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Postnatal Development of Brainstem Serotonin-Containing Neurons projecting to Lumbar Spinal Cord in Rats

Running Title: Development of 5-HT descending systems

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ABSTRACT

We quantified postnatal changes in brainstem serotonin

(5-hydroxytryptamine, 5-HT)-containing neurons projecting to lumbar spinal cord. The medulla-spinal cord descending neurons were identified by a retrograde neurotracer, choleratoxin B subunit (CTb), and 5-HT neurons were stained by immunohistochemistry. Double-labeled neurons were assumed to be 5-HT neurons projecting to the lumbar spinal cord, and were quantitatively analyzed in each raphe nucleus in the medulla. The following results were obtained: (1) At PND 3, numerous CTb-labeled neurons (CTLN) were already present in the raphe pallidus (B1), while few CTLN were seen in raphe obscurus (B2) and raphe magnus (B3). CTLN then rapidly increased in number and were separately distributed after PND 7 in B3 and after PND 14 in B2. (2) At PND 3, numerous 5-HT-containing neurons were already present in B1-B3, with 23.4% and 14.0% of them labeled with CTb in B1 and B2, respectively, while there were few double-labeled neurons in B3. From PND 3 to 28, although the proportion of double-labeled to 5-HT neurons remained unchanged in B1 and B2, that in B3 rapidly increased from 5.8% at PND 7 to 28.8% at PND 14. Previous studies have shown that the 5-HT neurons in B3 send fibers mainly to the dorsal horn, while those in B1 and B2 send fibers mainly to the ventral horn at all spinal cord levels. Taken together, the present findings suggest that the brainstem 5-HT systems influence the ventral horn of the spinal cord, where spinal motoneurons exist earlier than in the dorsal horn. The functional significance of these early 5-HT systems in motor development and/or disabilities is discussed.

Key Words: development, 5-HT, spinal cord, choleratoxin B subunit, rat

INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT)-containing neurons are among the earliest to be detected in the developing central nervous system [1-3], especially along the midline of brainstem corresponding to the raphe nuclei. In the rat [1], for example, 5-HT neurons are present as early as embryonic days 13 to 16 in the brainstem. Descending 5-HT fibers are already present at birth in the ventral horn of the lumbar spinal cord [4-6].

Accordingly, dysfunction in 5-HT systems during early life could lead to long-lasting structural and functional alterations. Thus, early in development, 5-HT appears to play important roles in differentiation and proliferation of target cells such as spinal motoneurons (MNs).

It is also known that 5-HT participates in the regulation of various functions, including those of the motor, somatosensory, and limbic systems, and is associated with a wide range of neuropsychiatric and neurological disorders [7]. Furthermore some experimental data support the role of 5-HT in modulating the brain development [8]. Recently, drugs affecting levels of 5-HT such as selective serotonin reuptake inhibitors and L-5-hydroxytryptophan have been used for the treatment of neuropsychiatric disorders in children [9,10]. Consequently, detailed studies of developmental changes in 5-HT systems are essential for understanding the pathophysiology of several neurological and psychological disorders in children.

In the present study, we quantified postnatal changes in brainstem 5-HT-containing neurons projecting to lumbar spinal cord. The branstem-spinal cord descending neurons were identified by a retrograde neurotracer, choleratoxin B subunit (CTb) [11], and brainstem 5-HT neurons were identified by immunohistochemistry. Double-labeled neurons were assumed to be 5HT-containing neurons projecting to the lumbar spinal cord, and were quantitatively analyzed in each raphe nucleus in the medulla.

