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GABA TRANSPORT AND NEUROINFLAMMATION ARE COUPLED IN MULTIPLE SCLEROSIS: REGULATION OF THE GABA TRANSPORTER-2 BY GANAXOLONE

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Abstract—Interactions between neurotransmitters and the immune system represent new prospects for understanding neuroinflammation and subsequent neurological disease. GABA is the chief inhibitory neurotransmitter but its actions on immune pathways in the brain are unclear. In the present study, we investigated GABAergic transport in conjunction with neuroinflammation in models of multiple sclerosis (MS). Protein and mRNA levels of γ-amino butyric acid transporter 2 (GAT-2) were examined in cerebral white matter from MS and control (Non-MS) patients, in cultured human macrophages, microglia and astrocytes, and in spinal cords from mice with and without experimental autoimmune encephalomyelitis (EAE) using western blotting, immunocytochemistry and quantitative real-time polymerase chain reaction (qRT-PCR). GABA levels were measured by HPLC. The GAT-2's expression was increased in MS patients' ($n = 6$) white matter, particularly in macrophage lineage cells, compared to Non-MS patients ($n = 6$) ($p < 0.05$). Interferon-γ (IFN-γ) stimulation of human macrophage lineage cells induced *GAT*-2 expression and reduced extracellular GABA levels ($p < 0.05$) but soluble GABA treatment suppressed *HLA-DRα*, *GAT*-2 and *XBP-1*/s expression in stimulated macrophage lineage cells ($p < 0.05$). Similarly, the synthetic allopregnanolone analog, ganaxolone (GNX), repressed GAT-2, JAK-1 and STAT-1 expression in activated macrophage lineage cells ($p < 0.05$). *In vivo* GNX treatment reduced *Gat*-2, *Cd*3ε, *Mhc*II, and *Xbp-1*/s expression in spinal cords following EAE induction ($p < 0.05$), which was correlated with improved neurobehavioral outcomes and reduced neuroinflammation, demyelination and axonal injury. These findings highlight altered GABAergic transport through GAT-2 induction during neuroinflammation. GABA transport and neuroinflammation are closely coupled but regulated by GNX, pointing to GABAergic pathways as therapeutic targets in neuroinflammatory diseases. © **2014 Published by Elsevier Ltd. on behalf of IBRO.**

Key words: multiple sclerosis, experimental autoimmune encephalitis, neuroinflammation, γ-amino butyric acid, γ-amino butyric acid transporter-2, ganaxolone.

INTRODUCTION

There is a growing recognition that innate and adaptive immune processes are involved in neuroinflammation and subsequent neurodegeneration, which contribute to Alzheimer's and Parkinson's diseases, HIV-associated neurocognitive disorders and multiple sclerosis (MS) (Eikelenboom et al., 2002; Frohman et al., 2006; Noorbakhsh et al., 2006; Tansey et al., 2008). MS is the prototype neuroinflammatory disease of the CNS defined by inflammatory demyelination and axonal loss (Stadelmann, 2011). Defects in GABA signaling have been implicated in MS and other inflammatory neurodegenerative diseases (Demakova et al., 2003; LeWitt et al., 2011). GABA has been reported also to regulate lymphoid and macrophage lineage cell activation (Stuckey et al., 2005; Nigam et al., 2010). GABA's reported effects on the immune system include the modulation of pro-inflammatory cytokine and chemokine production (Reyes-Garcia et al., 2007; Tian et al., 2011) and diminished T cell proliferation (Shiratsuchi et al., 2009; Dionisio et al., 2011; Soltani et al., 2011). GABA's actions have been studied in the MS animal model, experimental autoimmune encephalitis (EAE) in which GABA-specific therapeutics displayed differential outcomes, highlighting the complexity of the GABAergic system (Carmans et al., 2013).

The GABA (reuptake) transporters (GATs) are essential regulators of extra- and intra-cellular GABA levels (Conti et al., 2004). Moreover, GATs' expression is induced in macrophages and lymphocytes following immune activation, presumably leading to enhanced GABA cellular reuptake (Bhat et al., 2010; Dionisio et al., 2011). Neuroactive steroids are produced within the

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Abbreviations: BBB, blood–brain barrier; DMSO, dimethyl sulphoxide; EAE, experimental autoimmune encephalomyelitis; FBS, fetal bovine serum; GAT-2, γ-amino butyric acid transporter 2; GNX, ganaxolone; HFAs, human fetal astrocytes; HFMs, human fetal microglia; HPLC, high- performance liquid chromatography; IFN-γ, interferon-γ; MBP, myelin basic protein; MDMs, monocyte-derived macrophages; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; PBLs, peripheral blood lymphocytes; PBMCs, peripheral blood mononuclear cells; PBS, phosphate-buffered saline.

