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Persistent c-fos expression and NADPH-d reactivity in the medulla and the lumbar spinal cord in rat with short-term peripheral anosmia

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Abstract

Here we examine hypothesis that short-term peripheral $ZnSO_4$-induced anosmia can produce effects on c-fos expression within spinal cord and caudal medulla in male Wistar rats ($n = 4$). Fos-like-immunoreactive cells revealed by avidin-biotin-peroxidase method show a significant bilateral increase in the nucleus proprius (layers 3 and 4) and medial part of layers 5 and 6. In substantia gelatinosa (layer 2_i) and area 10 Fos-positive neurons were intermixed together with nicotinamide adenine dineucleotide phosphate-diaphorase (NADPH-d)-reactive cells. Short-term anosmia enhanced c-fos expression in ventral horn (layers 7 and 8), ventrolateral segment and dorsal part of the spinal trigeminal nuclei. In anosmic rats varicose fibres and numerous NADPH-d-stained neurons were present in the gelatinous layer of the spinal trigeminal nucleus caudalis, and a separate population of Fos-positive cells was detected within this layer. Nucleus tractus solitaris also contained a few NADPH-d-reactive, medium sized neurons intermixed with Fos-immunoreactive cells.

Keywords: Anosmia; C-fos expression; Sensitization; Anxiety; Grooming; Rat

Behavioral changes seen in animals with olfactory disturbance include hyperactivation, stereotypic grooming and aggression [11]. Bulbectomy (central anosmia) or repeated 3–4 weeks $ZnSO_4$ treatment (peripheral anosmia) have long been used for modeling animal depression [8]. In our recent experiments rats subjected to a short-term 7-days $ZnSO_4$ induced anosmia showed a different, anxiety-like profile with inhibition of exploration and increased urination, defecation and grooming [13]. The brain mechanisms underlying such behavioral changes in short-term anosmia are far from being understood. As such, there may be certain differences in basal level of neuronal activation between control and anosmic rats.

Analysis of an early-response gene c-fos activation has been proved to be a useful tool in the mapping, at the cellular level, of certain functional pathways activated following noxious stimulation or sensory deprivation [2]. Thus, we are examining the hypothesis that the short-term anosmia accompanied by behavioral disorders and excessive grooming can produce effects on c-fos expression and Fos protein production within spinal cord, spinal nucleus of the trigeminal nerve and other structures of caudal medulla connected with autonomic reactions.

Male Wistar rats (240–270 g) were anesthetized with ether. Short-term anosmia was induced by spraying 5% $ZnSO_4$ solution intranasally. In the control rats irrigation of the olfactory mucosa was produced with saline solution. Seven days after these procedures $ZnSO_4$-treated (group I, $n = 4$) and control rats (group II, $n = 4$) were deeply anesthetized with sodium pentobarbital (Sigma, USA) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Caudal medulla oblongata and lumbar spinal cord (L3–L5) were cut into coronal frozen sections (40 μm thick). Fos-like-immunoreactive (Fos-LI) cells in the various structures of the medulla and spinal cord layers 1–10 [16] in the lesioned and control rats were revealed by standard avidin-biotin-peroxidase method [4]. A rabbit polyclonal antibody directed against c-Fos protein (1:2000, Oncogene Res. CA, USA) and commercial Kit (1:200, Vectastain ABC, USA) were used. Diaminobenzidine-tetraHCl reaction was intensified by nickel ammonium

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sulfate. Immunostained sections were then processed for nicotin-amide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemistry [20] to reveal nitric oxide synthase (NOS)-containing neurons, which may be intermixed with Fos-LI or double-staining cells in lumbar spinal cord and medulla. It was reported that most of NADPH-d-reactive spinal neurons are also NOS-immunoreactive [19]. Fos-LI cells were counted in medulla and spinal cord bilaterally and data were used for both statistical analysis and graphic representation. Up to 20 medullar and 100 spinal cord sections per rat from lesioned and control groups were analyzed to reveal the regional Fos-LI distribution. Results

