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C-terminals in the mouse branchiomotor nuclei originate from the magnocellular reticular formation

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Abstract

Large cholinergic synaptic boutons called "C-terminals" contact motoneurons and regulate their excitability. C-terminals in the spinal somatic motor nuclei originate from cholinergic interneurons in laminae VII and X that express a transcription factor *Pitx2*. Cranial motor nuclei contain another type of motoneuron: branchiomotor neurons. Although branchiomotor neurons receive abundant C-terminal projections, the neural source of these C-terminals remains unknown. In the present study, we first examined whether cholinergic neurons express *Pitx2* in the reticular formation of the adult mouse brainstem, as in the spinal cord. Although *Pitx2*-positive cholinergic neurons were observed in the magnocellular reticular formation and region around the central canal in the caudal medulla, none was present more rostrally in the brainstem tegmentum. We next explored the origin of C-terminals in the branchiomotor nuclei by using biotinylated dextran amine (BDA). BDA injections into the magnocellular reticular formation of the medulla and pons resulted in the labeling of numerous C-terminals in the branchiomotor nuclei: the ambiguous, facial, and trigeminal motor nuclei. Our results revealed that the origins of C-terminals in the branchiomotor nuclei are cholinergic neurons in the magnocellular reticular formation not only in the caudal medulla, but also at more rostral levels of the brainstem, which lacks *Pitx2*-positive neurons.

Keywords

C-terminals; branchiomotor neurons; cholinergic neurons; reticular formation; brainstem

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Conflict of Interests

None of the authors have any conflicts of interest associated with this study.

1. Introduction

The somata and proximal dendrites of motoneurons are contacted by large cholinergic synaptic terminals [1, 2, 3, 4, 12, 17]. Those terminals have very thin membrane-bound structures—termed subsurface cistern—in the postsynaptic structure, and were hence named "C-terminals (or C boutons)" after the cisterns. The muscarinic m2 receptors are closely aligned with C-terminals on the postsynaptic motoneurons [9], and activation of the m2 receptors increases motoneuron excitability [16]. The genetic deletion of functional C-terminals causes impairment of the gastrocnemius muscle activation in a swimming task [26]. These physiological and behavioral studies indicate that C-terminal inputs into spinal motoneurons play a key role in the modulation of locomotor behavior. Spinal C-terminals originate from cholinergic interneurons located in Rexed laminae VII and X [16, 21, 26]. *Pitx2*, a homeodomain transcription factor, is essential for left-right asymmetry and normal development of eye, tooth, pituitary, heart and muscle, and required for neuronal migration and differentiation in the hypothalamus and midbrain [10, 15, 24]. In the spinal cord, *Pitx2* also labels the subtype of cholinergic interneuron called partition cells, which give rise to C-terminals [5, 26].

In the brain, C-terminals are present in some cranial motor nuclei [9, 17, 25]. The cranial motor nuclei that innervate skeletal muscles are categorized into 2 groups based on their differentiation: branchiomotor (trigeminal motor (Vm), facial (VIIIm), and ambiguus (Amb) nuclei) and somatic motor (oculomotor, trochlear, abducens, and hypoglossal (XIIIm) nuclei) [20]. We have previously identified the origin of C-terminals in the rat XIIIm, which is the rostral extension of spinal motor columns [8]—the motoneurons in the XIIIm receive C-terminal projections from neurons in the reticular formation (RF) near the target. Although the origin, and some functions, of C-terminals in the somatic motor nuclei have emerged, those in the branchiomotor nuclei have not yet been determined. Some studies using retrograde tracing have revealed that the brainstem RF contains cholinergic interneurons with direct projections to the branchiomotor nuclei [6, 7, 22]. These findings suggest that C-terminals in the branchiomotor nuclei may originate from a population of cholinergic premotor neurons in the RF.

