Neuroscience 250 (2013) 536–545

A NEW CONCEPT AFFECTING RESTORATION OF INFLAMMATION-REACTIVE ASTROCYTES ☆

L. BLOCK,[b] U. BJÖRKLUND,[a] A. WESTERLUND,[a] P. JÖRNEBERG,[c] B. BIBER[b] AND E. HANSSON[a]*

[a] *Department of Clinical Neuroscience and Rehabilitation, Institute of Neuroscience and Physiology, The Sahlgrenska Academy, University of Gothenburg, SE 413 45 Gothenburg, Sweden*

[b] *Department of Anaesthesiology and Intensive Care Medicine, Institute of Clinical Sciences, The Sahlgrenska Academy, University of Gothenburg, SE 413 45 Gothenburg, Sweden*

[c] *Department of Anaesthesiology and Intensive Care Medicine, Division of Pain Management, NU-Hospitals, SE 461 85 Trollhättan, Sweden*

Abstract—Long-lasting pain may partly be a consequence of ongoing neuroinflammation, in which astrocytes play a significant role. Following noxious stimuli, increased inflammatory receptor activity, influences in Na^+/K^+-ATPase activity and actin filament organization occur within the central nervous system. In astrocytes, the Ca^{2+} signaling system, Na^+ transporters, cytoskeleton, and release of pro-inflammatory cytokines change during inflammation. The aim of this study was to restore these cell parameters in inflammation-reactive astrocytes. We found that the combination of (1) endomorphin-1, an opioid agonist that stimulates the $G_{i/o}$ protein of the μ-opioid receptor; (2) naloxone, an opioid antagonist that inhibits the G_s protein of the μ-opioid receptor at ultralow concentrations; and (3) levetiracetam, an anti-epileptic agent that counteracts the release of IL-1β, managed to activate the $G_{i/o}$ protein and Na^+/K^+-ATPase activity, inhibit the G_s protein, and decrease the release of IL-1β. The cell functions of astrocytes in an inflammatory state were virtually restored to their normal non-inflammatory state and it could be of clinical significance and may be useful for the treatment of long-term pain.

Key words: μ-opioid receptor, μ-opioid agonist, μ-opioid antagonist, astrocyte networks, IL-β release, levetiracetam.

INTRODUCTION

During injury, pain can be dissociated from its normal physiological role and can persist for a long period of time, even after the primary noxious stimulus has passed (McMahon and Malcangio, 2009; Milligan and Watkins, 2009; Gao and Ji, 2010). The mechanisms behind insufficiently healed neuroinflammation and how the neuronal and non-neuronal activities evoked by painful stimuli and inflammation are processed in the brain and throughout the central nervous system (CNS) are not well understood.

Under conditions that lead to neuroinflammation in the nervous system, Ca^{2+} signaling in the astrocyte network is overactivated, which triggers the activation of astrocytes and microglia (Strokin et al., 2011). Several receptors are influenced, and the expression of some receptors, such as Toll-like receptor 4 (TLR4) (Forshammar et al., 2011; Stevens et al., 2013), are increased, whereas the responses of other receptors, such as opioid receptors (El-Hage et al., 2006; Block et al., 2012), are changed. In addition, there is an increased release of glutamate into the neural synapse, where astrocytes are the predominant players in clearing the extracellular space. The astrocyte uptake of excessive extracellular glutamate by membrane-bound glutamate transporters, such as GLAST and GLT-1, play a critical role in preventing glutamate excitotoxicity (Zhao et al., 2012). In addition, metabotropic and ionotropic glutamate receptors will be excited. The astrocyte metabotropic receptor mGluR5 activates G_q protein-coupled receptors, resulting in increased intracellular Ca^{2+} release (Hansson, 1994; Bradley et al., 2009), whereas the *N*-methyl-D-aspartic acid (NMDA) receptor subunit NR2B is expressed in states of increased neuronal excitability (Krebs et al., 2003) and in inflammation-reactive astrocytes (Lundborg et al., 2011; Gérard and Hansson, 2012). These changes in the glial cells can lead to pathogenic chronic neuroinflammation. Subsequently the neurons change their excitability and signaling.

Endomorphin-1, a selective endogenous μ-opioid receptor agonist, is capable of interacting with the pertussis toxin-sensitive G_i/G_0 protein (Connor and Christie, 1999), and increases the activity of Na^+/K^+-ATPase *in vitro* (Horvath et al., 2003). Similar results have been observed with morphine, which indirectly

*Corresponding author. Address: Department of Clinical Neuroscience and Rehabilitation, Institute of Neuroscience and Physiology, The Sahlgrenska Academy, University of Gothenburg, Per Dubbsgatan 14, 1tr, SE 413 45 Gothenburg, Sweden. Tel: +46-31-786-3363; fax: +46-31-342-2467.

E-mail address: elisabeth.hansson@neuro.gu.se (E. Hansson).

Abbreviations: ANOVA, analysis of variance; AUC, areas under the curve; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; GFAP, glial fibrillary acidic protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HHBSS, Hank's HEPES-buffered saline solution; LPS, lipopolysaccharide; MEM, minimum essential medium; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; Sap, saponine; SDS, sodium dodecyl sulfate; TBST, Tris-buffered saline; TLR4, Toll-like receptor 4.

