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Expression of Glial Cell-Derived Neurotrophic Factor Receptors Within Nucleus Ambiguus During Rat Development

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Objective: The nucleus ambiguus (NAmb) is a column of neurons in the medulla oblongata, involved in bulbar functions. Expression of Glial Cell-Derived Neurotrophic Factor (GDNF) and its receptors (GDNFR) is observed within the cell bodies during reinnervation following recurrent laryngeal nerve (RLN) injury. Little is known regarding GDNFR expression in the formation of the NAmb and the laryngeal innervation during embryogenesis. Understanding the timing and pattern of GDNFR expression in embryogenesis versus after RLN injury may provide insights into therapeutic targets for regeneration after RLN injury.

Study Design: Laboratory experiment.

Methods: Rat brainstems at E14.5/E16.5/E18.5/E20.5/adult were stained for GDNFR: GFRα-1/GFRα-2/GFRα-3/Ret. Islet1 and choline acetyltransferase were used as cell body markers. Sections were observed using fluorescent microscopy and quantified through manual cell counting.

Results: Expression of GFRα-1, GFRα-3, and Ret was identified within the NAmb, hypoglossal, and facial nuclei of the adult medulla. During development, GFRα-1 immunoreactivity was seen at E20.5. GFRα-2 expression was not observed at any timepoint. GFRα-3 expression began at E16.5. Ret expression within nerve fibers in the NAmb were observed beginning at E14.5, but never in the cell bodies.

Conclusion: Embryonic GDNFR expression in the NAmb differs from that of the adult after RLN injury. The developing brainstem experienced upregulation at discrete timepoints with signaling sustained through adulthood. In contrast, adult RLN-transected rats experienced patterns of up and down regulation. GFRα-1 may contribute to muscle targeting and neuromuscular junction maturation, GFRα-3 may contribute to both, as well as axon guidance. It is likely that GDNF is functioning via a Ret-independent pathway.

Key Words: GDNF, GFRalpha, larynx, nucleus ambiguus, recurrent laryngeal nerve, Ret, vocal fold paralysis.
Level of Evidence: NA

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INTRODUCTION

Transection of the recurrent laryngeal nerve (RLN) is a risk of surgeries involving neck or upper thorax pathology, such as thyroidectomies or cervical spine surgeries.[1–3] RLN injury causes ipsilateral vocal fold paralysis, resulting in dysphagia, respiratory distress, and dysphonia.[1,4] Nerve injury activates Wallerian degeneration of the distal segment of the RLN and regenerated axonal outgrowth from the proximal end of the RLN toward the intrinsic laryngeal muscles (ILM) of the larynx. However, motor reinnervation of the larynx is non-selective.[5] Consequently, synkinetic vocal fold movement persists due to aberrant reinnervation of the larynx, and normal motion is never restored.[4] Patients live with permanent dysphonia, among other clinical sequelae of laryngeal synkinesis.[1,3,4]

Synkinetic reinnervation of the larynx is guided by the expression of neurotrophic factors triggered by denervation of the ILM.[6–9] Neurotrophic factors are proteins whose expression promotes the differentiation, maturation, survival, and maintenance of motoneurons and neuromuscular junction (NMJ) during development and postnatal life.[6–9] Neurotrophic factors may also be expressed in denervated muscles, serving as long-distance cues for regenerating axons, as has been observed following RLN injury and during ILM reinnervation.[10]

Glial Cell-Derived Neurotrophic Factor (GDNF) is a neurotrophic factor originally isolated from rat glial cell lines.[5,11–13] It is a member of the transforming growth factor-β super family, which activates intracellular signals for axonal outgrowth and neuronal survival via the receptor tyrosine kinase, Ret, derived from the *Rearranged during Transfection* gene. Ret is activated via ligand binding to glycosylphosphatidylinositol-linked co-receptors of the GDNF Family Receptor Alpha family.[5,6,8,9,11,12,14] GDNF and its receptors, Ret and GFRα-1/2/3 are expressed within brainstem neurons

