Neonatal Maternal Separation Increases Brain-Derived Neurotrophic Factor and Tyrosine Kinase Receptor B Expression in the Descending Pain Modulatory System

Elaine K.Y. Chung a Zhao-Xiang Bian a Hong-Xi Xu b Joseph Jao-Yiu Sung c

a School of Chinese Medicine, Hong Kong Baptist University, b Hong Kong Jockey Club Institute of Chinese Medicine, and c Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong, SAR, China

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Abstract
Neonatal maternal separation (NMS) could trigger long-term changes in the central neuronal responses to nociceptive stimuli in rats. Stress-induced visceral hyperalgesia is closely associated with the dysfunction of descending pain modulatory systems. Brain-derived neurotrophic factor (BDNF) not only has an important role in long-term synaptic plasticity but also in facilitating descending pain. The present study aimed to investigate changes in the expression of BDNF and its receptor tyrosine kinase receptor B (TrkB) in the amygdala and the rostral ventromedial medulla (RVM) after NMS and colorectal distention (CRD) stimulation in rats. Male Wistar rat pups were subjected to 180 min of daily NMS or not handled for 13 consecutive days. Expression of BDNF and TrkB following NMS and CRD stimulation was determined using immunohistochemistry. The results revealed an increase in the expression of BDNF and TrkB in the amygdala and the rostral ventromedial medulla (RVM) after NMS and colorectal distention (CRD) stimulation in rats. Male Wistar rat pups were subjected to 180 min of daily NMS or not handled for 13 consecutive days. Expression of BDNF and TrkB following NMS and CRD stimulation was determined using immunohistochemistry. The results revealed an increase in the expression of BDNF and TrkB in the amygdala and the rostral ventromedial medulla (RVM) after NMS and colorectal distention (CRD) stimulation in rats. An interactive effect of NMS and CRD on the expression of TrkB, but not BDNF, was found in the RVM. Furthermore, a significant interactive effect of NMS and CRD on the colocalization coefficient of TrkB and phospho-extracelular signal-regulated kinase expression in both the amygdala and RVM were found. These data demonstrate that NMS increases BDNF and TrkB expression in the descending pain systems, which may contribute to the development of NMS-induced visceral hyperalgesia.

Introduction

Neonatal maternal separation (NMS) from postnatal days 2 to 14 has been shown to have a long-lasting influence on the development of the neural systems of rats [1–3]. In previous studies, we found that NMS rats developed visceral hyperalgesia and enhanced neuronal sensitivity to noxious visceral stimuli in the central brain nuclei [4, 5]. These nuclei, including the cingulate cortex, the thalamus and the amygdala, play important roles in the central pain matrix and are involved in generating emotional and autonomic responses [6, 7].

The amygdala, in particular, plays a central role in the emotional affective component of pain [8–10]. It has important roles in descending pain enhancement and inhibition control of the interneurons in the spinal cord through the relay site of the rostral ventromedial medulla (RVM) [11, 12]. Neuronal plasticity changes in the amygdala have been found to be involved in the genera-
tion and maintenance of central sensitization in animals and humans [8, 13, 14]. Strong evidence for long-term potentiation in the amygdala has been found in various pain models [15–19]. The extracellular signal-regulated kinases (Erks) have been identified as molecular substrates underlying this long-term potentiation plasticity change in the amygdala in an inflammation-induced pain model [20, 21]. The RVM forms the primary relay site and exerts a biphasic influence on visceral nociceptive transmission [22, 23]. The increased excitability in response to visceral stimuli in the spinal cord after NMS, observed in our previous studies [4, 24], reflects changes in plasticity in descending pain circuitry. Our recent work has shown differential regulation of phospho (p)-Erk and c-Fos expression in the central pain matrix [5]. However, the mechanisms underlying these changes in plasticity are unknown.

Brain-derived neurotrophic factor (BDNF) has been shown to contribute to the synaptic plasticity formation in learning and memory [25–27] as well as the long-term plasticity changes in the central pain matrix [28]. A previous study found that BDNF expression was downregulated in the hippocampus and the striatum but upregulated in the ventral tegmental area of adult rats following postnatal maternal separation [29]. A growing body of evidence has indicated that supraspinal BDNF signaling is a novel descending pain facilitation pathway in nociceptive processing in the spinal cord [30]. These findings suggest that BDNF is an endogenous stress-responsive mediator in the brain. Therefore, we hypothesize that changes in the expression of BDNF and its receptor tyrosine kinase receptor B (TrkB) in descending pain circuitry may contribute to the development of stress-induced visceral hyperalgesia. The present study aimed to map the changes in BDNF signaling in the amygdala and RVM in an animal model of NMS-induced visceral hyperalgesia.