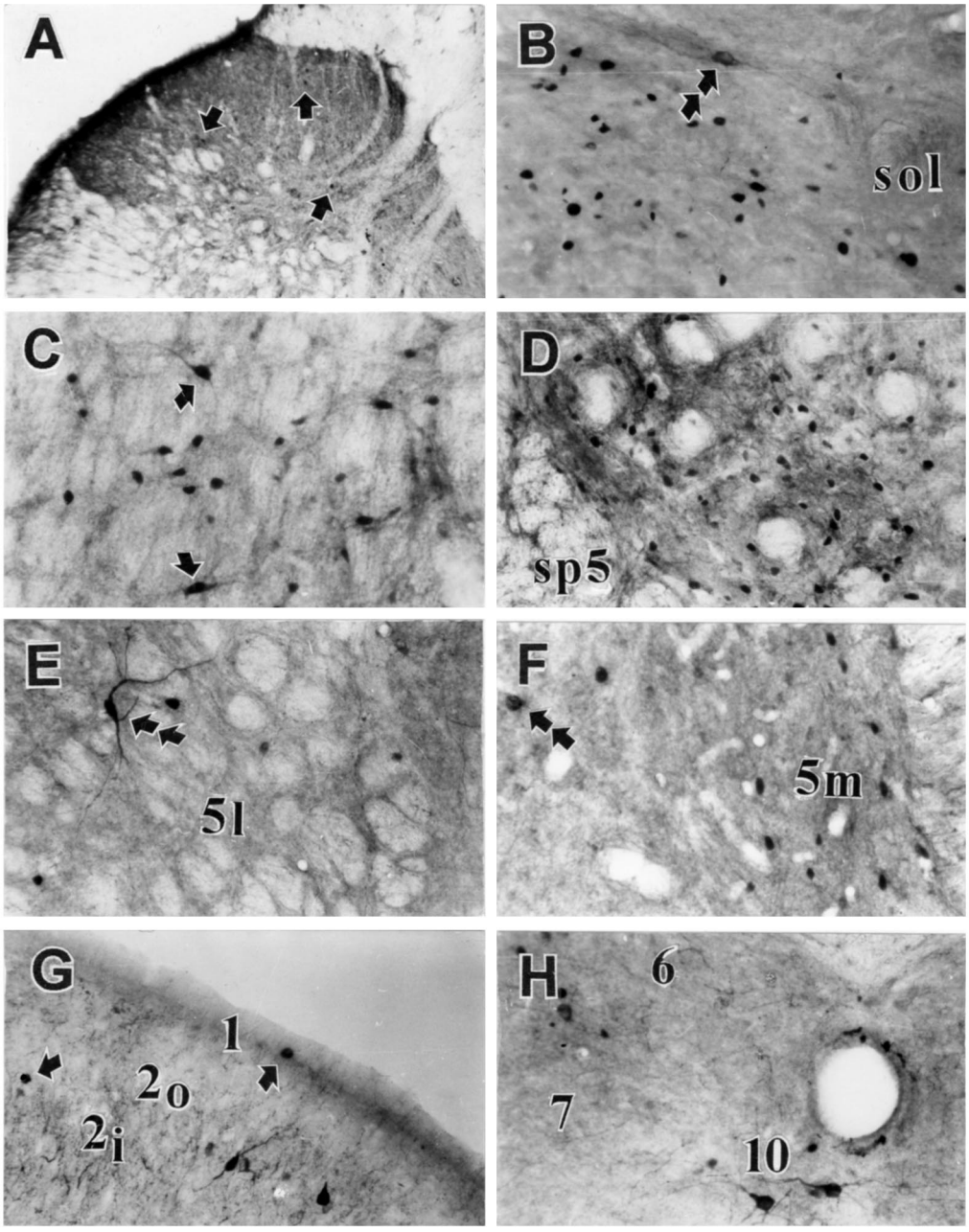

Fig. 1. (A) Lower magnification photomicrograph of the dorsal horn in the control rat. Note solitary Fos-LI neurons in the layers 2–4 (arrows). (B,C) Higher magnification photomicrographs of Fos-LI neurons and NADPH-d positive cells in the nucleus of the solitary tract and caudal ventrolateral part of medulla respectively. Note NADPH-d positive neuron (double arrow) and intensive Fos immunoreactivity in some cell bodies and dendrites (arrows). (D) Intensive Fos-immunoreactivy in the spinal trigeminal nucleus caudalis in anosmic rat. (E,F) examples of Fos-LI neurons and NADPH-d positive cells (double arrows) in the lateral and medial parts of layer 5 (5m, 5l). (G) solitary Fos-LI cells in layers 1 and 2_i (arrows) and clusters of NADPH-d positive cells. in layer 2_i. (H) Fos-LI neurons and NADPH-d positive cells within area 10 and intermediate zone (layers 6 and 7). Fos-LI and NADPH-d positive cells in (B,E–H) are intermixed, with no double-stained cells observed. Note small and elongated in shape nuclei in layer 5m (F) in comparison with large and round in shape nuclei in layers 5l (E) and 1 (G). sol, solitary tract; sp5, spinal trigeminal tract. Bar, 100 μm in (A); Bar, 50 μm in (H) and it is applicable for (B–G).

