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CHRONIC RESTRAINT STRESS DECREASES GLIAL FIBRILLARY ACIDIC PROTEIN AND GLUTAMATE TRANSPORTER IN THE PERIAQUEDUCTAL GRAY MATTER

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Abstract—Stress affects brain activity and promotes long-term changes in multiple neural systems. Exposure to stressors causes substantial effects on the perception and response to pain. In several animal models, chronic stress produces lasting hyperalgesia. Postmortem studies of stress-related psychiatric disorders have demonstrated a decrease in the number of astrocytes and the level of glial fibrillary acidic protein (GFAP), a marker for astrocyte, in the cerebral cortex. Since astrocytes play vital roles in maintaining neuroplasticity via synapse maintenance and secretion of neurotrophins, impairment of astrocytes is thought to be involved in the neuropathology. In the present study we examined GFAP and excitatory amino acid transporter 2 (EAAT2) protein levels in the periaqueductal gray matter (PAG) after subacute and chronic restraint stresses to clarify changes in descending pain modulatory system in the rat with stress-induced hyperalgesia. Chronic restraint stress (6 h/day for 3 weeks), but not subacute restraint stress (6 h/day for 3 days), caused a marked mechanical hypersensitivity and aggressive behavior. The chronic restraint stress induced a significant decrease of GFAP protein level in the PAG (32.0 ± 8.9% vs. control group, $p < 0.05$). In immunohistochemical analysis the remarkable decrease of GFAP was observed in the ventrolateral PAG. The EAAT2 protein level in the 3 weeks stress group (79.6 ± 6.8%) was significantly lower compared to that in the control group (100.0 ± 6.1%, $p < 0.05$). In contrast there was no significant difference in the GFAP and EAAT2 protein levels between the control and 3 days stress groups These findings suggest a dysfunction of the PAG that plays pivotal roles in the organization of strategies for coping with stressors and in pain modulation after chronic restraint stress.

Key words: descending system, restraint stress, hyperalgesia, GFAP, glutamate transporter.

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Abbreviations: BSA, bovine serum albumin; CNS, central nervous system; EAAT, excitatory amino acid transporter; GFAP, glial fibrillary acidic protein; IR, immunoreactivity; LPAG, lateral PAG; PAG, periaqueductal gray matter; PB, phosphate buffer; PLSD, protected least significant difference; PPARγ, peroxisome proliferator-activated receptor gamma; PWT, paw withdrawal threshold; RT, room temperature; RVM, rostral ventromedial medulla; SD, Sprague–Dawley; SDS, sodium dodecyl sulfate; SIH, stress-induced hyperalgesia; TBS, Tris-buffered saline; TTBS, Tris-buffered saline containing 0.1% Tween-20; VLPAG, ventrolateral PAG.

INTRODUCTION

Stress affects brain activity and promotes long-term changes in multiple neural systems. A variety of psychophysical stresses have been shown to induce both decrease and increase in pain sensitivity. These phenomena are termed as stress-induced analgesia (SIA) and stress-induced hyperalgesia (SIH), respectively (Imbe et al., 2006). Chronic stress induced by repeated exposure to cold environment (Satoh et al., 1992), restraint (da Silva Torres et al., 2003; Imbe et al., 2004) and forced swim (Quintero et al., 2000; Imbe et al., 2010) produces lasting hyperalgesia. Chronic psychoemotional stress and anxiety enhance pain sensitivity in humans (Ashkinazi and Vershinina, 1999; Rhudy and Meagher, 2000). Stress has also been found to exacerbate and could contribute to the etiology of chronic painful disorders, such as, fibromyalgia (Wood, 2004), irritable bowel syndrome (Delvaux, 1999), rheumatoid arthritis (Herrmann et al., 2000) and headache (Nash and Thebarge, 2006). However the mechanisms underlying SIH remain unknown.

The periaqueductal gray matter (PAG) is involved in various important functional activities, such as fear, anxiety, defensive reaction and autonomic regulation. It also plays a crucial role in pain modulation (Bandler and Shipley, 1994). The PAG receives projections from the cerebral cortices, amygdala and hypothalamus and in turn controls spinal nociceptive neurons through relays in the rostral ventromedial medulla (RVM) and dorsolateral pontine tegmentum (DLPT). These structures constitute neural circuits of ''descending pain modulatory system'' (Fields and Basbaum, 1999; Millan, 2002; Ren and Dubner, 2002). The descending inputs from this system exert bi-directional (facilitatory and inhibitory) control of nociception. In addition to well-known descending inhibition, it is now evident that descending facilitation increases neuronal responses in the spinal dorsal horn and contributes to persistent pain and hyperalgesia (Millan, 2002; Porreca et al., 2002; Ren and Dubner, 2002).

Astrocytes are crucially involved in neuronal energy supply and participate in the buffering effect of neurotransmitters and electrolytes in the extracellular space (Schousboe and Waagepetersen, 2006; Iadecola and Nedergaard, 2007). Additionally, in peripheral tissue injury astrocytes increase cytokine productions, which phosphorylate *N*-methyl-D-aspartate (NMDA) receptors on neurons and induce neuronal hyperexcitability (Guo et al., 2007). These glial–cytokine–neuronal interactions