Materials and Methods

Animals
Primiparous timed-pregnant female Wistar rats were obtained from the Laboratory Animal Services Center, The Chinese University of Hong Kong. Dams were housed individually in plastic cages and maintained on a 12-hour light/dark cycle with free access to food and water. The handling of rats and all procedures performed were approved in accordance with the Animals (Control of Experiments) Ordinance, Hong Kong, China.

Maternal Separation
On postnatal days 2–14, pups were moved from their maternity cages to adjacent cages of an identical type for 180 min daily, as described previously [31]. After the separation period, pups were returned to their maternity cages. Control groups were not exposed to handling [nonhandled (NH) rats] and were maintained in their maternity cages with the dams. On postnatal day 22, the sex of the pups was determined. Female pups were culled, and male pups were weaned and housed in individual cages. After weaning, pups were weighed weekly. Only male rats weighing 250–300 g were used in the present study.

Colorectal Distention
One group each of NMS rats and NH rats (n = 6 for each group) was randomly chosen for the colorectal distention (CRD) experiments. A series of twenty 20-second distensions of 80 mm Hg was performed, as previously described [32]. Intervals between balloon stimulations were 4 min. One hour after the final stimulation, rats were deeply anesthetized with an overdose of midazolam hydrochloride and transcardially perfused. The remaining groups of NMS and NH rats (n = 3 for each group) were lightly anesthetized with ether, without CRD stimulus, and then deeply anesthetized and perfused.

Immunohistochemistry
Tissue Preparation. The brain and the lumbar-sacral spinal cord segments were removed and postfixed in fixative overnight, postfixed in 30% sucrose in 0.1 M PBS at 4°C for approximately 3 days and then stored at −80°C with embedding matrix (Shandon Cryomatrix, Thermo Electron Corporation, Waltham, Mass., USA). Coronal sections (30 μm) were cut on a freezing cryostat.

Single Immunohistochemistry Staining. Immunohistochemistry was performed as previously described [24]. Sections were blocked with 20% normal horse serum in PBS for 1 h followed by incubation with rabbit polyclonal antibody solutions against BDNF (1:1,000 in 0.3% Triton X-100 containing 0.02% sodium azide in 2× PBS; Chemicon International, Billerica, Mass., USA) or TrkB (1:2,000 in 0.3% Triton X-100 in PBS; Chemicon International) for 48 h at 4°C. Following primary antibody incubation, the sections were rinsed 3 times for 5 min each in PBS and then incubated for 2 h with a biotinylated secondary antibody (1:1,200 in PBS; Vector Laboratories, Burlingame, Calif., USA) at room temperature. Following incubation in a solution containing avidin-biotin complex (Elite ABC kit, Vector Laboratories) for 45 min at room temperature and subsequent reaction with diaminobenzidine, sections were mounted on gelatin-coated slides, dehydrated in a series of graded alcohol and coverslipped.

Double Immunofluorescence Staining. Sections were incubated in a mixture of mouse monoclonal antibody against p-Erk1/2 (1:300; Cell Signaling Technology) with either rabbit polyclonal antibody against BDNF (1:1,000; Chemicon International) or TrkB (1:2,000; Chemicon International) in PBS containing 0.3% Triton X-100 for 48 h at 4°C. Following primary antibody incubation, sections were rinsed 3 times in PBS and then incubated for 2 h in a secondary antibody cocktail consisting of Alexa Fluor 488-conjugated secondary antibodies and Alexa Fluor 568-conjugated secondary antibodies (1:500; Molecular Probes, Eugene, Ore., USA). After the final incubation, sections were rinsed 3 times in PBS and then coverslipped with mounting medium (Vectashield, Vector Laboratories). Finally, sections were imaged with a laser scanning microscope (Olympus Fluoview FV1000). The absence of cross-reactivity was confirmed by a single-labeled control preparation.
Central Sensitization for Visceral Pain

Controls. Controls for the single and double immunohistochemistry trials were performed by omission of primary antibodies in original reaction sequences, as described in previous studies [4].

Densitometry Measurement

To measure the level of intensity of BDNF and TrkB immunoreactivity, digital images of the sections were obtained using a light microscope (Axiovert 200, Zeiss) under standard illumination conditions. Images at 200× magnification were then analyzed by an image analysis program (Image J, NIH). Measurements of the calibrated optical densities indicating the levels of BDNF and TrkB immunoreactivity in the amygdala and the RVM were determined. The data from at least 3 sections from each animal were averaged and expressed as the mean ± standard error of the mean (SEM).