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nervous system (Mellon et al., 2008) and bind to both intracellular steroid receptors and/or cell surface neurotransmitter receptors (Rupprecht et al., 1993; Robel and Baulieu, 1994; Rupprecht, 1997). Several neuroactive steroids are positive allosteric modulators of the $GABA_A$ receptor (GABA-A-R), including allopregnanolone, or they act to antagonize the GABA-A-R, such as DHEA-S (Rupprecht, 1997; Falkenstein et al., 2000; Hosie et al., 2006; Mellon et al., 2008). Allopregnanolone is a cholesterol-derived neuroactive steroid, which is synthesized by neurons and glia (Rupprecht et al., 1993; Belelli and Lambert, 2005; Houtchens, 2007). Importantly, Allopregnanolone levels were decreased in cerebral white matter from MS patients and treatment with allopregnanolone reduced neuroinflammation and demyelination in EAE (Noorbakhsh et al., 2011). Ganaxolone (GNX) is a synthetic analog of allopregnanolone (Reddy and Woodward, 2004) and binds to a GABA-A-R site (Carter et al., 1997). Studies in neurons indicate that this interaction enhances chloride (Cl^-) permeability of the GABA-A ionophore receptor complex, which results in hyperpolarization of the post-synaptic membrane (Carter et al., 1997; Jorgensen, 2005; Hosie et al., 2006). GNX has been investigated as an anti-epileptic drug in animal studies (Carter et al., 1997; Liptakova et al., 2000; Reddy and Rogawski, 2010) and in human studies for epilepsy treatment (Reddy, 2010) although its effects on the immune system remain unknown. Herein, we report the induction of γ-amino butyric acid transporter 2 (GAT-2) (*SLC6A12*) in the brains of MS patients, particularly on macrophage lineage cells, and also in animals with EAE. GNX suppressed GAT-2 expression in macrophage lineage cells leading to reduced neuroinflammatory gene expression with reduced disease severity in EAE.

EXPERIMENTAL PROCEDURES

Human brain tissue samples

Frontal brain white matter was collected at autopsy, with consent, from age and sex-matched MS subjects ($n = 6$; relapsing-remitting (RR-MS; $n = 3$), secondary progressive MS ($n = 3$)) and Non-MS patients ($n = 6$; sepsis, myocardial infarction, cancer, HIV/AIDS, all without neuropathological lesions), as previously reported (Deslauriers et al., 2011). These studies were approved by the University of Alberta Ethics Committee (Pro00002291).

Cell cultures

Human monocyte-derived macrophages (MDMs) and peripheral blood lymphocytes (PBLs) were prepared from blood collected from healthy donors as previously described (Tsutsui et al., 2004; Acharjee et al., 2011). Briefly, human peripheral blood mononuclear cells (PBMCs) were purified from healthy blood with Histopaque (Sigma–Aldrich, Oakville, ON, Canada) (Power et al., 1998). PBLs were isolated from PBMCs by removing adherent macrophages and maintained in RPMI 1640 medium with 15% fetal bovine serum (FBS) with phytohemagglutinin-P (PHA-P) (5 μg/ml) (Sigma–Aldrich, Oakville, ON, Canada) for 3 days. Cells were then harvested and cultured with anti-human anti-CD3 (5 μg/ml)-coated plates supplemented with recombinant human IL-2 (10^3 U/ml) (PeproTech, Rocky Hill, NJ, USA) for an additional 24 h with either GNX (100 μM) or dimethyl sulphoxide (DMSO) (Sigma–Aldrich, Oakville, ON, Canada) vehicle control in equal volume. MDMs were isolated from PBMCs, differentiated for 1 week (Maingat et al., 2009). MDMs were pretreated with GNX (100 μM) or DMSO in MEM containing 10% FBS (Life Technologies, Burlington, ON, Canada), 1% penicillin/streptomycin (Life Technologies, Burlington, ON, Canada) and 1% L-glutamine (Life Technologies, Burlington, ON, Canada), followed by recombinant human interferon-γ (IFN-γ) (400 U/mL) (PeproTech, Rocky Hill, NJ, USA) exposure for 24 h. Treated MDMs were lysed in either TRIzol® reagent (Life Technologies, Burlington, ON, Canada) followed by total RNA extraction or Laemmli buffer with 0.1% β-mercaptoethanol (Sigma–Aldrich, Oakville, ON, Canada) for protein isolation.

Human fetal astrocytes (HFAs) and microglia (HFMs) were prepared from 15–19-week-old fetal brains obtained, with consent (approved by the University of Alberta Ethics Committee, Pro00027660), as previously described (Zhu et al., 2009; Vivithanaporn et al., 2010). Briefly, fetal brain tissues were dissected, meninges were removed and digested for 30 min with 2.5% trypsin (Life Technologies, Burlington, ON, Canada) and 2 mg/ml DNase I (Roche Diagnostics, Mannheim, Germany) and passed through a 70-μm cell strainer (BD Biosciences, Mississauga, ON, Canada). Cells were washed twice with centrifugation at 14,000 rpm for 10 min and plated in T-75 poly-L-ornithine-coated flasks (Sigma–Aldrich, Oakville, ON, Canada) at 6–8 × 10^7 cells/flask with media. Following a week of incubation (37 °C at 5% CO_2), adherent cells (HFA) were separated from suspension cells (HFM) and re-plated for primary astrocytic or microglia differentiation (80% confluence). HFAs and HFMs were maintained in MEM containing 10% FBS (Life Technologies, Burlington, ON, Canada), 1% penicillin/streptomycin (Life Technologies, Burlington, ON, Canada), 1% L-glutamine (Life Technologies, Burlington, ON, Canada) and 10% dextrose (Life Technologies, Burlington, ON, Canada).

Animals

C57BL6 female mice were purchased from the Jackson Laboratory and maintained in the Health Sciences Laboratory Animal Services facility of the University of Alberta under conventional housing conditions. All experiments were approved by the University of Alberta Animal Care Committee (AUP00000317).

GNX preparation

GNX (5α-pregnan-3β-methyl-3α-ol-20-one) (Steraloids Inc., Newport, RI, USA) was solubilized in DMSO and further diluted in 1× phosphate-buffered saline (PBS) (Life Technologies, Burlington, ON, Canada) and stored at 4 °C. Active therapeutic concentrations of GNX were derived from previous studies (Ram et al., 2001). For

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