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Selective elimination of isolectin B4-binding trigeminal neurons enhanced formalin-induced nocifensive behavior in the upper lip of rats and c-Fos expression in the trigeminal subnucleus caudalis

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ABSTRACT

The functional significance of non-peptidergic C-fibers in orofacial pain processing is largely unknown. The present study examined the effects of the selective elimination of *isolectin* B4 (IB4)-binding ($IB4^+$) neurons on formalin-induced face rubbing behavior (FRB) in the upper lip of rats and c-Fos-immunoreactive (c-Fos-IR) cells in the trigeminal subnucleus caudalis (Vc). IB4 conjugated to neurotoxin, saporin (IB4-Sap), blank-saporin (Bl-Sap), or saline (Sal) was injected into the cisterna magna. IB4-Sap treatments significantly decreased $IB4^+$ terminals in lamina II of Vc and $IB4^+$ trigeminal ganglia neurons, whereas Sal- and Bl-Sap treatments did not. The number of formalin-induced FRB 15–30 min after the formalin injection was significantly higher in IB4-Sap-treated rats than in Sal- or Bl-Sap-treated rats, and was associated with an increase in c-Fos-IR cells. The systemic preadministration of the $GABA_A$ antagonist, bicuculline, and agonist, muscimol, had stronger decreasing effects on FRB and c-Fos-IR cells in IB4-Sap-treated rats than the preadministration of Sal, whereas the opposite effects were observed in Sal- and Bl-Sap-treated rats. These results indicate that $IB4^+$ neurons in the trigeminal nerve play antinociceptive regulatory roles in formalin-induced orofacial pain processing and that $GABA_A$ receptor functions at segmental and supratrigeminal sites have complex modulatory influences on antinociceptive roles.

1. Introduction

Small-sized sensory C-fibers, most of which, but not all are nociceptors, can be classified into non-peptidergic and peptidergic groups. Non-peptidergic fibers have affinity to *isolectin* B4 (IB4) and sensitivity to glial cell line-derived neurotrophic factor, while peptidergic fibers do not have affinity to IB4 or sensitivity to nerve growth factor (Bennett et al., 1996; Molliver et al., 1997). In adult rats, most non-peptidergic neurons innervate the epidermis and project to the inner lamina II of the dorsal horn, whereas peptidergic neurons mostly innervate viscera and project to the most superficial layer, lamina I of the dorsal horn (Ambalavanar and Morris, 1992; Lu et al., 2001; Joseph and Levine, 2010). Although these two types of nociceptors have been suggested to play different roles in nociceptive processing, their functional significance remains largely unknown.

Previous studies reported that peptidergic fibers in the spinal dorsal horn, such as neurokinin-1 receptor (NK-1)-bearing neurons, were essential for the perception of intense noxious stimuli and coding of graded mechanical stimuli (De Felipe et al., 1998; Suzuki et al., 2002, 2003; Rahman et al., 2007). Furthermore, c-Fos expression induced by an electrical noxious stimulation of the trigeminal ganglion (TG) was shown to be significantly reduced in the trigeminal subnucleus caudalis (Vc) after the elimination of NK-1-bearing neurons by an intra-cisterna magnal injection of substance P conjugated to saporin (Abe et al., 2005). The systemic preadministration of bicuculline, an antagonist of the major inhibitory neurotransmitter, γ-aminobutyric acid (GABA) acting at the type A ($GABA_A$) receptor, also increased the expression of c-Fos evoked in

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the Vc (Abe et al., 2005). Therefore, peptidergic fibers are essential for ascending nociceptive processing and the descending facilitatory modulation loop on dorsal horn neurons through $GABA_A$ receptors at the supraspinal/trigeminal level (Abe et al., 2005; Bester et al., 2001).

There have been some contradictory findings regarding the nociceptive functions of non-peptidergic IB4-binding ($IB4^+$) neurons. When neuropathic hyperalgesia was induced by a chronic constriction injury in the sciatic nerve, the density of the labeling of $IB4^+$ central terminals of non-peptidergic fibers (i.e., with affinity to IB4), rather than peptidergic C-fibers, decreased in the spinal dorsal horn (Bailey and Ribeiro-da-Silva, 2006). Other studies, in which IB4 conjugated with neurotoxin, saporin (IB4-Sap) was used to selectively eliminate $IB4^+$ neurons in the sciatic nerve (Joseph and Levine, 2010) and mental nerve (Taylor et al., 2012) as well as their terminals in the dorsal horn of the spinal cord and medulla, suggested that non-peptidergic neurons were essential for late onset chronic hyperalgesia. However, the selective elimination of the $IB4^+$ neurons in the sciatic nerve was found to elevate the nociceptive threshold to mechanical stimuli (Taylor et al., 2012). Information on the functional roles of $IB4^+$ non-peptidergic neurons in orofacial pain processing is limited.

Therefore, in order to determine the functional role of $IB4^+$ neurons associated with orofacial pain, we selectively eliminated $IB4^+$ neurons by pretreating the cisterna magna with IB4-Sap and examined formalin-induced nocifensive behavior and c-Fos expression in the Vc with/without systemic bicuculline or muscimol.