MATERIALS AND METHODS

All of the experimental procedures used in this study were approved by the Animal Studies Committee of Asahikawa Medical College in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Guide, revised 1996). Every attempt was made to minimize animal suffering and to reduce the number of animals used. Sprague-Dawley rats at postnatal days (PND) 3, 7, 14, and 28 were used, with four rats examined at each age. For labeling of brainstem-spinal cord descending neurons, fluorescent isothiocyanate (FITC)-labeled CTb (List Biol. Inc.) was used as a retrograde neurotracer. After the animals were anesthetized with sodium pentobarbital (18mg/kg b.w., i.p.), the skin of the back was incised and laminectomy was performed to expose the lumbar spinal cord. Using a Hamilton microsyringe, 5-10µl of 0.1 % CTb solution was injected into the lumbar enlargement. To avoid bias in injection sites due to technical factors, the spinal cord was completely crosscut by a surgical knife at the lumbar enlargement, and CTb was injected at the incision. Post-injection survival time was kept constant at a minimum of 24 hours. The CTb-treated rats were re-anesthetized with sodium pentobarbital (50mg/kg b.w., i.p.) and perfused transcardially with 0.01M phosphate-buffered saline (pH7.4), followed by 4% paraformaldehyde, 0.2% picrate, and 0.35% glutaraldehyde in 0.1M phosphate buffer (pH7.4) at 4°C. The brainstem was removed and postfixed overnight with 4% paraformaldehyde and 0.2% picrate in 0.1M phosphate buffer at

4°C, followed by immersion for at least four hours in 0.1M phosphate buffer containing 15% sucrose at 4°C. Then, 14µm-thick coronal sections were cut using a cryostat and subjected to the following steps of treatment: 1) incubation overnight with rabbit 5-HT antiserum (diluted 1:10000) at room temperature; 2) incubation for two hours with tetramethylrhodamine isothiocyanate (TRITC)-labeled goat anti-rabbit IgG (diluted 1:40, Vector) at room temperature; and 3) coverslipping with Vectashield (Vector Inc.). The sections were examined under a fluorescence microscope. Double-exposure photographs enabled identification of CTb-filled and TRITC-labeled neurons, indicative of 5-HT-containing neurons projecting to the lumbar spinal cord. The proportion of 5HT neurons projecting to the spinal cord to total number of 5HT neurons was calculated for each nucleus at each PND.

RESULTS

The CTb-labeled neurons (CTLN) in the medulla are shown in Fig. 1 for all postnatal ages examined. Medulla-spinal cord descending neurons were mainly distributed along the midline and in the ventral reticular formation. In the raphe pallidus (B1; Figs.1A), numerous CTLN were tightly distributed at PND 3. Between PND 7 and 28, CTLN gradually became located separately and distant from each other. In the raphe obscurus (B2; Figs.1B), few CTLN were observed at PND 3-7, and after PND 14 CTLN rapidly increased in number and were relatively

separate from each other in location. In the raphe magnus (B3; Figs.1C), few CTLN were observed at PND 3, resembling findings for B2, but had already rapidly increased in number by PND 7. Separation of CTLN was much more evident after PND 14 than in B1 and B2.

With the optimal filter setting, FITC labeling of CTLB and TRITC structures demonstrating 5-HT-containing neurons was recognizable separately (Figs. 2A and B) or simultaneously (Fig. 2C). Arrows indicate double-labeling indicative of 5-HT neurons projecting to the lumbar spinal cord. In B1 (Fig. 3A), numerous 5-HT-containing neurons were already tightly distributed and one-fourth of them were labeled with CTb at PND 3. From PND 3 to 28, while 5-HT neurons separated in stages, the proportion of double-labeled neurons to 5-HT neurons remained unchanged. Unlike B1, there were small numbers of double-labeled neurons in B2 and B3 at PND 3 (Figs. 3B and C). However, the proportion of double-labeled to 5-HT neurons in B3 rapidly increased from PND 7 to 14, but stayed unchanged in B2 during the same period. Fig. 4 demonstrates the distribution of 5-HT neurons including double-labeled neurons (open circles). Reference planes are based on the atlas of Paxinos and Watson [12]. No clear tendency was observed in location of double-labeled neurons, which were scattered throughout each raphe nucleus. The proportion of double-labeled to 5-HT neurons in each raphe nucleus is shown in Table 1. The proportion of 5HT neurons projecting to the lumbar spinal cord was relatively high in B1 and B2 at PND 3

and did not increase markedly from PND 3 to PND 28. In B3, however, this proportion was very low, 2.8%, at PND 3, rapidly increased from 5.8% at PND 7 to 28.8% at PND 14, but exhibited little change from PND 14 to 28.

