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PKA IS REQUIRED FOR THE MODULATION OF SPINAL NOCICEPTIVE INFORMATION RELATED TO EPHRINB–EPHB SIGNALING IN MICE

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Abstract—EphB receptors and their ephrinB ligands are implicated in modulating of spinal nociceptive information processing. Here, we investigated whether protein kinase A (PKA), acts as a downstream effector, participates in the modulation spinal nociceptive information related to ephrinB–EphB signaling. Intrathecal injection of ephrinB2-Fc caused thermal hyperalgesia and mechanical allodynia, which were accompanied by increased expression of spinal PKA catalytic subunit (PKAca) and phosphorylated cAMP-response element-binding protein (p-CREB). Pre-treatment with H89, a PKA inhibitor, prevented the activation of CREB by ephrinB2-Fc. Inhibition of spinal PKA signaling prevented and reversed pain behaviors induced by the intrathecal injection of ephrinB2-Fc. Furthermore, blockade of the EphB receptors by intrathecal injection of EphB2-Fc reduced formalin-induced inflammatory, chronic constrictive injury (CCI)-induced neuropathic, and tibia bone cavity tumor cell implantation (TCI)-induced bone cancer pain behaviors, which were accompanied by decreased expression of spinal PKAca and p-CREB. Overall, these results confirmed the important involvement of PKA in the modulation of spinal nociceptive information related to ephrinBs–EphBs signaling. This finding may have important implications for exploring the roles and mechanisms of ephrinB–EphB signaling in physiologic and pathologic pain.

Key words: EphB, ephrinB, protein kinase A, central sensitization, hyperalgesia.

INTRODUCTION

Eph receptors, named for their expression in an erythropoietin-producing human hepatocellular carcinoma cell line, are the largest known family of receptor tyrosine kinases (RTKs). EphB receptors and their ephrinB ligands play critical roles in modulating multiple aspects of physiology and pathophysiology, including epileptogenesis, the inflammation response, activity-dependent synaptic plasticity, and excitotoxic neuronal death (Gerlai, 2001; Yamaguchi and Pasquale, 2004; Goldshmit et al., 2006; Pasquale, 2008). Recent evidence indicates that ephrinB–EphB signaling is also involved in modulating nociceptive information processing at the peripheral and central levels (Vasileiou et al., 2013). Previous studies have demonstrated that EphB receptors and their ephrinB ligands are present in laminae I–III of the dorsal horn and on small- and medium-sized dorsal root ganglion (DRG) neurons, which are two important sites for the modulation of nociceptive information, but not on large-diameter neurons (Bundesen et al., 2003; Song et al., 2008a). The activation of spinal ephrinB–EphB signaling plays a critical role in the development and maintenance of inflammatory (Battaglia et al., 2003; Slack et al., 2008), neuropathic (Bundesen et al., 2003; Kobayashi et al., 2007), and bone cancer pain (Dong et al., 2011; Liu et al., 2011). Moreover, central sensitization is induced by the activation of spinal ephrinB–EphB signaling via an N-methyl-D-aspartate (NMDA) receptor-dependent mechanism (Battaglia et al., 2003; Slack et al., 2008). However, because activation of the NMDA receptor alone produces only transient increases in spinal cord neuronal excitability (Cumberbatch et al., 1994), it has been hypothesized that sustained hyperexcitability depends on changes in intracellular signaling systems (Tolle et al., 1996).

The protein kinase A (PKA) complex is composed of two catalytic and two regulatory subunits. The regulatory subunits bind cyclic adenosine monophosphate (cAMP) and the catalytic subunits phosphorylated cAMP-response element-binding protein (CREB) at Ser133 (Kobierski et al., 1999; Souza et al., 2011). Numerous studies have well documented that the activation of PKA

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Abbreviations: ANOVA, analysis of variance; cAMP, cyclic adenosine monophosphate; CCI, chronic constrictive injury; DMSO, dimethyl sulfoxide; DRG, dorsal root ganglion; NMDA, N-methyl-D-aspartate; CREB, cAMP-response element-binding protein; p-CREB, phosphorylated cAMP-response element-binding protein; PKA, protein kinase A; PKAca, PKA catalytic subunit; PWL, paw withdrawal latency; PWT, paw withdrawal threshold; RT, room temperature; RTK, receptor tyrosine kinase; TCI, tibia bone cavity tumor cell implantation.

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signaling is involved in the modulation of nociceptive information peripheral and central sensitization produced by intense noxious stimuli (Malmberg et al., 1997; Aley and Levine, 1999; Hu et al., 2001; Hang et al., 2012, 2013; Huang et al., 2012; Zhu et al., 2014). Moreover, several lines of evidence have shown that regulation of PKA is associated with RTK system activation (Akaneya et al., 2010; Caldwell et al., 2012). Because ephrin–Eph receptor signaling is the largest family of RTK system, so we want to know whether PKA, acts as a downstream effector, participates in the modulation of nociceptive information related to ephrinB–EphB signaling in the spinal cord level. We provide evidence to support this hypothesis.

