$_A$ receptors regulate microglia activation in response to other inflammatory cytokines and/or mediators. Treating cultures with stimuli other than LPS may lead to altered microglial activation profiles, as different stimuli activate different intracellular pathways.

Although my study showed pharmacological evidence that astrocytes express α5GABA$_A$ receptors and modulate astrocyte activity, definitive proof for the astrocytic expression of α5GABA$_A$ receptors is required. To address this, RT-PCR assay of the α5 subunit mRNA in highly purified astrocytes should be carried out. In addition, electrophysiological recordings of GABA-induce current in astrocytes in the absence and presence of L655,708 would also be useful for verification of the existence of α5GABA$_A$ receptors in microglia..

The molecular mechanisms of how α5GABA$_A$ receptors affect microglial expression of IL-1β still remain to be defined. Results from my experiments implied that astrocytes control LPS-induced microglial activation through soluble factor(s). Identifying the soluble factor(s) is crucial for understanding glial cell interaction in the process of brain inflammation. Of interest is the S100B protein and ATP, as both molecules are secreted by astrocytes and affect microglia activation (Bianchi et al., 2010; Guerra et al., 2011; Liu et al., 2011).

Future studies should also focus on the molecular mechanisms by which α5GABA$_A$ receptor regulates the intracellular signaling in astrocytes thus modulating microglia activity. Of interest, determining the effect of α5GABA$_A$ receptor inverse agonist on mobilization of astroctytic intracellular Ca^{2+} will help establish the signaling pathway.

4.4 The significance of this study

As stated before, an accumulating amount of evidence indicates that GABA is widely used as a signalling molecule by various types of cells in the CNS and peripheral organs. New studies indicate that GABA regulates the function of immune cells in the periphery. The role of GABAergic signalling in the regulation of brain inflammation has just emerged (Wang et al., 2012). Another new study indicates that brain ischemia impairs the function of GABA transporters, increasing extracellular GABA concentration and subsequently increasing $\alpha 5GABA_A$ receptor activity, resulting in brain injury (Clarkson et al., 2010). However, the underlying mechanism of how the increased $\alpha 5GABA_A$ receptor activity causing brain injury is unknown. My study showed that the $\alpha 5GABA_A$ receptor mediated tonic activity in astrocytes stimulates astrocytic secretion of IL-1β, providing a potential cellular/molecular mechanism of ischemic brain injury.

My study showed that $\alpha 5GABA_A$ receptor differentially regulates the production and secretion of IL-1β by astrocytes and microglia. Specifically, my results imply that in brain inflammation, the $\alpha 5GABA_A$ receptor mediates tonic activity in astrocytes, elevating astrocytic release of IL-1β, but restraining microglial secretion of IL-1β. A full understanding of the physiological significance of GABAergic regulation of glial cell activity awaits more studies.

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