0306-4522/13 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2013.07.033

enhances Na^+/K^+-ATPase activity by activating μ-opioid receptors and G_i/G_0 protein (Masocha et al., 2002). Naloxone in ultralow picomolar concentrations has potent antagonistic actions on the excitatory μ-opioid receptor G_s protein. Higher concentrations antagonize both excitatory G_s proteins, and inhibitory G_i/G_0 proteins. As a consequence ultralow picomolar concentrations of naloxone enhance the analgesic effects of μ-opioid receptor agonists (Crain and Shen, 1995; Wang et al., 2005; Tsai et al., 2009). Naloxone also modulates the Na^+/K^+-ATPase activity (Forshammar et al., 2011). Ultralow concentrations of naloxone stimulate the activity of Na^+/K^+-ATPase through activation of phospholipase C resulting in increases in intracellular Ca^{2+} release. Higher concentrations have antagonistic effects on the Na^+/K^+-ATPase activity (Zhang et al., 2008; Forshammar et al., 2011).

Levetiracetam, an effective anti-epileptic drug, has in inflammation-reactive astrocyte models been shown to restore functional gap junction coupling (Stienen et al., 2010) by increasing the expression of connexin 43, the predominant gap junction protein, and decrease the enhanced IL-1β level (Haghikia et al., 2008).

The aim of this study was to use endomorphin-1, naloxone, and levetiracetam to evaluate if they could counteract the increased astrocyte Ca^{2+} signaling, downregulation of the Na^+/K^+-ATPase activity, disorganization of the cytoskeleton, and increased release of IL-1β. These agents might have a potential to restore cell functions that are altered by inflammation in astrocytes back to a normal non-inflammatory level.

EXPERIMENTAL PROCEDURES

In this study, the *in vitro* model involved astrocytes co-cultured with brain endothelial cells (Hansson et al., 2008). The biological rationale for co-culturing astrocytes with endothelial cells is that astrocytes are affected by substances that are released from the capillary endothelial cells of the BBB (blood–brain barrier) (Huber et al., 2001). These interactions are essential for a functional neurovascular system, where also the neurons take part (Abbott et al., 2006; Willis and Davis, 2008). The endothelial cells are not directly in physical contact with the astrocytes *in vitro*, and the interaction in the model is induced by the shared medium. The co-cultured astrocytes are morphologically differentiated by long, slender processes, and they exhibit greater Ca^{2+} responses and cytokine release than monocultured astrocytes. The μ-opioid receptor is also better expressed in the co-cultured astrocytes (Hansson et al., 2008), as well as the TLR4 (Forshammar et al., 2011). Furthermore, the cultures have very few microglial cells, visualized with OX42 (Fig. 1A), as well as after treatment with lipopolysaccharide (LPS) for 24 h (Fig. 1B).

Chemicals

All chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise stated.

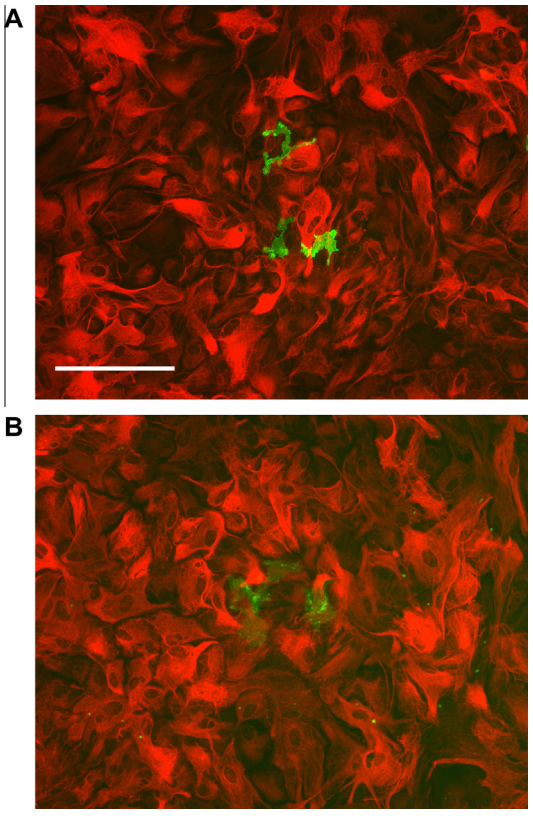

Fig. 1. Culture stained for OX42, a marker for microglial cells (green), and for GFAP, a marker for astrocytes (red). (A) Very few microglia were found in the co-cultured astrocytes. (B) Also very few microglia were found after treatment with LPS for 24 h. Scale bar = 50 μm. Representative images are presented. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The experimental protocols were approved by the Ethics Committee in Gothenburg for Laboratory Animals (Nos. 205-2010; 211-2010).

Primary astrocyte cultures

The primary astrocyte cultures were prepared from newborn rat cerebral cortices (Charles River, Sulzfeldt, Germany) and cultured on glass coverslips (Nr 1, diameter 20 mm, BergmanLabora, Stockholm, Sweden) as described by Hansson et al. (2008).

Microvascular endothelial primary cultures

Brain capillary fragments were isolated from male Sprague–Dawley rats, cerebral cortices (Charles River, 225–250 g), and the endothelial cells were cultured according to the protocol of Hansson et al. (2008).

Astrocytes co-cultured with adult rat brain microvascular primary cultures

The experimental astrocytes were obtained after co-cultivation with primary brain microvascular endothelial cultures and primary astrocyte cultures. Astrocyte cultures at 6 d *in vitro* were co-cultured with newly prepared microvascular cultures. The endothelial cells