EXPERIMENTAL PROCEDURES

Experimental animals, anesthesia, drugs, and administration

All experiments were performed in accordance with the guidelines of the International Association for the Study of Pain and approved by the Zhejiang Animal Care and Use Committee. Adult male C57BL/6 mice (weighing 20–25 g) were obtained from the Experimental Animal Center of Zhejiang University and were housed with a 12:12 light–dark cycle and a room temperature (RT) of 23 ± 1 °C. The animals received food and water *ad libitum*. All surgeries were performed under anesthesia with sodium pentobarbital (50 mg/kg, intraperitoneal).

All reagents used in the present study, including the EphB1 receptor activator ephrinB2-Fc chimera, the EphB1 receptor blocking reagent EphB2-Fc chimera, and the PKA inhibitor N-[2-(p-Bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89), were purchased from Sigma (St. Louis, MO, USA). EphrinB2-Fc chimera can combine with EphB1–B4 receptors and activate their downstream signals. EphB2-Fc chimera can combine with endogenous ephrinB1–B3. Thus, it can competitively inhibit endogenous EphB1–4 and 6, thereby inhibiting EphB receptor-mediated signaling. The drugs were dissolved in normal saline (ephrin2-Fc and EphB2-Fc) or 1% dimethyl sulfoxide (DMSO) (H89), and then 5 μl was injected intrathecally (i.t.) via lumbar puncture at the intervertebral space of L4–5, and L5–6 for multiple injections. The following drug doses were used: ephrinB2-Fc, 0.5 μg; EphB2-Fc, 0.5 μg; control Fc for ephrinB2-Fc and EphB2-Fc, 0.5 μg; and H89, 2.5 μg.

Model of inflammatory pain

To produce inflammatory pain, a mouse model of formalin-induced inflammatory pain was used in this study (Hunskaar and Hole, 1987). Approximately 30 min before testing, mice were individually placed in perspex observation chambers for adaptation. Then, the animals were taken out of the chamber, and 10 μl of 1% formalin in 0.9% saline was injected subcutaneously into the dorsal surface of the right hind paw. Immediately after formalin injection, each mouse was returned to the observation chamber. The amount of time spent licking and flinching the injected paw was measured from 0 to 10 min (the first phase) and from 10 to 45 min (the second phase) after formalin injection and was considered as indicative of nociception.

Model of neuropathic pain

We used a mouse model of chronic constrictive injury (CCI) in this study to produce peripheral nerve injury (Bennett and Xie, 1988). The skin of the right hind limb was sterilized with iodine tincture, followed by 75% alcohol, and the right sciatic nerve was exposed at the mid-thigh level by blunt dissection of the biceps femoris. Three silk thread (5-0) ligatures were tied loosely around the nerve approximately 1 mm apart, proximal to its trifurcation. For sham surgery, the sciatic nerve was isolated but not ligated. After CCI or sham surgery, the overlying muscles and skin were closed in layers and dusted with antibiotic power.

Model of bone cancer pain

To produce bone cancer pain, a mouse model of tibia bone cavity tumor cell implantation (TCI)-induced bone cancer pain was used in this study (Curto-Reyes et al., 2010). B16-F10 mouse melanoma cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). TCI was achieved by injecting the tumor cells (1×10^5 cells/μl, 5 μl) into the intramedullary space of the right tibia to induce bone cancer in mice. Sham surgery was done using similar procedures by injecting boiled cells.

Assessment of thermal hyperalgesia

Thermal hyperalgesia was assessed by measuring the paw withdrawal latency (PWL) in response to a radiant heat source using the Hargreaves' test (Hargreaves et al., 1988). Briefly, rats were placed individually in perspex observation chambers on an elevated glass platform, and a radiant heat source was applied to the glabrous surface of the paw through the glass plate. The nociceptive endpoints included the characteristic lifting or licking of the hind paw. The heat was maintained at a constant intensity, which produced a stable PWL of 12–15 s in normal animals. A 20-s cutoff was used to prevent tissue damage. After acclimation to the test chambers, both hindpaws were tested independently with 3-min intervals between trials.

Assessment of mechanical allodynia

Mechanical allodynia was assessed by measuring the paw withdrawal threshold (PWT) in response to a calibrated series of von-Frey hairs ranging from 0.31 to 4 g. Animals were placed individually into wire mesh-bottom cages and allowed to acclimatize for 1 h. The filaments were presented in ascending order of strength and perpendicular to the plantar surface with sufficient force to cause slight bending against the paw and held for 2 s. Brisk withdrawal or paw flinching was considered a positive response. PWT was determined by sequentially increasing and decreasing the stimulus strength (the ''up-and-down'' method), and the data