DISCUSSION

The present study demonstrated that the 5-HT fibers originating from B1 and B2 had already reached the lumbar spinal cord at PND 3, nearly the same level as at PND 28, but that those from B3 had not yet descended completely. From PND 7 to PND 14, the 5-HT fibers originating from B3 rapidly descended and reached the lumbar spinal cord. Skagerberg et al. [13] studied brainstem 5-HT neurons projecting to the spinal cord using True Blue as a retrograde neurotracer with immunohistochemistry for 5-HT in adult rats, and classified the descending 5-HT system into three pathways: (1) the dorsal pathway originating mainly from B3 and terminating in the dorsal horn at all spinal cord levels; (2) the intermediate pathway originating mainly from B1, B2 and the arcuate cell group, and terminating in the intermediate grey at thoracolumbar and upper sacral levels; and (3) the ventral pathway originating mainly from B1 and B2, and terminating in the ventral horn at all spinal cord levels. Taken together, the findings of the present study provide indirect evidence that the brainstem 5-HT systems innervate and modulate the ventral horn of the spinal cord, where MNs are present earlier than in the dorsal horn (Fig.

5). Several histological studies [4-6,14] have shown that the development of intraspinal 5-HT fibers exhibits a ventro-dorsal gradient, consistent with the results of the present study as summarized in Figure 5.

In our study, only about 20-30% of 5-HT neurons in B1-B3 had axons reaching the lumbar spinal cord even by PND 28. This finding suggests that individual 5-HT neurons do not project their axons to the entire spinal cord independently, but to appropriate levels separately. From PND 3 to 28, the proportion of double-labeled neurons to 5-HT neurons remained unchanged in B1. However, it is known that the density of 5-HT fibers in the ventral horn of lumbar spinal cord rapidly increases after PND 3 [6]. This discrepancy may be accounted for by the finding that 5-HT neurons first projected their axons to the lumbar cord in early stages, and that the density of 5-HT fibers then increased there. We previously examined the development of intraspinal noradrenaline (NA) fibers using immunohistochemistry for tyrosine hydroxylase [15], and found that the descending NA fibers first reached the spinal MNs on their soma, and then the extended dendrites of MNs. Many developmental studies of intraspinal NA [16] and dopaminergic [17] fibers have revealed a pattern similar to that of the development of 5-HT fibers in both sequence and time course. It may be that 5-HT fibers first innervate MNs and then exert a trophic effect on them by increasing in density and length of the dendrites along with the growth of MNs, as observed for catecholamine fibers.

Several studies have been made of the neurotrophic effects of 5-HT on target neurons in in vivo and in vitro preparations. Lauder et al. [18] found that administration of p-chlorophenylalanine, an inhibitor of 5HT synthesis [19], to pregnant rats slowed the onset of neuronal differentiation selectively in those regions of fetal brain which will, in the adult, receive 5HT terminals. The morpho-functional development of the visual cortex of newborn rats and the stimulating effects of 5-HT on it were demonstrated in tissue culture by Chubakov et al. [20]. On the other hand, 5-HT systems exert important effects on background excitability of MNs in mature animals, which may lead to the modification of such functions as locomotor activity [21]. Electrical stimulations of the raphe nucleus facilitate spinal cord activity, such as monosynaptic reflexes and/or excitability of MNs [22,23]. In other words, the brainstem 5-HT neurons innervating to the spinal MNs may play critical roles on the development of motor functions such as muscle tone and locomotor activities by maturing MNs and modulating the excitability of MNs in early developmental periods.