2. Materials and methods

All efforts were made to minimize the number of animals used and their suffering. All surgical procedures were reviewed and approved by the Osaka University Graduate School of Dentistry Intramural Animal Care and Use Committee (24-016-0), and conformed to the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.1. Animals and surgical operation

Experiments were conducted on 54 male Sprague-Dawley rats weighing 150–180 g (Keari Co. Ltd., Osaka, Japan). They were housed in pairs on a 12-hour light/dark cycle and were given free access to food and water. To minimize animal suffering, the number of animals used was based on the minimum required for statistically valid results. Rats were anesthetized intraperitoneally (i.p.) by the administration of sodium pentobarbital (50 mg/kg). They were fixed in a stereotaxic apparatus (Narishige, Tokyo, Japan). IB4-Sap (3 μM, 5 μl) (Advanced Targeting Systems, San Diego, CA, USA), non-targeted peptide (blank)-saporin (Bl-Sap; Advanced Targeting Systems, San Diego, CA, USA), or physiological saline (Sal) was injected into the cerebellomedullary cistern (cisterna magna) through the posterior atlanto-occipital membrane via a 26-G needle connected to a Hamilton syringe. The incision was closed with 4-0 vicryl sutures. IB4-Sap-, Bl-Sap-, or Sal-treated rats were used 2–4 weeks later for behavioral testing and histochemistry. Each treatment group (IB4-Sap, Bl-Sap, or Sal) consisted of 18 animals.

2.2. GABAergic drug

(+)-Bicuculline ($n = 6$ each for 3surgical paradigms; 2 mg/kg, i.p.; Sigma, MO, U.S.A.), muscimol ($n = 6$ each for 3surgical paradigms; 1 mg/kg, i.p.; Research Organics, OH, U.S.A.), or Sal for the control ($n = 6$ each for 3surgical paradigms) was injected in a volume of 1 ml/kg body weight 10 min before the formalin injection. This dose was sufficient for binding to $GABA_A$ receptors (Sawynok, 1987; Takemura et al., 2000; Abe et al., 2005; Sugiyo et al., 2009). All drugs used were diluted in saline and administered in a volume of 1 ml/kg body weight.

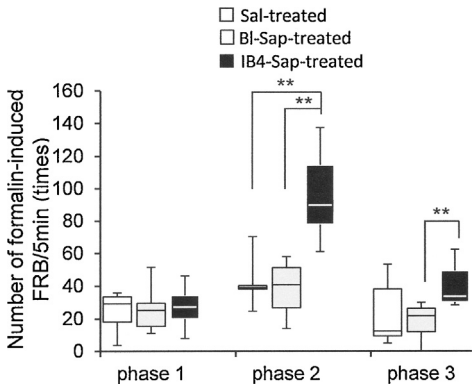

Fig. 1. Face rubbing behavior (FRB) in phases 1 (0–15 min), 2 (15–30 min), and 3 (30–45 min) after the subcutaneous injection of formalin into the upper lip of rats that received either the saline (Sal), blank saporin (Bl-Sap), or IB4 conjugated with saporin (IB4-Sap) treatment with the preadministration of Sal. Data were expressed as medians (horizontal line) with first and third quartiles (boxes), and maximal and minimal values (vertical lines). No significant differences were observed in the number of FRB in the three phases between Sal- and Bl-Sap-treated controls. The number of FRB in phase 2 was significantly higher in IB4-Sap-treated rats than in Sal-treated rats and Bl-Sap-treated rats. However, no significant difference was observed in the median number of FRB in phase 1 between Sal-, Bl-Sap-, and IB4-Sap-treated rats. All groups, $n = 6$ animals. $^{**}P < 0.01$.

2.3. Behavioral test (formalin test)

Animals were placed in Plexiglas testing chambers (30 cm × 25 cm × 15 cm) and allowed to acclimate for at least 60 min. A mirror was placed behind the chamber to facilitate the observation of orofacial nocifensive behaviors (facial rubbing behavior). After acclimation, the GABAergic drug (bicuculline or muscimol), or Sal for the control was injected i.p., then 50 μl of 5% formalin (1.85% of formaldehyde) was injected subcutaneously 10 min later into the left upper lip of the rat. Following the formalin injection, rats were immediately placed back into the testing chamber and the frequency of face rubbing behavior (FRB) with the fore- or hind-paw was counted as nocifensive behavior every 5 min over a period of 45 min. Formalin-induced FRB was constructed for phase 1 (0–15 min), phase 2 (15–30 min), and phase 3 (30–45 min) after the injection of formalin, respectively (Sugiyo et al., 2009).

2.4. Histochemistry

Two hours after the formalin injection, all animals were euthanized with an overdose of sodium pentobarbital (100 mg/kg, i.p.), and perfused intracardially with 100 ml of 0.02 M phosphate-buffered saline (PBS; pH 7.4) followed by 500 ml of a freshly prepared fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). The caudal brainstem and left TG were removed, postfixed in the same fixative overnight at 4 °C, and then immersed in 30% sucrose in 0.1 M PB at 4 °C until they sank.

The caudal brainstem was sectioned into serial coronal sections (60-μm-thick) on a freezing microtome and divided into two sets. One set of sections in all experimental series was stained with IB4, while the other set was stained with c-Fos. The TG was sectioned into plane parallel sections (60-μm-thick) to their long axis. All TG sections were stained with IB4.

One set of sections of the caudal brainstem and sections of the TG were incubated in 0.3% hydrogen peroxide for 20 min to inactivate

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