were expressed as mean number (±SEM) of Fos-LI cells per section for the side of each nucleus or layer. Student's *t*-test for unpaired data was used for comparison between experimental groups with analysis of variance (ANOVA).

It was reported previously [1,2], in line with our study, that the basal level of c-fos expression in the lumbar spinal cord is very low, less than 5 Fos-LI cells per 40 μm thick section of non-stimulated freely moving rats (Fig. 1A). However, in anosmic rats we recorded a significant bilateral increase in the number of Fos-LI neurons in the nucleus proprius (layers 3 and 4) and medial part of layers 5 and 6. In substantia gelatinosa (layer 2_i) and area 10 Fos-LI neurons were intermixed together with NADPH-d-reactive cells with no double-labeled cells in those areas. In addition, short-term anosmia enhanced c-fos expression in ventral horn (layers 7 and 8) (Fig. 1E–H and Fig. 2A).

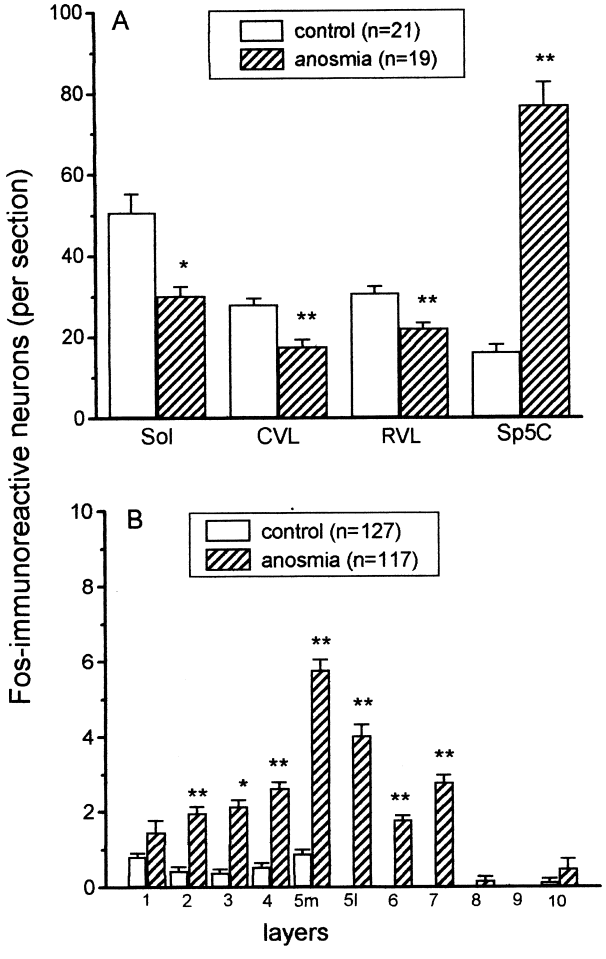

Fig. 2. (A) diagrams showings a mean number (±SEM) of Fos-LI cells in the nucleus of solitary tract (Sol), caudal and rostral parts of ventrolateral medulla (CVL, RVL) and the spinal trigeminal nucleus caudalis (Sp5C). Asterisks represent significance levels between anosmic and control rats (*$P < 0.01$, **$P < 0.001$). (B) Diagrams showing mean number (±SEM) of Fos-LI neurons in different layers of the lumbar enlargement (L4/L5) in anosmic vs. control rats. Asterisks represent significance levels between anosmic and control rats (*$P < 0.01$, **$P < 0.001$).