In this study, our aim was to identify C-terminal origins in the mouse branchiomotor nuclei. We first investigated *Pitx2* expression in cholinergic neurons in the brainstem RF to determine C-terminal projection candidates. Whereas cholinergic neurons in the RF of the caudal medulla were positive for *Pitx2*, no *Pitx2* expression was detected in the rostral portion of the RF, which has been shown previously to contain premotor neurons [6, 7, 22]. We next performed anterograde tracing experiments to confirm whether these cholinergic neurons gave rise to C-terminals. Our results show that C-terminals on branchiomotor neurons originate from the magnocellular reticular formation not only in the caudal medulla, but also at more rostral levels of the brainstem, which lacks *Pitx2*-positive neurons.

2. Materials and methods

Adult male and female ICR (SLC, Shizuoka, Japan) and ICR-C57BL/6J mixed-background mice weighing 25–40 g were used for in situ hybridization (n = 3) and anterograde tract-tracing study (n = 21). All procedures performed on animals were approved by the Animal Care and Use Committee of the National Defense Medical College.

2.1. In situ hybridization

An RNA probe corresponding to the 3' UTR of *Pitx2* was used for in situ hybridization [14]. Mice were deeply anesthetized using chloral hydrate (600 mg/kg) and perfused

transcardially by using Bouin's solution. The brains were removed and embedded in paraffin.

Tissue sections (8- μ m thickness) were deparaffinized, and pretreated with 0.2 M HCl. After washes, the sections were digested with 20 μ g/ml proteinase K and post-fixed in 4% paraformaldehyde (PFA) before treatment with 0.2% glycine. The sections were pre-hybridized and then hybridized with a Dig-labeled probe for 14 hours at 70°C. Unbound probes were washed with 1 $\times$ and 0.1 $\times$ saline-sodium citrate buffer. The signals were detected using anti-Dig alkaline phosphatase-conjugated antibody (1:500 dilution; Roche Diagnostics, Mannheim, Germany) and NBT/BCIP.

To compare the distribution between the *Pitx2*-positive and cholinergic neurons, the adjacent sections were subjected to immunohistochemical analysis against choline acetyltransferase (ChAT) (1:200; Merck Millipore, Billerica, MA). The distribution of *Pitx2*- and ChAT-positive cells was plotted using adjacent 3 sections in the same series. Images showing co-distribution of *Pitx2* and ChAT in single neurons were produced by overlaying images of the adjacent sections by using Photoshop (Adobe Systems, San Jose, CA).

2.2. Anterograde tract-tracing combined with immunohistochemical analyses

The animals were anesthetized using chloral hydrate (500 mg/kg) and placed in a stereotaxic apparatus (Narishige, Tokyo, Japan). Biotinylated dextran amine (BDA) (3000 MW; Molecular Probes, Eugene, OR) was used as an anterograde tracer. Unilateral injections of 10% BDA in distilled water were made iontophoretically through a glass micropipette by using a positive current pulse for 20 minutes (4 μ A, 8 s on/8 s off). The stereotaxic coordinates were determined using a mouse brain atlas by Paxinos and Franklin (2001) [18]

After a 7-day survival period, the mice were deeply anesthetized and perfused transcardially with 4% PFA in 0.1 M phosphate buffer (PB) (pH 7.4). The brains were cryoprotected in 20% glycerin in PB and frozen on dry ice. Serial frozen sections 40 μ m thick were cut coronally using a sliding microtome (HM440E; Carl Zeiss, Jena, Germany) and separated into 4 series. For anterograde axonal labeling, free-floating sections of one series were incubated with avidin-biotin-peroxidase complex (ABC) solution (3 drops per 10 ml TBS; Vector, Burlingame, CA) overnight at 4°C and developed with diaminobenzidine-nickel solution. Sections were mounted on gelatin-coated slides and counterstained with thionin for cytoarchitectonic identification.