We previously examined the effects of neonatal hypoxia on cells of the central nervous system such as cholinergic neurons [24], medulla-spinal cord descending neurons including the raphe-spinal neurons [25], and spinal MNs in rats [26]. In the studies on the spinal MNs [26], we noted that the dendrites of lumbar MNs at PND 14 were shorter and less extensive in rats with hypoxic insult at birth than in rats without such insult,

though no significant difference was observed in cervical MNs between rats with and without hypoxia. This difference in effect of hypoxic insult on the development of MNs between cervical and lumbar cord may reflect the clinical observation that neurological manifestations in preterm infants involve motor deficits with greater effect on the lower than on the upper limbs. We speculated that this observation is closely related to the development of 5-HT systems with a rostro-caudal gradient [27]. This speculation will allow us to design future experiments about the effects of hypoxia on the development of 5-HT systems in relation to the development of other neural networks for further understanding of the clinical manifestations of neonatal hypoxic insults.

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Figure legends

Fig. 1 Photomicrographs of CTb-labeled brainstem-lumbar spinal cord descending neurons in raphe pallidus (A), raphe obscurus (B), and raphe magnus (C) at PND 3 (a), 7 (b), 14 (c), and 28 (d). At PND 3, numerous CTb-labeled neurons are already present in the raphe pallidus, while few CTb-labeled neurons are observed in the raphe obscurus and raphe magnus. CTb-labeled neurons rapidly increase in number after PND 7 in the raphe magnus and after PND 14 in the raphe obscurus. Bar=100μm.

Fig. 2A: CTb-labeled neurons in B3 at PND 14. Neurons labeled with CTb indicating brainstem-lumbar spinal cord descending neurons. B: 5HT-containing neurons labeled with TRITC in the same section. C: A double-exposure photomicrograph showing both CTb- and TRITC-positive fluorescent structures. Arrows indicate double-labeled 5-HT neurons projecting to the lumbar spinal cord. Bar=100μm.

Fig. 3 Double-exposure photomicrograph showing both CTb- and TRITC-positive fluorescent structures in the raphe pallidus (A), raphe obscurus (B), and raphe magnus (C) at PND 3 (a), 7 (b), 14 (c), and 28 (d). Arrows indicate some of double-labeling indicative of 5-HT neurons projecting to the lumbar spinal cord. At PND 3, about one-fourth of the 5-HT-containing neurons are labeled with CTb in B1 and B2, while

there are few double-labeled neurons in B3. From PND 3 to 28, although the proportion of double-labeled to 5-HT neurons remains unchanged in B1 and B2, that in B3 rapidly increases. Bar=100μm.

Fig. 4 Distribution of 5-HT-containing neurons (closed circles) and double-labeling (open circles) indicative of 5-HT neurons projecting to the lumbar spinal cord at PND 3 (A), 7 (B), 14 (C), and 28 (D). Reference planes are based on the atlas of Paxinos and Watson [11]. Small a and b indicate planes corresponding approximately to the level of Bregma -11.8 and -12.3, respectively. Abbreviations: RM: nucleus raphe magnus, RO: nucleus raphe obscurus, RP: nucleus raphe pallidus.

Fig. 5 Schematic illustrations of developmental changes in the 5-HT brainstem-spinal cord descending systems based on the findings of the present study. By PND 3, the 5-HT fibers originating from B1 and B2 have already reached mainly the ventral portion of the lumbar spinal cord, but those from B3 have not yet completely descended. From PND 7 to PND 14, the 5-HT fibers originating from B3 rapidly descend and reach mainly the dorsal portion of lumbar spinal cord.

Table 1 Proportion of the 5HT Neurons Projecting to the Spinal Cord to the 5HT neurons in the Raphe nuclei

	Double labeled neurons / 5HT immunoreactive neurons (%)			
	PND3	PND7	PND14	PND28
B1	29/124=23.4%	36/130=27.7%	40/126=31.7%	41/144=28.5%
B2	12/86=14.0%	14/77=18.2%	19/87=21.8%	21/101=20.8%
B3	4/145=2.8%	6/104=5.8%	19/66=28.8%	21/78=26.9%

Number of neurons on ten specimens was counted in each nucleus at each postnatal day

Figure 1 Tanaka H. et al.

