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Research report

Repeated forced swim stress affects the expression of pCREB and ΔFosB and the acetylation of histone H3 in the rostral ventromedial medulla and locus coeruleus

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ABSTRACT

The rostral ventromedial medulla (RVM) and locus coeruleus (LC) play crucial roles in descending pain modulation system. In the present study we examined the expression of phospho-cAMP response element-binding protein (pCREB) and ΔFosB and the acetylation of histone H3 in the RVM and LC after forced swim stress (FS) and complete Freund's adjuvant (CFA) injection to clarify changes in descending pain modulatory system in a rat model of stress-induced hyperalgesia. FS (day 1, 10 min; days 2–3, 20 min) induced a significant increase in the expression of pCREB and ΔFosB and the acetylation of histone H3 in the RVM, whereas the FS induced a significant increase only in the acetylation of histone H3 in the LC. CFA injection into the hindpaw did not induce a significant change in those expression and acetylation. Quantitative image analysis demonstrated that the numbers of pCREB-, acetylated histone H3- and ΔFosB-IR cells in the RVM were significantly higher in the FS group than those in the naive group. The CFA injection after the FS did not affect the FS-induced increases in the expression of pCREB and ΔFosB and the acetylation of histone H3 in the RVM even though nullified the increase in the acetylation of histone H3 in the LC. These findings suggest different neuroplasticities between the RVM and LC after the FS, which may be involved in activity change of descending pain modulatory system after the CFA injection.

1. Introduction

The rostral ventromedial medulla (RVM), including the nucleus raphe magnus (NRM), is the main source of serotonergic projection to the spinal dorsal horn and constitutes a core element of the descending pain modulatory system. These descending inputs from the RVM are functionally bi-directional, producing either pain inhibition or pain facilitation (Millan, 2002; Ren and Dubner, 2002). Descending inhibition is universally recognized and descending facilitation is now accepted as an important contributor to spinal neuronal hyperactivity and behavioral hyperalgesia (Millan, 2002; Porreca et al., 2002; Ossipov et al., 2010). Descending facilitation from the RVM is also reported in human. RVM activity is visible during heat allodynia and positively correlates with spinal dorsal horn activity (Rempe et al., 2015). The locus coeruleus (LC) is the main source of noradrenergic projection to the spinal dorsal horn and constitutes another core element of descending pain modulation systems. Although descending inputs from the LC has little influence in baseline pain sensitivity, those inputs contribute to pain inhibition in peripheral tissue injury (Fields and Basbaum, 1999; Millan, 2002; Tsuruoka et al., 2012; Pertovaara, 2013).

Under some experimental conditions, psychophysical stresses can elicit hyperalgesia instead of analgesia (Imbe et al., 2006). Especially repeated and prolonged stresses such as forced swim (Quintero et al., 2000; Imbe et al., 2010, 2014; Imbe and Kimura, 2015) and restraint stresses (da Silva Torres et al., 2003; Imbe et al., 2004, 2012, 2013) enhance nociceptive response and decrease pain threshold. These finding is known as stress-induced hyperalgesia (SIH), while the mechanism underlying SIH remains to be elucidated.

Abbreviations: ABC, avidin-biotin-peroxidase complex; BSA, bovine serum albumin; CBP, CREB-binding protein; CCK, cholecystokinin; CFA, complete Freund's adjuvant; CREB, cAMP response element-binding protein; FS, forced swim stress; GAD, glutamic acid decarboxylase; HAT, histone acetyltransferase; HDAC, histone deacetylases; IR, immunoreactivity; LC, locus coeruleus; NRM, nucleus raphe magnus; PB, phosphate buffer; pCREB, phospho-CREB; PFA, paraformaldehyde; RT, room temperature; RVM, rostral ventromedial medulla; SIH, stress-induced hyperalgesia; TBS, Tris-buffered saline; TTBS, Tris-buffered saline containing 0.1% Tween-20.

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cAMP response element-binding protein (CREB) is a transcription factor and its phosphorylation (pCREB) at serine 133 leads to activation of gene transcription (Carlezon et al., 2005). CREB is a critical mediator of neuroplasticity, which has been involved in behavioral responses to chronic stress and hyperalgesia (Hoeger-Bement and Sluka, 2003; Qi et al., 2008). ΔFosB is the truncated form of FosB, which lacks most of the C-terminal transactivation domain. However ΔFosB can form heterodimer with Jun family members and activate transcription at AP-1 site (McClung et al., 2004). Additionally, since ΔFosB persists in the brain for a long time compared with other Fos and Jun family members, it has lasting effects on gene transcription (Nestler et al., 2001; McClung et al., 2004). Induction of ΔFosB seems to be implicated in susceptibility to chronic stress (Vialou et al., 2014). Several transcriptional regulators, such as CREB-binding protein (CBP) that binds pCREB, possess intrinsic histone acetyltransferase (HAT) and histone deacetylase (HDAC) activity. Histone acetylation is catalyzed by HAT and removed by HDAC. Histone acetylation neutralizes the positive charge and reduces the affinity of histone for DNA, resulting in unwrapping DNA from histone. Under this condition transcriptional factors and RNA polymerase II gain access to DNA, allowing gene transcription. Thus an increase in histone acetylation is associated with activation in gene transcription (Chen et al., 2001; Ito and Adcock, 2002). Histone acetylation has been shown to play an important role in pain perception and behavioral adaptation to chronic stress (Denk and McMahon, 2012; Stankiewicz et al., 2013).