To elucidate whether the BDA-labeled terminals in the motor nuclei were cholinergic, one series of sections were processed both for BDA and immunofluorescence staining for vesicular acetylcholine transporter (VACHT). In brief, the sections were blocked with 5% normal donkey serum in Tris-buffered saline (TBS) (pH 7.5) containing 0.5% Triton-X, and incubated in goat anti-VACHT antibody (1:1600; Merck Millipore, Billerica, MA). Then the sections were washed with TBS and incubated in Cy3-conjugated anti-goat IgG (1:400; Jackson ImmunoResearch, West Grove, PA) and Cy2-conjugated streptavidin (1:600; Jackson ImmunoResearch) for BDA detection. After washing, the sections were mounted and cover slipped with DPX. Fluorescence images were acquired with a confocal microscope (LSM 510; Carl Zeiss, Jena, Germany). We identified putative C-terminals based on cholinergic specificity (VACHT immunoreactivity), size (length ranging from 3 to 7 μ m) and proximity to the motoneurons. In a series of double immunolabeled sections, we typically observed Vm on 5 sections, VIIIm on 6 sections, Amb on 10 sections, and XIIIm on 11 sections, respectively. The number of C-terminals was counted on the all sections which include each motor nucleus using the confocal images.

3. Results

3.1. Cholinergic neurons and *Pitx2* expression

We first investigated *Pitx2* expression to confirm whether presumptive C-terminal origins in the RF have similar genetic characteristics as those in the spinal cord (Fig. 1). At the level of the pyramidal decussation, *Pitx2*⁺ neurons were present around the central canal and the IRt (Fig. 1A, 1B-left). At the caudal level of the XIIIm, *Pitx2*⁺ neurons were also located laterally in the IRt and in the MdV, and their number decreased rostrally (Fig. 1C, 1D-left). In contrast, no *Pitx2* expression was found in the RF neurons more rostrally than the middle of the XIIIm (bregma -7.56).

We next determined whether *Pitx2*⁺ neurons in the caudal medulla were cholinergic by comparing the distribution of *Pitx2*- and ChAT-positive neurons. Fig. 1A and 1C show the locations of *Pitx2*⁺ (black dots) and ChAT⁺ (green dots) neurons at 2 different rostro-caudal levels. In the adjacent sections processed for ChAT immunohistochemical analysis (Fig. 1B-, 1D-center), ChAT⁺ neurons were observed, consistent with the results of a previous study [11]. Merging the images showed that ChAT⁺ neurons overlapped the distribution of *Pitx2*⁺ neurons around the central canal and the IRt in the caudal medulla (Fig. 1B-right). A comparative observation between adjacent sections indicated few *Pitx2*⁺ neurons clearly labeled with ChAT on the other half of cells (Fig. 1E, 1F). Colocalization of *Pitx2* and ChAT were not found at more rostral levels, although neurons expressing these 2 markers were closely situated (Fig. 1D-right). In contrast with the disappearance of *Pitx2*⁺ neurons in the RF of the rostral medulla, pons, and midbrain, ChAT⁺ neurons were present in the magnocellular RF of the IRt, Gi, and LPGi (Fig. 1G–J).

3.2. Identification of the C-terminal origins projecting to the branchiomotor nuclei

In order to identify whether cholinergic neurons in the RF were origins of the C-terminals in the branchiomotor nuclei, we used anterograde tracing in combination with immunohistochemical analyses for VACHT. Cholinergic neurons in the laterodorsal and pedunculopontine tegmental nuclei were excluded from the injections, as the results of our previous study revealed that BDA injections into these nuclei label only small-size terminals in the rat motor nuclei [8].

Table 1 shows the BDA injection sites and the number of double-labeled terminals with BDA and VACHT in each motor nucleus. Eighteen injections resulted in BDA labeling of large cholinergic terminals, putative C-terminals, in the Vm, VIIIm, Amb, and XIIIm. Schematic drawings and photomicrographs of the representative injection sites are shown in Figure 2A–2F.

In 3 experimental cases (M568–570), the injections involved the regions containing *Pitx2*⁺ cholinergic neurons at the level of the pyramidal decussation. In M568, which had an injection centered on the IRt and MdV (Fig. 2F), BDA labeling was detected on both small and large terminals in the XIIIm, Amb, VIIIm, and Vm (Fig. 2G). To assess whether BDA-labeled large terminals were cholinergic, we performed a double-labeling for BDA and VACHT (Fig. 2H). BDA-labeled terminals overlapped with VACHT on the large cholinergic terminals that contacted the somata and proximal dendrites of the motoneurons, indicating anterograde labeling of C-terminals. In the motor nuclei, we identified a total of 1154 double-labeled terminals on both the ipsilateral (43.7% of the total labeled terminals) and contralateral (56.3%) sides. In the VIIIm, labeled C-terminals were distributed predominantly in the intermediate subnucleus. In the remaining 2 mice (M569 and M570), we also found nuclei with anterogradely labeled C-terminals among the XIIIm, Amb, VIIIm, and Vm.