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and exhibit dynamic expression during development, in adulthood and post RLN injury in the rat and mouse brainstem.[9] In the periphery, GDNF and its receptors play a role in NMJ formation as differential activation of these receptors induces intracellular signaling for axonal guidance and innervation of muscle targets.[11,12,14,15] The nucleus ambiguus (NAmb) is one such location where GDNF may play a role in axonal guidance and survival.[9,16]

NAmb is a long rostrocaudally oriented column of motoneurons located in the ventral aspect of the lower medulla oblongata that innervates structures within the head, neck, thoracic, and abdominal cavity.[6,9,11,12,17,18] The NAmb is divided into three somatotopically distinct segments, with ILM-innervating motoneurons located in the caudal one-third of the column.[18–22] Development of NAmb begins during medulla oblongata formation on Day 12 of rat development.[23] GFRα-1/2/3 and Ret have shown immunoreactivity in cranial nuclei in the medulla oblongata, suggesting that the GDNF receptors may play a role during development.[9]

Although expression of GDNF and its receptors have been studied in murine and human brainstem development, differential expression of GDNF receptors at key timepoints during ILM innervation is a novel area of research.[9] This study aims to describe the developmental expression of GDNF receptors within the NAmb during rat development and functional laryngeal innervation, and to correlate this with the known timing of GDNF receptor expression within the NAmb in adult rats after RLN injury and non-functional reinnervation. Discrepancies between the two may suggest further avenues of research in search of a therapeutic treatment for RLN injury.

MATERIALS AND METHODS

Animals

This study was conducted in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act (7 U.S.C. et seq.). The Institutional Animal Care and Use Committee of Columbia University Medical Center approved the animal use protocol. Rat brainstems were analyzed at four different development stages as well as adulthood. Embryos were obtained at E14.5/E16.5/E18.5/E20.5 days (4 animals per group, Table I). Adults and pregnant rats were sacrificed via intraperitoneal injection of xylazine (20 mg/ml) and ketamine hydrochloride (100 mg/ml). Euthanasia was confirmed via thoracotomy.

Tissue Extraction

Following euthanasia, adult animals underwent transcardiac perfusion with 0.1 M phosphate buffer saline (PBS) (200 ml) followed by 4% paraformaldehyde in PBS (250 ml). To access the brainstem, the skull was removed, and the rat brainstem was dissected caudally at the spinomedullary junction and rostrally at the junction of the midbrain and diencephalon. Brainstems were post-fixed with 4% paraformaldehyde in PBS at 4°C overnight. The brainstems were then immersed in 15% sucrose in PBS until the tissue sank. The pieces were transferred to 30% sucrose in PBS until sunk prior to being submerged in a cryostat embedding medium in a mold and frozen at −8°C.

In gestational stages, embryos were extracted from euthanized pregnant rats at different developmental periods as mentioned above. Embryos were placed in vials of 4% paraformaldehyde in PBS at 4°C overnight. Embryos were submerged in sucrose at 15% and 30% prior to processing. Embryos were dissected at the base of the neck. The head was then positioned at a 50°–60° angle in relation to the horizontal axis of the mold, covered with a cryostat embedding medium, and frozen at –8°C.

TABLE I.
Number of Animals Used in This Study.