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also have a great impact on neuronal activities in the RVM in descending pain modulatory system (Wei et al., 2008). Glial fibrillary acidic protein (GFAP) is a well-studied astrocyte-specific protein, which makes up the intermediate filaments in the cytoskeleton of differentiated astrocytes. Although many studies have shown that chronic stress decreases GFAP in the cerebral cortices, amygdala and hippocampus (Czeh et al., 2006; Leventopoulos et al., 2007; Banasr and Duman, 2008; Gosselin et al., 2009; Ye et al., 2011), no study has examined GFAP protein level in the lower brainstem such as the PAG after chronic stress. It is generally accepted that major proportion of glutamate released from presynaptic terminals are taken up not by neurons but by surrounding astrocytes that encase the presynaptic terminal. In the plasma membrane of these cells the excitatory amino acid transporters (EAATs), high-affinity glutamate transporters, are located and play a key role in the control of glutamate clearance and its availability. Five EAATs have been identified in the mammalian nervous system: EAAT1 (Glast), EAAT2 (Glt1), EAAT3 (EAAC1), EAAT4 and EAAT5. EAAT1 and EAAT2 are expressed primarily in glial cells. EAAT3 is thought to be the major neuronal transporter in most regions of the central nervous system (CNS). EAAT4 and EAAT5 are restricted largely to the cerebellar cortex and retina, respectively (Tzingounis and Wadiche, 2007; Eulenburg and Gomeza, 2010).

We have previously demonstrated that chronic restraint stress induces thermal hyperalgesia and affects the activity of extracellular signal-regulated kinase (ERK) in descending pain modulatory system (Imbe et al., 2004). Recently we have also demonstrated that descending pain modulatory system is involved in the enhancement of formalin-evoked nocifensive behavior following the forced swim stress (Imbe et al., 2010). In the present study we examined GFAP and EAAT2 protein levels in the PAG after subacute and chronic restraint stresses to clarify changes in descending pain modulatory system in the rats with SIH.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague–Dawley (SD) rats (Japan SLC, Shizuoka, Japan) weighing 143–330 g ($n = 90$) were used in all experiments. The animals were housed two per cage, maintained under a 12-h light–dark cycle, and allowed free access to food and water. The experiments were approved by the Animal Care Committee of the Wakayama Medical University. All experiments conformed to the National Institutes of Health Guide for the Care and Use of the Laboratory Animals (NIH Publications No. 99-158 revised 2002). All efforts were made to minimize the number of animals used for experiments and their suffering.

Stress protocol

The animals were stressed by restraint for 6 h daily. In the subacute stress model, they were repeatedly exposed to daily restraint for 3 days (3 days stress group). In the chronic stress model, they were repeatedly exposed to daily restraint for 3 weeks (3 weeks stress group). Restraint was carried out by wrapping each animal with soft wire mesh and adhesive tape (Imbe et al., 2004). The restraint procedure was started between 8:00 and 11:00. Restraint was performed in the experimental room during the light phase. During restraint the animals were observed about once an hour and were unable to eat or drink. The control group did not receive any stress procedure.

Behavioral test

Mechanical test. Animals (3 days stress group $n = 8$; 3 weeks stress group $n = 8$; control group $n = 8$) were placed on a wire mesh floor and covered with an inverted clear plastic cage ($22 \times 16 \times 12$ cm). The von Frey monofilaments were then applied from underneath the wire mesh floor to the plantar surface of the right hindpaw. Paw withdrawal threshold (PWT) was taken as the lowest force that evoked a brisk withdrawal response to one of five repetitive stimuli (Imbe et al., 2003). Mechanical thresholds were measured three times for each animal, and the mean values were designated as PWTs. In the 3 weeks stress group the mechanical test was performed at 1 h after stress session on the 18th day of the restraint stress. In the 3 days stress group it was performed at 1 h after stress session on the 3rd day. The investigator was blinded to the treatment conditions. Kruskal–Wallis test with Steel test for intergroup comparisons was performed. Differences were considered statistically significant at $p < 0.05$.

Aggressive behavior. To evaluate aggressive behavior, animals in the stress groups (3 days stress group $n = 8$; 3 weeks stress group $n = 8$) were returned to the home-cage immediately after the last restraint session. Then the home-cages were transferred from the experimental room to the animal room that was quiet in dim illumination. Aggressive behavior was quantified as the time spent on standing on top of the supine partner, biting the neck or back of the partner and chasing behaviors for a 10-min observation period. The time spent on those behaviors was recorded for 10 min using a stopwatch in the animal room. Animals in the control group ($n = 8$) were exposed to novel environment (a clear plastic box in the experimental room for 2–3 min) and returned to the home-cage. Then the home-cages were transferred from the experimental room to the animal room. Aggressive behavior was quantified in the same manner as the stressed animals. The person scoring the behaviors was blinded to the treatment conditions. In the observation period other than the period when rats showed aggressive behavior, the rats spent most of their time on grooming, roaming and crouching in the home-cage. The results were expressed as means ± S.E.M. Kruskal–Wallis test with Steel test for intergroup comparisons was performed. Differences were considered statistically significant at $p < 0.05$.

Western blot analysis

Animals in the stress groups (3 days stress group $n = 8$; 3 weeks stress group $n = 8$) were euthanized with CO_2 gas immediately after the last restraint session. The control animals ($n = 15$) were euthanized at the same time (14:00–17:00) as the stressed ones. The brains were rapidly removed. The PAG (from −5.30 to −9.30 mm to bregma) was microdissected using brain slicer (RBSC-01, Aster, PA, USA) and fine razor blades under zoom stereomicroscope. The tissues were homogenized using RIPA Lysis buffer (Upstate, Lake Placid, NY, USA), containing protease inhibitor cocktail (Nacalai, Kyoto, Japan), 50 mM NaF, 1 mM Na_3VO_4 and 50 μM Na_2MoO_4, and centrifuged (15,000 rpm 4 °C). The protein concentration of each sample (supernatant) was determined using the Pierce BCA Protein Assay (BCA-1, Sigma, St. Louis, MO, USA). The proteins (5 μg/lane) were separated by electrophoresis on 5–20% sodium