In 13 experimental cases, the injections involved the IRt, MdV and Gi between -7.8 and -5.4 mm rostrocaudally. In these cases, double-labeling was detected on 4–60 terminals per case in 1 to 4 nuclei of the XIIIm, Amb, VIIIm, and Vm. In M467, the injection was centered on the IRt and Gi at the level rostral to the XIIIm (Fig. 2D) and 19 double-labeled terminals (I: 52.6%; C: 47.4%) were identified in the bilateral XIIIm, VIIIm, and Vm and in the contralateral Amb. Although a small number of labeled C-terminals were revealed in this case, they were all found in the XIIIm, Amb, VIIIm, and Vm. This result suggests the existence of C-terminal origins that project throughout some of the branchiomotor nuclei and the XIIIm. In M580, the injection involved the MdV with some spread into the IRt at the level of XIIIm (Fig. 2E). A total of 60 C-terminals (I: 28.3%; C: 71.7%) were labeled with a contralateral predominance in the XIIIm, Amb, and Vm. In M429, which had a BDA injection centered on the IRt and PnC in the pons (Fig. 2A), 7 double-labeled terminals were found only in the Vm (I: 14.3%; C: 85.7%). M468 also showed double-labeling of terminals, probably due to tracer spread into the IRt.

In 2 experimental cases (M583, 584), the injections involved the LPGi and VIIIm. In these cases, the terminals in the ipsilateral medial subnuclei of VIIIm were excluded from the counts, because these terminals were located within the injection sites and masked by very dense labeling. In M583 (Fig. 2B), 203 double-labeled terminals (I: 79.6%; C: 20.4%) were found in the bilateral XIIIm, Amb, and Vm and in the ipsilateral VIIIm. In another case (M584), 39 labeled C-terminals were observed in the bilateral XIIIm, Amb, VIIIm, and Vm.

In 3 cases (M426–428), BDA injections were administered into the subcoeruleus nucleus (SubC) and the caudal part of the pontine reticular nucleus (PnC), which are the rostral extensions of the IRt and Gi (Table 1), respectively. These cases showed no BDA labeling on C-terminals, whereas BDA-labeled axons and small terminals were found in the branchiomotor nuclei.

4. Discussion

Branchiomotor neurons play an important role in intricate orofacial motor behaviors, such as mastication, deglutition, suckling, and phonation. In the present study, we have demonstrated that C-terminals in the branchiomotor nuclei originate from cholinergic interneurons in the magnocellular RF of the medulla and pons in the mouse. Our findings suggest that excitability of neurons that control orofacial behaviors may be modulated by these interneurons that provide excitatory input to motoneurons via C-terminal synapses.

Our data on *Pitx2* expression show both similarities and differences between C-terminal origins in the caudal medulla and those in the spinal cord. C-terminals in the spinal cord originate from a cluster of cholinergic interneurons in Rexed laminae VII and X [16, 21, 26]. These neurons are identified as a small *Pitx2*-positive neuronal subset of the V0 population, and they are distributed close to the central canal and along the rostrocaudal axis of the spinal cord [5, 26]. Our present data revealed the presence of a *Pitx2*⁺ population in the magnocellular RF of the caudal medulla, a similar distribution to the spinal population. This result suggests that *Pitx2*⁺ cholinergic neurons in the caudal medulla may belong to the equivalent subset in the spinal cord. Our BDA injections also showed that the magnocellular RF of the caudal medulla, which contained both *Pitx2*⁺ and *Pitx2*[−] cholinergic neurons, was a source of C-terminals in the brainstem motor nuclei. Together, in the caudal medulla, both *Pitx2*⁺ and *Pitx2*[−] cholinergic neurons are likely to be C-terminal origins.