Experiments	Groups					Post-fixation	Section thickness	Immunohistochemistry incubation	
	E14	E16	E18	E20	Adult			Primary antibody	Secondary antibody
Islet1/GFRα1/DAPI	4	4	4	4	4	48 h	14 μm	Mouse anti-Islet 1 [39.4D5; DSHB, Iowa City, IA] (1:50) Goat anti-GFRα-1 R&D Systems, Minneapolis, MN (1:100)	DaM-AF488 [Lot#: 23099139. Invitrogen, Waltham, MA] (1:200) DaG-AF594 [Lot#: 2309139. Invitrogen] (1:200)
Islet1/GFRα2/DAPI	4	4	4	4	4	48 h	14 μm	Mouse anti-Islet 1 [39.4D5; DSHB] (1:50) Goat anti-GFRα-2 [R&D Systems] (1:100)	Ab2: DaM-AF488 [Invitrogen] (1:200) DaG-AF594 [Invitrogen] (1:200)
Islet1/GFRα3/DAPI	4	4	4	4	4	48 h	14 μm	Mouse anti-Islet 1 [39.4D5; DSHB] (1:50) Goat anti-GFRα-3 [R&D Systems] (1:100)	Ab2: DaM-AF488 [Invitrogen] (1:200) DaG-AF594 [Invitrogen] (1:200)
Islet1/Ret/DAPI	4	4	4	4	4	48 h	14 μm	Mouse anti-Islet 1 [39.4D5; DSHB] (1:50) Goat anti-Ret [R&D Systems] (1:100)	Ab2: DaM-AF488 [Invitrogen] (1:200) DaG-AF594 [Invitrogen] (1:200)
ChAT/DAPI	—	—	—	—	4	72 h	40 μm	Ab1: Goat anti-ChAT [Chemicon] (1:100)	DaG-AF594 (1:200) [Invitrogen]

Islet-1 = RRID:AB_2314683; GFRα-1 = (R and D Systems Cat# AF560, RRID:AB_2110307); GFRα-2 = (R and D Systems Cat# AF429, RRID:AB_2294621); GFRα-3 = (R and D Systems Cat# AF2645, RRID:AB_2110295); Ret = (R and D Systems Cat# AF482, RRID:AB_2301030).

ChAT = anti-acetylcholine transferase; DaM-AF488 = Donkey anti-Mouse conjugated to Alexa Fluor 488; DaG-AF594 = Donkey anti-Goat conjugated to Alexa Fluor 594; DaG-AF594 = Donkey anti-Goat conjugated to Alexa Fluor 594; DAPI = 4′,6-diamidino-2-phenylindole; GFRα = GDNF Family Receptor Alpha; Ret = rearranged during Transfection gene receptor.

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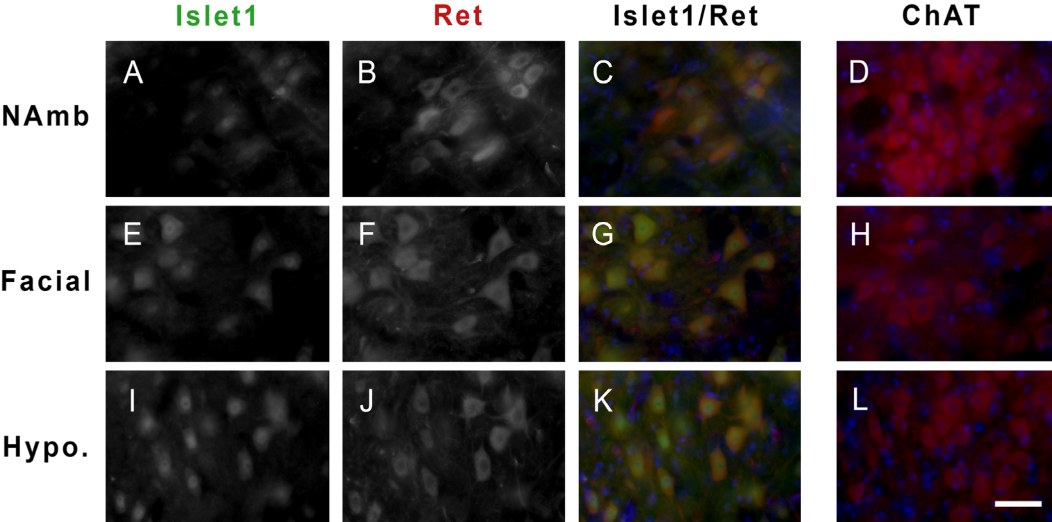