To determine the percentage of colocalization of p-Erk1/2 and either BDNF or TrkB immunoreactivity, images from double-immunostained sections were captured under the same parameters in the confocal microscope (Fluoview FV1000, Olympus) at high magnification (using a 60× lens). The pinhole was set to 1 Airy unit, and sections were scanned 3 times to reduce noise. Images acquired at a digital size of 1,024 × 1,024 were used to determine the percentage of colocalization using image analyzing software (Image J, NIH). The ‘image correlator plus’ plugin of Image J was used to generate the Pearson’s colocalization coefficient. This was done by measuring the overlap of pixels from 2 channels of each image. Then the coefficients from each group were averaged and expressed as the mean ± SEM.

Statistical comparisons between groups were conducted using two-way ANOVA with repeated measures followed by a post hoc test with Bonferroni correction.

Results

Upregulation of BDNF and TrkB Expression in the Central Nuclei of the Amygdala after NMS

BDNF immunoreactivity was seen in the neuropil of the amygdala (fig. 1). The neuronal cell bodies were clearly stained in each experimental group of rats (fig. 1A–D). Semiquantitative analyses of the density of immunoreac-

Fig. 1. Immunoreactivity for BDNF is detected in the neuropil of the CeA of NH (A, B) and NMS (C, D) rats with or without CRD. Arrows indicate intense staining of BDNF in close proximity to cell-like structures (A–D). Higher expression of BDNF is observed in the NMS rats (C, D) compared to the NH rats (A, B). Scale bar = 500 μm (A–D).

Fig. 2. A significant NMS effect on the expression of BDNF in the CeA was found (n = 3; p = 0.002, F = 18.18; two-way ANOVA). Bars represent the mean ± SEM of the average optical density of the immunoreactivity for BDNF (BDNF-ir).
Activity for BDNF in a fixed area within the central nuclei of the amygdala (CeA) revealed a significant effect of NMS on the level of expression of BDNF (p = 0.002, F = 18.18; n = 3). Upregulation of BDNF expression in the CeA was found after NMS (fig. 2).

A similar pattern was found for TrkB expression. TrkB-positive cell bodies were observed in the CeA of each group of rats (fig. 3A–D). Semiquantitative analyses revealed a significant effect of NMS, but not CRD, on the expression of TrkB in the CeA (p = 0.037, F = 5.98; n = 3). Upregulation of TrkB expression was found after NMS (fig. 4).

**Interactive Effect of NMS and CRD on TrkB Expression in RVM**

In the RVM, TrkB immunoreactivity was observed in the neurons (fig. 5A–D). TrkB expression increased significantly after CRD stimulation in NMS rats (fig. 6). Semiquantitative analyses of the density of TrkB immunoreactivity indicate that there was a significant interactive effect of NMS and CRD on the expression of TrkB in the RVM (p = 0.006, F = 12.192; n = 3).

**Increased Colocalization of TrkB on p-Erk1/2-Positive Neurons in the CeA and RVM in NMS Rats**

Double immunofluorescence revealed staining for p-Erk1/2 and TrkB localized in the same neuronal perikarya in the CeA (fig. 7). A strong correlation between p-Erk1/2 and TrkB expression was found in all groups of rats (fig. 7A–D). Analyses of the colocalization coefficient of the 2 immunoreactive products revealed a significant interactive effect of NMS and CRD on the proportion of colocalization of TrkB and p-Erk1/2 in the CeA (p = 0.041, F = 4.77; n = 6). Under CRD stimulation, a higher colocalization coefficient of TrkB on p-Erk1/2-positive neurons was found in NMS rats as compared to NH rats (fig. 8).
In the RVM, TrkB- and p-Erk1/2-positive neurons were observed (fig. 9). A significant interactive effect of NMS and CRD on the proportion of colocalization of TrkB and p-Erk1/2 was found \( (p = 0.01, F = 8.16; n = 6) \). Under CRD stimulation, the proportion of TrkB expression in p-Erk1/2-positive neurons was found to be higher in NMS rats as compared to NH rats (fig. 10).

**Discussion**

In the present study, BDNF and TrkB expression was upregulated in the CeA after NMS. An interactive effect of NMS and CRD on the expression of TrkB in the RVM was found, which was demonstrated by an upregulation of TrkB expression in the NMS rats that underwent CRD stimulation. Double immunostaining revealed a significant interactive effect of NMS and CRD on the proportion of colocalization of TrkB with p-Erk1/2 in both the CeA and RVM. In summary, the present results provide evidence of changes in BDNF and TrkB expression within the descending pain circuitry, and these changes may play an important role in the development of visceral hyperalgesia in NMS rats.

