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SEX-DEPENDENT ACTIVITY OF THE SPINAL EXCITATORY AMINO ACID TRANSPORTER: ROLE OF ESTROUS CYCLE

JAHANGIR SAJJAD, [a] **VALERIA D. FELICE,** [a,b†] **ANNA V. GOLUBEVA,** [b†] **JOHN F. CRYAN** [a,b] **AND SIOBHAIN M. O'MAHONY** [a,b*]

[a] *Department of Anatomy and Neuroscience, University College Cork, Ireland*

[b] *APC Microbiome Institute, University College Cork, Ireland*

Abstract—Females are more likely to experience visceral pain than males, yet mechanisms underlying this sex bias are not fully elucidated. Moreover, pain sensitivity can change throughout the menstrual cycle. Alterations in the glutamatergic system have been implicated in several pain-disorders; however, whether these are sex-dependent is unclear. Thus, we aimed to investigate sex differences in the spinal cord glutamate uptake and how it varies across the estrous cycle. The activity of the glutamate transporters, excitatory amino acid transporters (EAATs) was assessed using an *ex vivo* aspartate radioactive uptake assay in the lumbosacral spinal cord in Sprague–Dawley male and female rats. The gene expression of EAATs, glutamate receptor subunits NR1 and NR2B and the estrogen receptors ERα & ERβ in the spinal cord were also analyzed. EAAT activity was lower in females, particularly during the estrus phase, and this was the only cycle stage that was responsive to the pharmacological effects of the EAATs activator riluzole. Interestingly, EAAT1 mRNA expression was lower in high-estrogen and high-ERα states compared to diestrus in females. We conclude that the Spinal EAAT activity in females is different to that in males, and varies across the estrous cycle. Furthermore, the expression levels of estrogen receptors also showed a cycle-dependent pattern that may affect EAATs function and expression.

Key words: visceral pain, estrous cycle, EAATs, estrogen, glutamatergic system, spinal cord.

BACKGROUND

In general, women are more sensitive to pain than men, and some painful disorders such as irritable bowel syndrome (IBS) (Chang and Heitkemper, 2002; Mogil and Bailey, 2010), chronic pelvic pain (Scialli, 1999), temporomandibular disorder (Cairns, 2010), fibromyalgia (Yunus, 2002), biliary colic (Palsson and Sandblom, 2015), esophagitis (Krarup et al., 2013), and rheumatoid arthritis (Brennan and Silman, 1995) are more prevalent in women. Similar associations have been reported in rodents, where female rats showed exaggerated viscero-motor responses to colorectal distension (Holdcroft et al., 2000; Ji et al., 2006, 2012; Winston et al., 2014; Guo et al., 2015) as well as other types of pain (Mogil and Bailey, 2010). On the other hand, there are some reports of increased visceral and somatic hypersensitivity in male rats exposed to early life stress as compared to female rats (Prusator and Greenwood-Van Meerveld, 2015). Significant gender differences in incidence, symptomatology, and therapeutic outcome in various pain disorders indicate the necessity for gender-tailored treatment approaches. However to achieve this it is necessary to have a comprehensive understanding of the molecular mechanisms responsible for these pain-related gender differences.

IBS is the most common functional gastrointestinal disorder with symptoms of altered bowel habit and visceral hypersensitivity (Kennedy et al., 2014; Hyland et al., 2015). The majority of women with IBS who seek health care are of reproductive age. Population surveys have reported that the prevalence of IBS declines after the age of 40 years (Hungin et al., 2003); suggesting a role of female sex hormones in the etiology of IBS. Studies on gender differences in visceral pain are increasingly being carried out, yet few of these have evaluated the impact of menstrual cycle on pain perception (Houghton et al., 2002). Moreover, there is a paucity of information on the potential mechanism underlying sex differences in visceral pain processing. Of these, plasma levels of cytokines have been shown to vary depending on the cycle stage (O'Brien et al., 2007). Furthermore, there is growing evidence of the involvement of gonadal hormones (e.g. estrogen) in visceral and somatic pain sensitivity; however, conflicting results have been reported. Some studies have shown ovarian hormones (e.g. estrogen) to be antinociceptive (Bradshaw et al., 1999; Sanoja and Fernando, 2005; Heitkemper and Chang, 2009), whereas others indicate that these hormones may be pro-nociceptive (Ji et al., 2003; Lu et al., 2009; Chaloner and Greenwood-Van Meerveld, 2013). Noteworthy, visceromotor response to colorectal distension was shown to be higher in proestrus (high-estrogen state) than in

*Correspondence to: Siobhain M. O'Mahony. Office 4.113, Department of Anatomy and Neuroscience, Western Gateway Building, Ireland.
E-mail address: somahony@ucc.ie (S. M. O'Mahony).
† These authors contributed equally to the manuscript.
Abbreviations: Ct, cycle threshold; EAATs, excitatory amino acid transporters; ER, estrogen receptor; ERK, extracellular signal-regulated kinase; IBS, irritable bowel syndrome; MAPK, mitogen-activated protein kinase.

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metestrus/diestrus (low-estrogen states) (Ji et al., 2008; Peng et al., 2008). A need, therefore, arises to assess the underlying mechanisms that are implicated in visceral pain with regard to the changing levels of gonadal hormones across the female cycle.