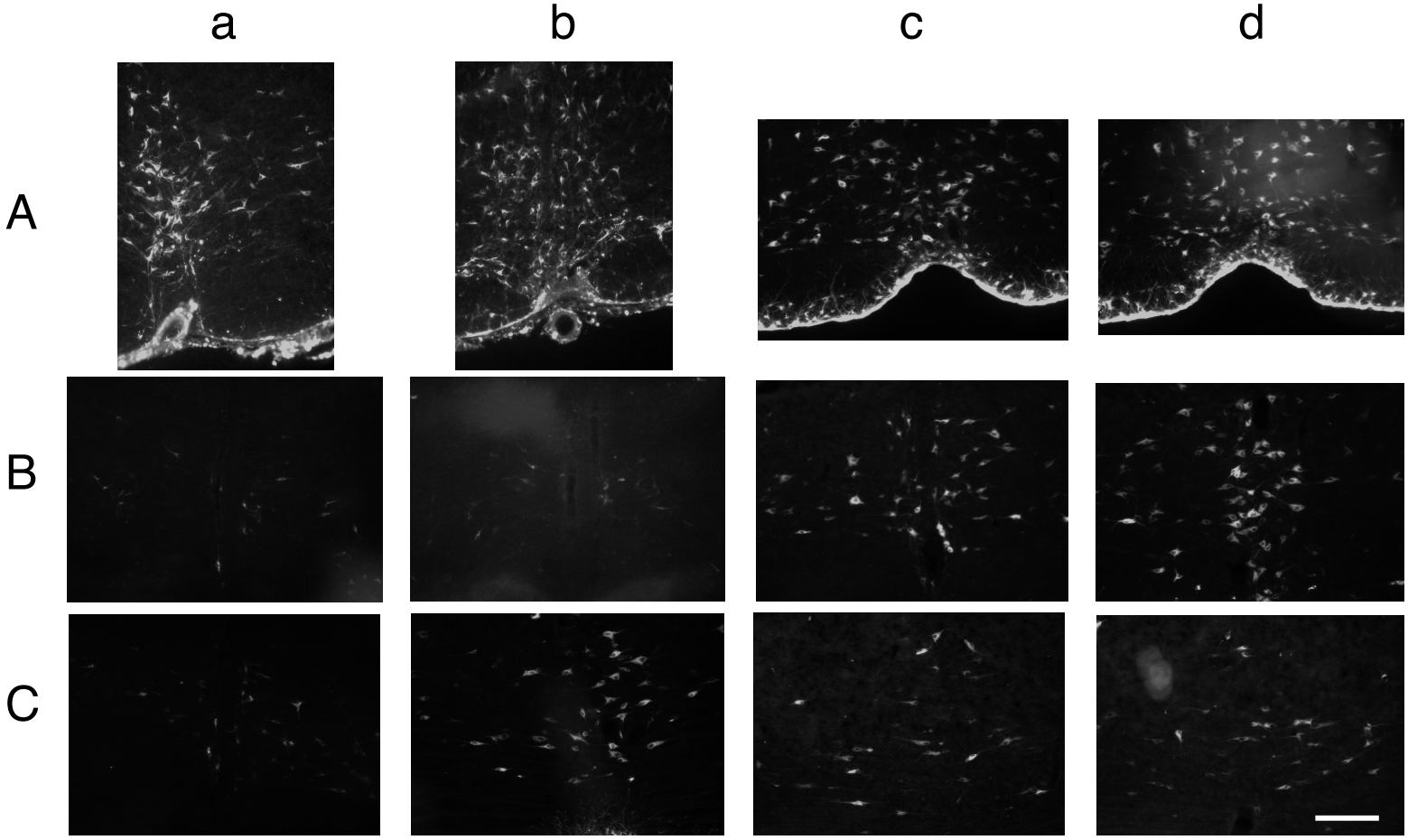

Figure 2 Tanaka H. et al.