In anosmic rats the number of Fos-LI neurons compared to the control group was markedly lower ($P < 0.01$), in the nucleus tractus solitarius (Sol), caudal and rostral parts of ventrolateral medulla (CVL, RVL), i.e. functionally related areas of pressor reflex pathways (Fig. 1B–C and Fig. 2B). Our previous data [12] show that catecholamine-containing neurons located within Sol, CVL RVL (noradrenaline and adrenaline-containing cells of the groups A2, A1, A1/C1, respectively) play a crucial role in cardiovascular reactions and supraspinal control over outflow through its direct descending projections to the spinal cord. These groups of catecholaminergic neurons may be involved in circulatory regulation and inhibitory control via different autonomic centers of the brain, including the hypothalamus and locus coeruleus [3] which also send direct projections to the spinal cord [9]. The results of our study show that neuronal activity within Sol, CVL and RVL may be attenuated by short-term anosmia. The latter is in line with finding that blood pressure and heart rate 5–10 days after olfactobulbectomy were significantly lower than those in intact rats [7].

Several other nuclear groups in caudal medulla (area postrema, external cuneate nuclei, vestibular nuclei and parvocellular division of the lateral reticulal nucleus) demonstrate a considerable basal level of Fos-LI. However, our data demonstrate that short-term anosmia does not enhance or reduce c-fos expression in these areas.

In the spinal trigeminal nucleus caudalis (Sp5C), the area involved in the transmission of orofacial sensory information, only scarce Fos-LI neurons were seen in the control animals. By contrast, we recorded a significant increase in c-fos expression within ventrolateral segment and dorsal part of the Sp5C in anosmic rats (Fig. 2B). Fos-LI neurons were densely packed throughout its extension up to its interpolar part. Positive cells were located mainly in layers 2 and 3 (Fig. 1D). A plexus of varicose fibres and numerous NADPH-d-stained neurons in the lesioned rats were present in the gelatinous layer of the Sp5C, and a separate population of Fos-LI cells was detected within this layer. Sol also contained a few NADPH-d-reactive, medium sized neurons intermixed with Fos-LI cells.

Summarizing our data, we notice that in case of short-term anosmia when enhanced grooming behavior is often observed, a considerable c-fos expression in substantia gelatinosa, nucleus proprius and medial part of the neck of the dorsal horn of the lumbar spinal cord was found. It is known [6,15] that these regions receiving non-nociceptive cutaneous afferents from skin and hair receptors, contain cells that respond to the tactile stimulation. We speculate that, in addition to other somatic afferents, anosmia-induced grooming can lead to particular tactile stimulation of orofacial afferents. It is possible that the latter can also provoke c-fos expression within Sp5C (see similar data obtained after corneal tactile stimulation in [14]). Neurons located ventrally to the nucleus proprius in medial part of the neck of the dorsal horn (layer 5m) receive large diameter non-noxious primary afferents from muscles and joints [15].

In the pioneer study [5] it was emphasized that both noxious and non-noxious peripheral stimulation evoke c-fos expression in the spinal cord neurons, and the lamellar distribution of Fos-LI cells is related to the nature of sensory stimulation. Thus, we suggested that the pattern of c-fos expression induced in medulla and lumbar spinal cord of the $ZnSO_4$-treated rats with enhanced grooming behavior is not associated with pain. After short-term anosmia we found no statistically significant activation of c-fos expression in layer 1, in the outer part of substantia gelatinosa (layer 2_o), or in lateral reticular portion of layer 5 (5l), i.e. regions that contain cells predominantly responsive to noxious stimulation [1,17]. It is a well-known fact that nuclei of the caudal medulla might be involved in eliciting of the cardio-vascular reactions produced by acute pain [10]. However, it is possible to speculate that patterns of Fos-LI neuron distribution in dorsal and ventral horns may relate to central sensitization and hyperalgesia [15,18] produced by anosmia. Other possible functional explanation can be that attenuation of neuronal activity within CVL, RVL and Sol under short-term anosmia would tend to increase, rather then suppress, activity of spinal neurons. Importantly, the question arises whether anosmia-induced persistent neuronal activation in the brainstem is specific (i.e. olfactory-related) or present rather general mechanism accompanying sensation. Further experiments will be necessary to study mechanisms of such activation as well as possible biological rationale behind this phenomenon.