During pain signal transmission, glutamate is released into the synaptic cleft, which, in turn, causes activation of N-methyl-D-aspartate (NMDA) receptors and hence neurotransmission across the synapse occurs. The synaptic concentration and resultant activity of glutamate are controlled by excitatory amino acid transporters (EAATs) which transport glutamate from the synaptic cleft into the glial cells. In the spinal cord, EAAT1 and EAAT2 are the most efficient mediators of glutamate clearance at synaptic cleft (Danbolt, 2001; Holmseth et al., 2012), and their gene expression has an important role in visceral sensitivity (Lin et al., 2009; Gosselin et al., 2010). It has been shown that intrathecal administration of a glutamate transporter antagonist results in increased sensitivity to colorectal distension in rats (Gosselin et al., 2010). Moreover, transgenic mice overexpressing EAAT2 demonstrated a twofold increase in the glutamate uptake across these transporters and a significant reduction in the visceromotor response to colorectal distension (Lin et al., 2009). Furthermore, maternally separated rats have shown an increase in visceral sensitivity associated with a selective reduction in spinal EAAT1 expression (Gosselin et al., 2010). However, the majority of preclinical studies assessing the role of EAATs in pain have been carried out in male animals.

Estrogen levels fluctuate during the phases of the menstrual cycle (humans) and estrous cycle (rodents) (Marcondes et al., 2001; Stricker et al., 2006) and were shown to alter the activity of glutamate receptors (Tang et al., 2008). Moreover, NMDARs showed higher activity in dorsal root ganglia (DRGs) in female rats as compared to males, and this effect was further potentiated in females by exogenous estrogen (McRoberts et al., 2007). Co-localization of estrogen receptor alpha (ER-α) and NR1 (a subunit of NMDAR) in the dorsal horn of the lumbosacral spinal cord suggests a direct modulation of glutamate receptor activity by estrogen through ER-α (Tang et al., 2008). Furthermore, it has been shown that the threshold for visceral pain is lower in high-estrogen proestrus phase (Ji et al., 2008).

Thus, we aimed to investigate if the activity of spinal glutamate transporters/or efficiency of glutamate uptake differs between sexes, across the estrous cycle and in response to the EAATs activator riluzole. We further sought to determine if subunits of the NMDA and estrogen receptors are differentially expressed in males and females, as well as throughout different phases of the estrous cycle.

EXPERIMENTAL PROCEDURES

Animals

Adult male and female Sprague–Dawley rats weighing 250–300 g (Harlan, UK) were housed in a local animal facility with food (2018 Teklad Global 18% Protein Rodent, Envigo) and water ad libitum, on a 12:12-h dark–light cycle (lights on at 7:00 AM) with the temperature at 20 °C ± 1 °C. Animals were group-housed by four to five per cage in plastic cages with sawdust bedding, shredded paper, and a cardboard roll. They were allowed to habituate in the new environment for a week before the commencement of experiments. One cohort of 10 males and 30 female rats was used for the aspartate uptake studies. Another cohort of 10 males and 29 females was used for gene expression analysis of glutamate receptors subunit and estrogen receptors in the spinal cord. All experiments were in full accordance with the European Community Council Directive (2010/63/EU) and approved by Animal Experimentation Ethics Committee of University College Cork.

Vaginal smearing

Females were vaginally lavaged with saline, and cells were immediately viewed under the microscope at round 08:00 am on the day of the experiment. The stage of estrous cycle was determined as previously described (Ji et al., 2008). Briefly, the metestrus was characterized by the appearance of small leukocytes mixed with round nucleated epithelial cells, whereas, diestrus smear had fewer leukocytes along with cornified epithelial cells. The smears from proestrus rats had a predominance of round nucleated cells of uniform size, with a complete absence of leukocytes. Finally, in estrus, nonnucleated cornified epithelial cells were predominant. Since metestrus only lasts for a short period (5–6 h) and the plasma estrogen concentration in metestrus does not differ largely from that in diestrus, data from these two groups of rats were pooled. Animals were lavaged immediately before euthanasia, to determine estrous stage accurately for all tissue collected.

Aspartate uptake

Aspartate uptake on brain slices has been previously described (Thomazi et al., 2004); here we optimized this technique to be used on spinal cord slices.

Reagents

Aspartic acid, D-[2,3-^{3}H] (specific gravity 12.9 Ci/mmol) was purchased from Perkin-Elmer, USA. RIPA buffer and Pierce BCA protein assay kit were purchased from Fisher Scientific Ireland. (3*S*)-3-[[3-[[4-(Trifluoromethyl) benzoyl] amino] phenyl] methoxy]-L-aspartic acid (TFB-TBOA), 2-amino-6-trifluoromethoxybenzothiazole (Riluzole) and 17-β-estradiol-3-benzoate were purchased from Tocris UK. All other reagents were purchased from Sigma–Aldrich. Hank's balanced salt solution (HBSS) was prepared in-house containing (in mM): 137 NaCl; 0.63 Na_2HPO_4; 4.17 $NaHCO_3$; 5.36 KCl; 0.44 KH_2PO_4; 1.26 $CaCl_2$; 0.41 $MgSO_4$; 0.49 $MgCl_2$ and 1.11 glucose, pH 7.2. In sodium-free HBSS, NaCl was replaced by 137 mM N-methyl-D-glucamine.