We have previously demonstrated that the forced swim stress (FS) prior to the formalin or complete Freund's adjuvant (CFA) injection enhances inflammatory hyperalgesia (Imbe et al., 2010, 2014; Imbe and Kimura, 2015). We have also shown that RVM lesion prevent the enhancement of inflammatory hyperalgesia after the FS in this SIH animal model (Imbe et al., 2010). In the present study we examined the expression of pCREB and ΔFosB and the acetylation of histone H3 in the RVM and LC at 7–8 days after the CFA injection and FS to further clarify changes in descending pain modulatory system in the same SIH animal model.

2. Material and methods

2.1. Animals

Male Sprague-Dawley rats (Japan SLC, Shizuoka, Japan) weighing 225–251 g at the beginning of experiment were used. They were housed individually under a 12-h light-dark cycle, and maintained on food and water *ad libitum* in standard plastic cages. All experimental procedures were approved by Wakayama Medical University Animal Care Committee and carried out in accordance with the National Institutes of Health Guide for the Care and Use of the Laboratory Animals (NIH Publications No. 99–158 revised 2002). The experiments were designed to minimize the number of animals used and their suffering.

2.2. Stress and inflammation

Rats were subjected to FS (for 10 min on day 1, for 20 min on days 2–3) by placing them in the plastic cylinder that contained water at 24–26 °C. After swimming sessions, each rat was carefully dried with a new towel (Imbe et al., 2010, 2014; Imbe and Kimura, 2015).

Inflammation was induced in the right hindpaw by injection of CFA (Mycobacterium tuberculosis, Sigma, St Louis, MO, USA) suspended in an oil:saline solution (1:1) emulsion (0.05 mL, 50 μg) (Imbe et al., 2014; Imbe and Kimura, 2015).

Rats were divided into the following 4 groups: 1) The naïve group did not receive the FS and CFA injection. The rats (n = 8) was euthanized with CO_2 gas. 2) The CFA group received CFA injection into the right hindpaw. The rats (n = 8) was euthanized with CO_2 gas at 7 days after the CFA injection. 3) The FS group received the FS. The rats (n = 7) were euthanized with CO_2 gas at 8 days after the last stress session. 4) The FS + CFA group received CFA injection into the right hindpaw at 1 day after the last stress session. The rats (n = 8) were euthanized with CO_2 gas at 7 days after the CFA injection.

2.3. Immunohistochemical analysis

After the euthanasia with CO_2 gas, the rats were perfused transcardially with 100 mL of saline solution followed by 500 mL of ice-cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) at pH 7.4. The brains were removed, post-fixed overnight in the same fixative and then immersed in 30% sucrose (w/v) in 0.1 M PB for cryoprotection for 3 days. Brain 30 μm thick sections were cut in a cryostat and transferred serially to multiwell tissue culture plates containing 0.1 M Tris-buffered saline (TBS). The sections (−9.32, −9.50, −9.68, −9.86, −10.40, −10.58, −10.76 and −10.94 mm from bregma) were selected according to the cytoarchitectonic atlas (Paxinos and Watson, 1998).

Immunohistochemical staining using diaminobenzidine was conducted in keeping with standard protocol, as described previously (Imbe et al., 2014; Imbe and Kimura, 2015). In brief, endogenous peroxidase activity was blocked by 30 min incubation in 1% H_2O_2 in TBS containing 0.1% Tween-20 (TTBS). The sections were then incubated in TTBS containing 3% bovine serum albumin (BSA) for 1 h at RT to prevent nonspecific binding. The following primary antibodies were used: pCREB (rabbit polyclonal antibody, Cat # 06-519, dilution 1:15000, Millipore, Temecula, CA, USA), FosB and ΔFosB (rabbit polyclonal antibody, Cat # sc-48, dilution 1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and acetylated histone H3 (Ac(Lys9)-Ac(Lys 14)-H3, rabbit polyclonal antibody, Cat # 06-599, dilution 1:2500, Millipore). The incubation with primary antibody was performed in TTBS with 3% BSA for 24 h at 4 °C. The sections were incubated with biotinylated secondary antibodies (goat anti-rabbit antibodies, Cat # BA-1000, dilution 1:400, Vector Laboratories, Burlingame, CA, USA) for 1 h at RT. They were subsequently treated with avidin-biotin-peroxidase complex (Elite ABC kit, Vector Laboratories) for 1 h at RT. The ABC reaction was developed in TBS containing 0.05% 3,3′- diaminobenzidine tetrahydrochloride, 0.2% nickel sulfate and 0.01% H_2O_2. The sections were reacted for standardized lengths of time (pCREB 3 min; ΔFosB 2 min; acetylated histone H3 3 min) to obtain uniform staining intensity in all experimental groups. The brain sections were mounted on slides and air-dried. After dehydration in ethanol and clearing in xylene, they were coverslipped.

pCREB-IR, ΔFosB-IR or acetylated histone H3-IR cells in the sections from each animal were counted using a light microscope (BZ-9000, Keyence, Osaka, Japan) and a computer-based image analysis system (BZ-II, Keyence) as described previously (Imbe et al., 2014; Imbe and Kimura, 2015). By dynamic cell count in this image analysis system, brightness range was set such that only specific pCREB-IR, ΔFosB-IR or acetylated histone H3-IR nuclei were accurately discriminated from the background in the outlined areas. Area's boundaries were defined according to the cytoarchitectonic atlas (Paxinos and Watson, 1998) and the previous studies (Imbe et al., 2009, 2013). The number of positive cells in the outlined area was divided by the area of the outlined area (/mm^2). The values (number of positive cell per unit area) per section in each area were calculated per animal [RVM, −10.40, −10.58, −10.76 and −10.94 mm to bregma, 4 sections; LC, −9.32, −9.50, −9.68, −9.86 mm, 4 sections]. Then the group means ± S.E.M. were determined. The LC is the average value of right and left. Statistical significance was determined using one-way ANOVA with Tukey for intergroup comparisons. When no homogeneous variance among