[1] Buritova, J., Honore, P., Chapman, V. and Besson, J.-M., Carrageenan oedema and spinal Fos–LI neurones are reduced by piroxicam in the rat, NeuroReport, 6 (1995) 1257–1260.
[2] Herdegen, T., Rudiger, S., Mayer, B., Bravo, R. and Zimmermann, M., Expression of nitric oxide synthase and colocalisation with Jun, Fos and Krox transcription factors in spinal cord neurons following noxious stimulation of the rat hindpaw, Brain Res. Mol. Brain Res., 22 (1994) 245–258.
[3] Hodge Jr., C.J., Apkarian, A.V., Owen, M.P. and Hanson, B.S., Changes in the stimulation of locus coeruleus and nucleus raphe magnus following dorsal rhizotomy, Brain Res., 288 (1983) 325–329.
[4] Hsu, S.-M., Raine, L. and Fanger, H., Use of avidin–biotin–peroxidase complex (ABC) in immunoperoxidase techniques, , J. Histochem. Cytochem, 29 (1981) 577–580.
[5] Hunt, S.P., Pini, A. and Evan, G., Induction of c-fos-like protein in spinal neurons following sensory stimulation, Nature, 328 (1987) 632–634.
[6] Jasmin, L., Gogas, K.R., Ahlgren, S.C., Levine, J.D. and Basbaum, A.I., Walking evokes a distinctive pattern of Fos-like immunoreactivity in the caudal brainstem and spinal cord of the rat, Neuroscience, 58 (1994) 275–286.
[7] Kawasaki, H., Watanabe, S. and Ueki, S., Changes in blood pressure and heart rate following bilateral olfactory bulbectomy in rats, Physiol. Behav., 24 (1980) 51–56.
[8] Kolunie, J.M. and Stern, J.M., Maternal aggression in rats: effects of olfactory bulbectomy, $ZnSO_4$-induced anosmia, and vomeronasal organ removal, Horm. Behav., 29 (1995) 492–518.
[9] Kuypers, H.G.J.M. and Maisky, V.A., Retrograde axonal transport of horseradish peroxidase from spinal cord to brainstem cell groups in the cat, Neurosci. Lett., 1 (1975) 9–14.
[10] Lanteri-Minet, M., Weil-Fugazza, J., de Pommery, J. and Menetrey, D., Hindbrain structures involved in pain processing as revealed by the expression of c-Fos and other immediate early gene proteins, Neuroscience, 58 (1994) 287–298.
[11] Leonard, B.E., The olfactory bulbectomized (OB) rat as a model of depression, Pol. J. Pharmacol. Pharm., 36 (1984) 561–569.
[12] Maisky, V.A. and Doroshenko, N.Z., Catecholamine projections to the spinal cord in the rat and their relationship to central cardiovascular neurons, J. Auton. Nerv. Syst., 34 (1991) 119–128.
[13] Makarchuk, N.E. and Kalueff, A.V., Olfaction and Behavior, CSF, Kiev, 2000 134 pp.
[14] Meng, I.D. and Bereiter, D.A., Differential distribution of the Fos-like immunoreactivity in the spinal trigiminal nucleus after noxious and innoxious thermal and chemical stimulation of rat cornea, Neuroscience, 72 (1996) 243–254.
[15] Millan, M., The induction of pain: an integrative review, Progr. Neurobiol., 57 (1999) 1–164.
[16] Paxinos, G. and Watson, C., The Rat Brain in Stereotaxic Coordinates, Academic Press, New York, 1997, pp. 34–56.
[17] Presley, R.W., Menetrey, D., Levine, J.D. and Basbaum, A.I., Systemic morphine supresses noxious stimulus-evoked Fos protein-like immunoreactivity in the rat spinal cord, J. Neurosci., 10 (1990) 323–335.
[18] Rohde, D.S., Detweiler, D.J. and Basbaum, A.I., Spinal cord mechanisms of opioid tolerance and dependence: Fos-like immunoreactivity expression increases in subpopulations of spinal cord neurons during withdrawal, Neuroscience, 72 (1996) 233–242.
[19] Saito, S., Kidd, G.J., Trapp, B.D., Dawson, T.M., Bredt, D.S., Wilson, D.A., Travstman, R.J., Snyder, S.H. and Hanley, D.F., Rat spinal cord neurons contain nitric oxide synthase, Neuroscience, 59 (1994) 447–456.
[20] Vincent, S.R. and Kimura, H., Histochemical mapping of nitric oxide synthase in the rat brain, Neuroscience, 46 (1992) 755–784.