A growing body of evidence has suggested that stress-induced neuronal plasticity changes in the brain are associated with modulations in BDNF signaling. Psychological stresses increase BDNF expression in the hippocampus, striatum and ventral tegmental area in rats \([29, 33]\), and chronic stress applied to adult rats causes upregulation of BDNF in the amygdala \([34, 35]\). These data point to the fact that BDNF is an important endogenous mediator of stress responses within the brain. The current results further support this notion by demonstrating that stress-induced changes in BDNF and TrkB expression within the descending pain circuitry may play a role in the development of visceral hyperalgesia in NMS rats.
Fig. 7. Fluorescent micrographs of the amygdala of rats double immunostained to reveal immunoreactivity for TrkB and p-Erk. TrkB and p-Erk colocalize on the same neurons, as indicated by the overlapping signals (merge) which appear prominently in NH (A), NMS (B), NH + CRD (C) and NMS + CRD (D) rats. Scale bar = 200 μm.

Fig. 8. A significant interactive effect of NMS and CRD on the expression of TrkB in p-Erk-positive neurons was found (n = 6; p = 0.041, F = 4.772; two-way ANOVA). Bars represent the mean ± SEM of the colocalization coefficient for TrkB and p-Erk immunoreactivity (TrkB-ir; p-Erk1/2-ir) in the CeA.
Fig. 9. Fluorescent micrographs of the RVM of rats double immunostained to reveal immunoreactivity for TrkB and p-Erk. TrkB and p-Erk colocalize, as indicated by the overlapping signals (Merge) which appear prominently in NH (A), NMS (B), NH + CRD (C) and NMS + CRD (D) rats. Scale bar = 200 μm.

Fig. 10. A significant interactive effect of NMS and CRD on the colocalization coefficient of TrkB and p-Erk immunoreactivity (TrkB-ir; p-Erk1/2-ir) in the perikarya in the RVM was found (n = 6; p = 0.01, F = 8.162; two-way ANOVA). Bars represent the mean ± SEM of the colocalization coefficient for TrkB and p-Erk.
rent data clearly demonstrate that BDNF and TrkB expression is upregulated in the amygdala after NMS. BDNF-induced synaptic plasticity, such as long-term potentiation in the amygdala, is mediated by Erks and plays a central role in the learning of fear [21]. Activation of Erk signaling in the amygdala was found to be necessary to induce long-lasting tactile hypersensitivity in an inflammation-induced pain model [20]. These changes in the amygdala may contribute to the increase in neuronal activity in NMS-induced visceral hyperalgesia.

Our recent study showed that p-Erk expression increased in the amygdala in NMS rats [5]. The present results provide evidence for the colocalization of p-Erk-positive neurons and TrkB immunoreactivity. An analysis of the colocalization coefficient revealed that the proportion of p-Erk-positive neurons displaying TrkB immunoreactivity was significantly increased in the amygdala under the interactive effect of NMS and CRD. These findings suggest that BDNF signaling may be involved in the plasticity change in the amygdala that contributes to the NMS-induced visceral hyperalgesia. Given the well-established role of the amygdala in both emotional responses and pain processing, it is possible that neuromodification in the amygdala is involved in central hyperalgesia in NMS rats.

A previous study demonstrated that the nuclei of the amygdala are involved in the RVM nociceptive modulatory systems [12]. Environmental stimuli likely trigger modifications in the descending pain modulatory systems through the neuronal circuits linking the amygdala and RVM. Enhanced activation of TrkB receptors in the RVM was previously found to contribute to descending facilitation of inflammatory pain [30]. Abundant behavioral pharmacological studies have demonstrated the functional role of TrkB in RVM neurons in pain facilitation through the effect of intra-RVM injection of anti-BDNF antiserum or TrkB-IgG fusion protein in attenuating central hyperalgesia. In the present study, we demonstrated a significant increase in TrkB expression in the RVM, the relay site between the amygdala and the spinal cord, under the joint influence of NMS and CRD. Study of the colocalization coefficient also revealed a significant interactive effect of NMS and CRD on the proportion of p-Erk-positive neurons expressing TrkB immunoreactivity. These data provide evidence for the modification of BDNF and TrkB in the amygdala-RVM circuit and neuronal response to CRD in NMS rats. These findings could serve as a basis for further investigations on the pharmacological and functional roles of BDNF in synaptic activity related to pain modulation in NMS-induced visceral hyperalgesia.

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