We did not find *Pitx2* expression in C-terminal origins that were located at more rostral levels of the brainstem RF. In the developing hindbrain, *Pitx2*⁺ neuronal populations in ventral rhombomere 1 differentiate into GABAergic neurons [23]. At more rostral levels in

the midbrain, *Pitx2*⁺ populations contribute to GABAergic neurons in the superior colliculus and glutamatergic neurons in the tegmentum [15, 24]. These observations suggest that a transmitter phenotype of *Pitx2*⁺ neurons may depend on the rostrocaudal and dorsoventral axes of the neural tube. Thus, in the brainstem more rostral to the XIIIm, we were not able to subclassify cholinergic interneurons on the basis of *Pitx2* expression.

The results of this anterograde tracing study show that cholinergic neurons in the magnocellular RF are origins of C-terminals in the branchiomotor nuclei and XIIIm. We found that BDA injections centered in the magnocellular RF generally labeled bilateral C-terminals in multiple orofacial motor nuclei. This labeling suggests that C-terminal origins have bilateral and simultaneous connections to motoneurons in multiple orofacial motor nuclei. In fact, case M467 (Fig. 2D), in which the tracer was taken up only by a small number of cholinergic neurons, showed C-terminal labeling in the bilateral XIIIm, VIIIm, and Vm as well as in the contralateral Amb. Our results are in agreement with previous retrograde double-labeling studies showing dual projections to Vm and VIIIm, to Vm and XIIIm, or to VIIIm and XIIIm [13, 19, 22]. In the spinal cord, cholinergic partition cells that are known as the origin of C-terminals show a strong preference for establishing synaptic connections with functionally equivalent or related motoneuron pools bilaterally [21]. Bilateral and simultaneous projection of C-terminals therefore may be involved in symmetric coactivation of the orofacial muscles. Although our results indicate that C-terminals projecting into the branchiomotor nuclei have to work in a coordinated manner, their precise nature has not been established within each motor nucleus. Further studies are needed to clarify preferential C-terminal connections with functionally segregated motoneurons in a nucleus, allowing more insight into the C-terminal function in modulating motor neuronal behavior including orofacial actions.

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Abbreviations

Vm	trigeminal motor nucleus
VSpC	caudal part of spinal trigeminal nucleus
VSpI	interpolar part of spinal trigeminal nucleus
VIIIm	facial nucleus
VIIIn	facial nerve
Xd	dorsal motor nucleus of vagus nerve
XIm	nucleus of the accessory nerve
XIIIm	hypoglossal nucleus
Amb	ambiguous nucleus
AP	area postrema
cc	central canal
Cu	cuneate nucleus
Gi	gigantocellular reticular nucleus

Gr	gracile nucleus
IO	inferior olive
IRt	intermediate reticular nucleus
LPGi	lateral paragigantocellular nucleus
LRt	lateral reticular nucleus
MdD	dorsal part of the medullary reticular nucleus
MdV	ventral part of the medullary reticular nucleus
PCRt	parvicellular reticular nucleus
PnC	caudal part of pontine reticular nucleus
Pr	prepositus nucleus
py	pyramidal tract
pyx	pyramidal decussation
RF	reticular formation
SO	superior olive
Sol	nucleus of the solitary tract
VeM	medial vestibular nucleus
VeSp	spinal vestibular nucleus

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Highlights

- C-terminals in branchiomotor nuclei originate from brainstem cholinergic neurons.
- These C-terminal origins include both Pitx2-positive and Pitx2-negative populations.
- C-terminals show simultaneous bilateral projections to branchiomotor nuclei.
- C-terminals may function in the modulation of synchronized orofacial behaviors.

