Expression of nischarin, an imidazoline 1 receptor candidate protein, in the ventrolateral medulla of newborn rats

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ARTICLE INFO

**Keywords:**
Immunohistochemistry, Parafacial respiratory group/retrotrapezoid nucleus, Respiratory control, Retrotrapezoid nucleus

**Abstract**

The activation of imidazoline 1 (I₁) receptors is suggested to stimulate the respiratory drive in newborn rats. Here, we immunohistochemically examined whether nischarin, an I₁ receptor candidate protein, is expressed in the ventrolateral medulla, where cardiorespiratory centers are located. Newborn rats (age, 3–5 days) were deeply anesthetized with isoflurane; the brainstem was dissected, sectioned sagittally, and labeled with nischarin. Nischarin-associated signals were observed broadly throughout the newborn rat brainstem, including at motor nuclei (motor trigeminal nucleus and facial nucleus), sensory nuclei (lateral superior olive, medial and spinal vestibular nuclei, cuneate nucleus, spinal trigeminal nucleus, and solitary nucleus), and the rostral and caudal ventrolateral medullar regions. In particular, the rostral ventrolateral medulla included a layer of aggregated nischarin expression along the ventral surface, and the layer was in close contact with GFAP-positive processes. In addition, some Phox2b-positive neurons were positive for nischarin in the region. Our results reveal nischarin expression in the newborn rat brainstem and suggest that I₁ receptor activation at the level of the ventrolateral medulla contributes to central chemoreception and respiratory control in newborn rats.

1. Introduction

Imidazoline receptors are divided into three subtypes (I₁, I₂, and I₃) [1], and the action of I₁ receptors in rostral ventrolateral medulla (RVLM) [2] is considered to exert a central sympatoexcitatory effect and suppress arterial pressure and heart rate [1]. In support of this idea, previous immunohistochemical studies using imidazoline receptor binding protein (IRBP) [3] or nischarin [4] demonstrated the expression of imidazoline receptors in adult rat brainstem. Nischarin is an I₁ receptor candidate protein that was first identified as the mouse homolog of human imidazoline receptor antisera-selected protein (IRAS) [4,5]. IRBP was originally isolated from bovine adrenal chromaffin cell membranes, and IRAS is thought to be either the human homolog of IRBP or a protein closely related to it [5].

In a previous study in newborn rats, we suggested that mean inspiratory flow (V̇<sub>T</sub>/T<sub>I</sub>, where V̇<sub>T</sub> is tidal volume and T<sub>I</sub> is inspiratory time), an index of respiratory drive, is preserved during the administration of dexmedetomidine (an α<sub>2</sub>-adrenoceptor/I₁ receptor agonist [1,6]) through I₁ receptor activation [7]. Dexmedetomidine is used clinically as a sedative and an analgesic and only minimally suppresses respiration in humans [8], including a child with congenital central hypoventilation syndrome [9]. Hence, we hypothesized that I₁ receptor stimulation enhances respiratory drive at the level of the ventrolateral medulla (VLM) in newborn rats at dexmedetomidine administration [7]. However, because I₁ receptor expression has not previously been confirmed in the brainstem of newborn rats [3,10], we here examined immunohistochemically whether I₁ receptor are present in the newborn rat brainstem, including regions related to the respiratory centers of the VLM.

2. Material and methods

The experimental protocol was reviewed and approved by the Animal Research Committee of the Nippon Dental University, School of Life Dentistry at Tokyo, Japan (17-08; approved in July 2017). The animals were treated in accordance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences [11] and the ARRIVE guideline [12]. All efforts were made to minimize animal pain and distress and the number of animals used.

Pregnant Wistar rats (n = 6) were obtained from CLEA Japan Inc. (Tokyo, Japan) and maintained in the Nippon Dental University’s animal center. Newborn rats (age, 3–5 days) (7 males and 8 females, body weight 7.2–12.1 g) were deeply anesthetized with isoflurane (Forane

**Abbreviations:** I<sub>1</sub> receptor, imidazoline 1 receptor; IRAS, imidazoline receptor antisera-selected protein; IRBP, imidazoline receptor binding protein.

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https://doi.org/10.1016/j.neulet.2021.136113
Received 13 May 2021; Received in revised form 24 June 2021; Accepted 9 July 2021
Available online 13 July 2021
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granular layer; MoCh, molecular layer of cerebellum; PK, Purkinje cell layer.