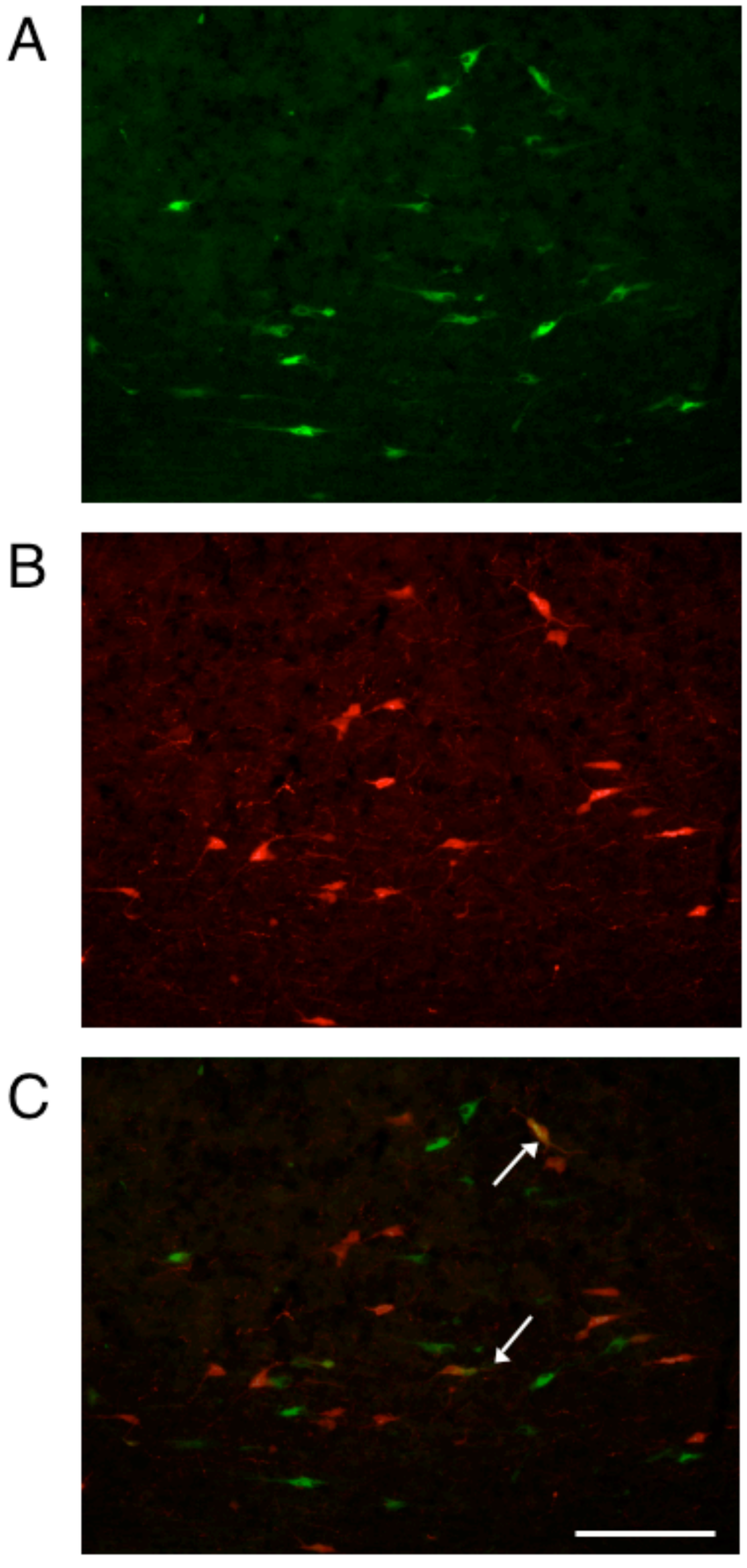

Figure 3 Tanaka H. et al.