Fig. 1. Distribution of *Pitx2*- and ChAT-positive neurons in the magnocellular reticular formation. (A, C) Line-drawings show the locations of *Pitx2*⁺ and ChAT⁺ neurons as black and green dots, respectively. (B, D) Images from adjacent sections following *Pitx2* in situ hybridization (left) and choline acetyltransferase (ChAT) immunohistochemistry (center) were merged (right). The positions of *Pitx2*⁺ neurons (arrows) overlapped with cholinergic neurons (arrowheads) in the intermediate reticular nucleus (IRt) and region around the central canal (cc). (E, F) On a comparison of adjacent sections, a *Pitx2*⁺ neuron also exhibited ChAT immunoreaction. (G–J) ChAT single-positive neurons were present in the magnocellular reticular formation such as the IRt (H), the gigantocellular reticular nucleus (Gi; I), and the lateral paragigantocellular nucleus (LPGi; J). The *Pitx2*⁺ neuron disappeared

in regions more rostral than the middle level of the hypoglossal nucleus (C, D). Scale bar: (A–D, G) 200 μ m, (E, F, H–J) 20 μ m.

Fig. 2.
C-terminals originated from cholinergic neurons located in the magnocellular reticular formation. (A–F) Schematic drawings (insets) and photomicrographs show representative locations of BDA injection sites in the magnocellular reticular formation. Dark green and light green areas indicate the core and halo of injection sites, respectively. (G, H) In M568 (F), BDA-labeled large terminals (arrowheads in G) were detected bilaterally in the hypoglossal (XIIIm), ambiguous (Amb), facial (VIIIm), and trigeminal motor (Vm) nuclei. Immunostaining with BDA (green) and VACHT (red) resulted in double-labeling (yellow; arrowheads in H) of the large cholinergic terminals in the motoneurons putative C-terminals. Scale bar: (A–F) 200 μm, (G, H) 20 μm.

Table 1

Injection sites and number of double-labeled 1 terminals, putative C-terminals, in each motor nucleus

Mouse#	Injection site		Vm		VIIIm		Amb		XIIm	
	Core	Halo ¹	Ipsi ²	Cont ³	Ipsi	Cont	Ipsi	Cont	Ipsi	Cont
427	SubC, PhC	-	-	-	-	-	-	-	-	-
426	SubC	PhC	-	-	-	-	-	-	-	-
428	PhC	IRt	-	-	-	-	-	-	-	-
429	IRt, PhC	-	1	6	-	-	-	-	-	7
583	LPGi, VIIIm	IRt, Gi	60	18	93	-	18	1	12	1
584	LPGi, VIIIm	IRt, Gi	5	4	9	1	1	2	9	8
416	IRt	Gi, PCRt, Sol, VeM	3	-	4	-	-	-	2	-
469	IRt	Gi, PCRt, Sol, VeM	5	1	15	1	-	-	6	-
468	Sol	IRt, PCRt, VSpO	-	-	4	-	-	1	-	-
418	IRt, VeM	Gi, PCRt, Sol, VeSp	3	1	1	1	-	-	8	1
467	IRt, Gi	PCRt	5	1	4	1	-	4	1	3
466	IRt	Gi, PCRt, Sol, VeM	-	5	-	-	-	-	4	5
495	IRt, Gi	PCRt, VeM	-	2	6	-	-	-	-	-
496	IRt	Gi, PCRt, Sol, VSpI	2	2	1	1	-	-	-	1
497	IRt, Gi	PCRt, VeM	2	-	-	-	-	-	2	-
580	MdV	IRt	-	22	-	-	2	3	15	18
581	MdV	IRt	10	-	-	-	-	-	-	-
579	MdV, IO	IRt	12	-	-	12	1	-	5	12
570	IRt, MdV	MdD, Cu	12	51	192	190	-	-	159	43
568	IRt, MdV	MdD, Sol, Cu, Xd, XIIm	227	263	107	212	5	1	165	174
569	IRt, MdV, MdD	Cu	69	24	1	27	1	-	4	7

For anterograde tracing, we injected BDA into the brainstem reticular formation. Eighteen injections resulted in BDA-labeled C-terminals in the trigeminal motor (Vm), facial (VIIIm), ambiguous (Amb), and hypoglossal (XIIm) nuclei.

¹ Halo: adjacent areas where the tracer might be taken up and cause anterograde labeling

² Ipsi: ipsilateral side of injection

³ Cont: contralateral side.