We noted extensive nischarin immunoreactivity throughout the brainstem and cerebellum (D), nischarin staining was less prominent and occurred diffusely and in scattered cells and axons in the facial nucleus (Fig. 2), and rostral RVLM (Fig. 1 B, E, and Fig. 3 C, D). In contrast, nischarin immunoreactivity was present (from rostral to caudal) in the most rostral area near the facial nucleus in the RVLM (hereafter, we refer to this region as the rostral RVLM and CVLM). In low-power photomicrographs of the VLM, dense immunoreactivity was present (from rostral to caudal) in the most rostral area near the facial nucleus in the RVLM (hereafter, we refer to this region as the rostral RVLM and CVLM) and used TH as a marker to identify catecholamine neuron groups [17], including the adrenergic C1 and noradrenergic A1 (or C1/A1 in newborn rat brain [15]) in the VLM (Fig. 1 C, F). The distributions of C1 and A1 neurons nearly match the anatomical locations of the RVLM and CVLM, respectively [19], or when present as C1/A1 neurons, of the newborn rat brain [15]) in the VLM (Fig. 1 C, F). The distributions of C1 and A1 neurons nearly match the anatomical locations of the RVLM and CVLM, respectively [19], or when present as C1/A1 neurons, of the newborn rat brain [19]. During this study, we referred to the Purkinje cells in the cerebellar cortex of the cerebellum (anatomical location is shown in the magnified inset in Fig. 1 D) as a positive control to identify nischarin immunoreactivity [16] (Fig. 2 B), applied GFAP as a marker to identify astrocyte [17], and used TH as a marker to identify catecholamine neuron groups [18], including the adrenergic C1 and noradrenergic A1 (or C1/A1 in newborn rat brain [15]) in the VLM (Fig. 1 C, F). The distributions of C1 and A1 neurons nearly match the anatomical locations of the RVLM and CVLM, respectively [19], or when present as C1/A1 neurons, of the newborn rat brain [15]) in the VLM (Fig. 1 C, F). The distributions of C1 and A1 neurons nearly match the anatomical locations of the RVLM and CVLM, respectively [19], or when present as C1/A1 neurons, of the newborn rat brain [15]) in the VLM (Fig. 1 C, F). The distributions of C1 and A1 neurons nearly match the anatomical locations of the RVLM and CVLM, respectively [19], or when present as C1/A1 neurons, of the newborn rat brain [15]).

3. Results

During this study, we referred to the Purkinje cells in the cerebellar cortex of the cerebellum (anatomical location is shown in the magnified inset in Fig. 1 D) as a positive control to identify nischarin immunoreactivity [16] (Fig. 2 B), applied GFAP as a marker to identify astrocyte [17], and used TH as a marker to identify catecholamine neuron groups [18], including the adrenergic C1 and noradrenergic A1 (or C1/A1 in newborn rat brain [15]) in the VLM (Fig. 1 C, F). The distributions of C1 and A1 neurons nearly match the anatomical locations of the RVLM and CVLM, respectively [19], or when present as C1/A1 neurons, of the newborn rat brain [15]). We noted extensive nischarin immunoreactivity throughout the newborn rat brainstem (Fig. 1 B, E), including motor nuclei (motor trigeminal and facial nuclei), sensory nuclei (lateral superior olive, medial and spinal vestibular nuclei, cuneate and external cuneate nuclei, solitary nucleus, and spinal trigeminal nucleus), and neurons in the VLM (RVLM and CVLM). In low-power photomicrographs of the VLM, dense immunoreactivity was present (from rostral to caudal) in the most rostral area near the facial nucleus in the RVLM (hereafter, we refer to this region as the rostral RVLM), facial nucleus, inferior olive (dorsal nucleus), and lateral recticular nucleus (Fig. 1 B, E). In high-power photomicrographs, nischarin immunoreactivity occurred not only in cell bodies but also in axons and dendrites, in the facial nucleus (Fig. 2 E), inferior olive (dorsal nucleus) (Fig. 2 G), lateral recticular nucleus (Fig. 2 H), and rostral RVLM (Fig. 1 B, E, and Fig. 3 C, D). In contrast, nischarin staining was less prominent and occurred diffusely and

...dissected. The tissues were then prepared according to standard techniques and embedded in paraffin. Sagittal sections were cut at a thickness of 5 μm. We applied Nissl staining to every 10th consecutive slide to identify anatomical structures [13].

For immunohistochemical examination, deparaffinized tissue sections were incubated with Deaparaffinization/Antigen Retrieval Solution pH6 (catalog no.415281, Nichirei Biosciences, Tokyo, Japan) for 40 min at 95–99°C for antigen retrieval and washed in PBS. The tissue sections were then incubated at room temperature (RT) in 3% H2O2 to block endogenous peroxidase activity, washed with PBS, and incubated in Blocking One Histo (Nacalai Tesque, Kyoto, Japan) as a blocking treatment.