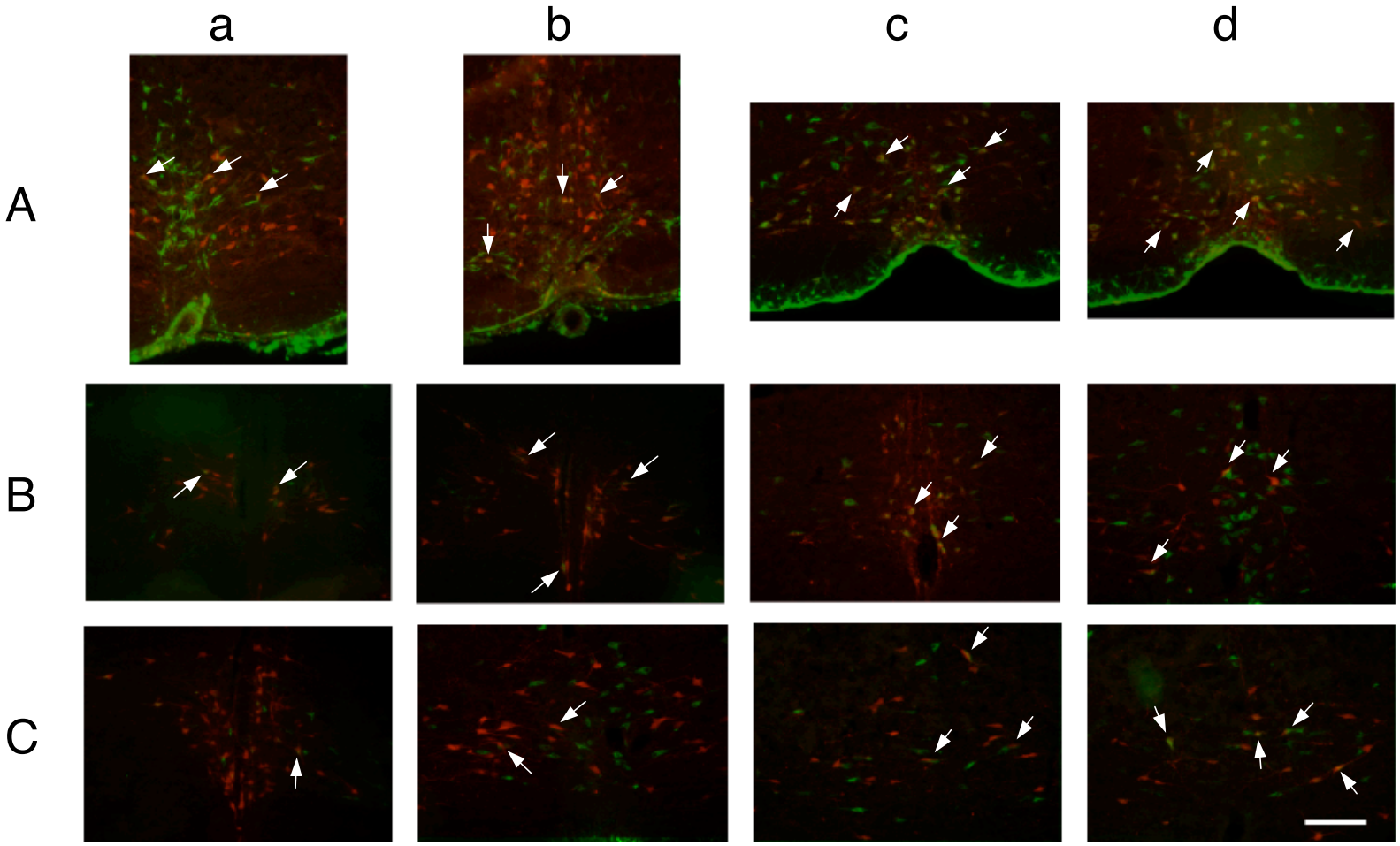

Figure 4 Tanaka H. et al.