Blocked sections were treated at 4°C with antibody specific for nischarin (dilution, 1:20; Rabbit Polyclonal Anti-NISCH antibody, catalog no. HPA023189, Atlas antibodies, Bromma, Sweden) for 3–6 days, or tyrosine hydroxylase (TH; dilution, 1:2000; catalog no. ab129991, Mouse Monoclonal Anti-TH antibody, Abcam, Cambridge, UK) or GFAP (dilution, 1:400; catalog no. bsm-33065M, Mouse Monoclonal Anti-GFAP Antibody, Bioss Antibodies, Woburn, MA) overnight. Primary antibodies were diluted in PBS containing 5% Blocking One Histo (Nacalai Tesque, Kyoto, Japan) as a blocking treatment. After sections were washed in PBS, they were incubated at RT with detection reagents comprising amino acid polymers, peroxidase, and Fab’ fragments of goat anti-rabbit IgG antibody (catalog no. 414191, Histofine Simple Stain Rat MAX-PO (R), Nichirei Biosciences) or goat anti-rabbit antibody and anti-mouse IgG antibody (catalog no. 414181, Histofine Simple Stain Rat MAX-PO (MULTI), Nichirei Biosciences). After the slides were washed in PBS, DAB substrate solution (catalog no. 425011, DAB Substrate Kit, Nichirei Biosciences) was applied at RT to reveal the antibody staining reaction. The tissue sections were then dehydrated in ethanol, cleared in xylene, and mounted. The stained tissue sections were examined under a light microscope (Axio Imager.M2, Carl Zeiss, Oberkochen, Germany) and anatomical structures were identified with the aid of rat stereotaxic atlases [14,15].
Fig. 2. High-power photomicrographs of Nissl staining (A) and nischarin immunoreactivity (B) in the cerebellar cortex. In developing rats, the cerebellar cortex coexists of three layers; (from outside to inside) external granular layer, molecular layer of cerebellum, and Purkinje cell layer [15]. Dense nischarin immunoreactivity occurred exclusively in Purkinje cells. Low-power photomicrograph of Nissl stain (C) and nischarin immunoreactivity (D) in the rostral ventrolateral medulla. The numbers with asterisks (*1–5) indicate blood vessels that commonly appeared in the images (C and D). High-power photomicrograph of nischarin immunoreactivity in the facial nucleus (E), a part in the rostral ventrolateral medulla (F) (*3 and *5 are those indicated in panel D), inferior olive (dorsal nucleus) (G), and lateral reticular nucleus (H). Nischarin immunoreactivity was present not only in neuronal cell bodies but also in surrounding tissues including axons and dendrites in the facial nucleus, inferior olive (dorsal nucleus), and lateral reticular nucleus. Scale bars = 100 μm (C, D) or 20 μm (A, B, E–H).
Fig. 3. Medium- and high-power photomicrographs of immunoreactivity to GFAP (A, B), nischarin (C, D), and tyrosine hydroxylase (E, F) and Nissl staining (G, H) in the rostral part of the rostral ventrolateral medulla. The serial sections were cut from the most medial (A, B) to the most lateral (G, H). The numbers with asterisks (*1–4) indicate blood vessels that commonly appeared in the images. The characteristic layer-like nischarin immunoreactivity along the ventral surface is due to nischarin-positive aggregated neurons (D) and surrounding astrocytes (B). This pattern of immunoreactivity was not obtained by using tyrosine hydroxylase (E, F). Scale bars = 50 μm (A-H). Abbreviation (based on [14,15]): 7N, facial nucleus.
Fig. 4. Medium-power photomicrographs of immunoreactivity to nischarin (A) and Phox2b (B) in the rostral ventrolateral medulla and the merged image (C). The serial sections were cut from medial (A) to lateral (B). In (C), Phox2b-positive nuclei are shown as violet. The numbers with asterisks (*1–4) indicate blood vessels that commonly appeared in the images. In some neurons, Phox2b and nischarin immunoreactivities overlapped (indicated by arrows). Scale bars = 50 μm. Abbreviation (based on [14, 15]): 7N, facial nucleus.
sporadically in the neurons comprising the reticular formation in the VLM (Fig. 2 D, F). Furthermore, in the rostral RVLM, the nischarin-positive aggregated neurons seemed to constitute a layer-like structure along the ventral surface (Fig. 3 C, D), where we found abundant GFAP-positive processes (Fig. 3 A, B). Interestingly, the staining pattern of the nischarin-immunoreactive layer at the ventral surface differed from that obtained by staining for TH, where the positive signal was primarily associated with axons (Fig. 3 E, F). In the rostral RVLM, which corresponds to the anatomical locations of the retrotrapezoid nucleus (RTN) [21] or, when present, the ventral parafacial region [22], we observed Phox2b-positive cells (Fig. 4 B), some of which were also positive for nischarin (Fig. 4 A, B, C).

4. Discussion

Our results from nischarin immunohistochemistry of the brainstem of newborn rats indicate 1) the possible presence of I1 receptors in the VLM, and 2) an I1-immunoreactive neuronal layer along the ventral surface of the RVLM.

The finding from the present study suggest that I1 receptors are broadly expressed throughout the newborn rat brainstem. Although our observation was limited to a nonquantitative evaluation of a small area of the lateral medulla, our results were compatible with an immunohistochemical observation of the adult rat brainstem that used IRBP [3] and with the results of a study of adult rat brain, in which imidazoline binding sites occupied 36.5% of the medulla oblongata [23]. In the VLM of newborn rats, various nuclei—namely, the facial nucleus, inferior olive (dorsal nucleus), and the lateral reticular nucleus—showed particularly prominent nischarin immunoreactivity. In rat cell line (PC-12), nischarin is expressed in Map-2-positive neuronal cell bodies and dendrites [16]. In addition, electron microscopy of adult rat brain showed IRBP in nerve termini and astrocytes [24]. Taken together, these previous findings suggest that the nischarin immunoreactivity in the facial nucleus, inferior olive (dorsal nucleus), and lateral reticular nucleus may be composed of axons, dendrites, and astrocytes (Fig. 2 E, F, G, H), and this same description can be applied to the layer characterized by I1 immunoreactivity along the ventral surface of the rostral RVLM (Fig. 3 C, D). Interestingly, a light microscopic examination of transverse sections of adult rat brain revealed IRBP-immunolabeled processes on the surface of the VRLM, although IRBP expression in the lateral reticular nucleus was low or negligible [3,24].

In the RVLM and CVLM, respiration-related regions coexist with cardiovascular sympathetic and parasympathetic circuities [19,25]. In particular, the lateral reticular nucleus contains sympathetic neurons [1], and microinjection of clonidine (an α2-adrenoceptor/I1 receptor agonist) into this region decreases blood pressure in adult cats [26]. Hence, the dense nischarin immunoreactivity that we noted in the lateral reticular nucleus of newborn rats (Fig. 1 B, E, Fig. 2 H) is compatible with the earlier observations [1,26]. Whether the areas showing high-density I1 receptor immunoreactivity change by the species and/or with age, as seen in the catecholamine cell groups in the brainstem [27], is an interesting question.

In the RTN (also known as the parafacial respiratory group/retrotrapezoid nucleus), Phox2b-positive neurons and astrocytes are sensitive to CO2 and may enhance active expiration and ventilatory responses [17,21,22,25]. Hence, our present results (Fig. 3 A-D and Fig. 4) suggest that I1 receptors also contribute to the central chemoreception. We observed TH immunoreactivity (Fig. 3 E, F) and a layer of nischarin immunoreactivity (Fig. 3 C, D) along the ventral surface of the rostral RVLM. A previous study in newborn kittens showed TH-positive cell bodies and varicosities near the ventrolateral surface of VLM (longitudinally, approximately from the lateral reticular nucleus to facial nucleus); the authors explained this structure as a tributary of a major longitudinal catecholamine bundle, which courses in a dorsolateral position through the entire brainstem tegmentum [28]. Considering these previous findings in light of our current observations reminds us of the area reticularis superficialis ventrolateralis, which is important for cardiorespiratory control and the dysfunction of which has been implicated in sudden infant death syndrome [29]. Although I1 agonist has been reported to increase second messengers concentrations in the rat VLM and causes hypotension [4], contribution of I1 receptors on neurons seems rather complex in the central nervous system [1,5], and in light of our present observations, further investigation regarding the functional role of I1 receptor in the brainstem is warranted.

5. Conclusions

Our current results confirm I1 receptor expression in the VLM, including the rostral RVLM, of newborn rats. Our findings are noteworthy because the RVLM plays crucial roles in central chemoreception and the ventilatory responses.

Author contributions

C.S. and N.S.H. conceived of the present idea. Y.N. carried out the experiment. C.S. wrote the manuscript with input from all authors. T.I. supervised the project. C.S. and R.I. drafted the manuscript, and designed and processed the figures. All authors discussed the results and contributed to the final manuscript.

Funding

Department resources funded the study.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors are grateful to Prof. Masanori Nasu (Research Center for Odontology, School of Life Dentistry at Tokyo, The Nippon Dental University, Tokyo) for the technical assistance.

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