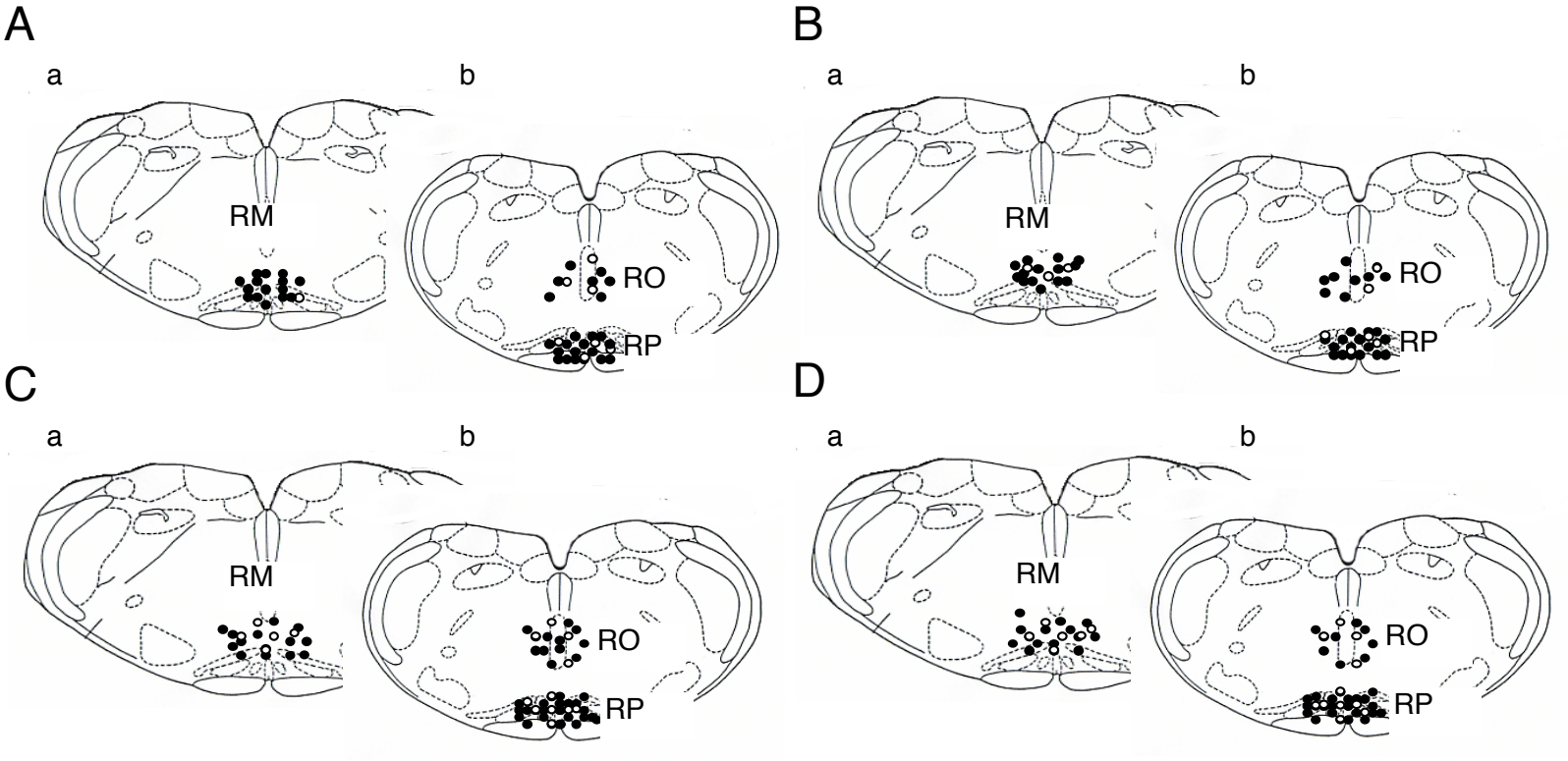

Figure 5 Tanaka H. et al.