Fig. 1. Motoneurons identified with choline acetyltransferase (ChAT) express Glial Cell-Derived Neurotrophic Factor Receptors within cranial nerve nuclei of the Adult medulla oblongata (A–D), Facial nucleus (E–H), and Hypoglossal nucleus (I–L). ChAT labeling of the adult NAmb (A), Facial nucleus (E), and Hypoglossal nucleus (I). Labeling with Ret within the adult NAmb (B), Facial nucleus (F), and Hypoglossal nucleus (J). Merged image of ChAT (green) and Ret (red) in the adult NAmb (C), Facial nucleus (G), and Hypoglossal nucleus (K). Merged imaged of ChAT (Acetylcholine Transferase) in red within the adult NAmb (D), Facial nucleus (H), and Hypoglossal nucleus (L). DAPI expression observed as blue nuclei in merged images (C, D, G, H, K, L). Scale bar = 50 μm.

Sectioning

Molds were placed in a −20°C cryostat for 30 min. Adult brainstems were sectioned in coronal sections at 40 μm caudal to rostral and placed serially in 24 multiwell plates containing PBS. Embryos were sectioned in coronal sections from caudal level at the area of the larynx and cervical spinal cord to cranial level at the cerebellar pontine angle in the rostral hindbrain. All embryos were sectioned at 14 μm. Coronal sections of the embryos were placed onto gelatin slides for immunostaining.

Immunohistochemistry

Two separate immunohistochemistry protocols were utilized for this study. The immunohistochemistry protocol was carried out in floating sections for adult brainstems and sections on gelatin slides for the embryos. Brainstems were stained for motoneuron marker Islet-1, as well as choline acetyltransferase (ChAT) in adult rats, and for GDNF receptors (Table I).

Adult brainstem sections were kept in 24 multiwell plates. They were divided into six columns. Each column was blocked for 30 min with 1% donkey serum in 0.3% triton in PBS (PBST) and then incubated in 1% donkey serum in PBST with anti-ChAT or Islet-1 antibodies plus another primary antibody for GDNF receptors as summarized in Table I. The sections were incubated on a BioRocker 2D shaker in primary antibody solutions for 72 h at 4°C. Sections were washed twice with PBS for 5 min prior to incubation in the secondary antibody solutions for 2 h, as stated in Table I. Floating sections were captured onto gelatin slides in serial order from caudal to rostral brainstem and mounted with 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) (Table I).

For the embryological groups, sections were captured onto gelatin slides. ImmEdge™ Pen (Burlingame, CA) was used for blocking the staining area, and slides were placed in a slide box to dry. Sections were postfixed in 4% paraformaldehyde in PBS for 10 min at room temperature, followed by a wash with PBS for 5 min. Antigen retrieval was performed via incubation in sodium citrate buffer solution 0.1 M (pH = 6) for 45 min at 60°C. After a PBS wash, sections were blocked as stated above, before incubation with the primary antibody solution consisting of Islet 1 plus another primary marker as stated in Table I. Slides were incubated in the 4°C refrigerator for 48–72 h. After two 5 min PBS washes, sections were incubated in secondary antibody

TABLE II.
Data Summary—Qualitative.

	GFRα1 neurons	GFRα1 nerve fibers	GFRα2 neurons	GFRα2 nerve fibers	GFRα3 neurons	GFRα3 nerve fibers	Ret neurons	Ret nerve fibers
E14.5 ($N = 4$)	−	+	−	−	−	−	−	+
E16.5 ($N = 4$)	−	+	−	−	+	−	−	+
E18.5 ($N = 4$)	−	+	−	+	+	−	−	+
E20.5 ($N = 4$)	+	+	−	+	+	−	−	+
Adult ($N = 4$)	+	+	−	+	+	−	+	+

Summary of Glial Cell-Derived Neurotrophic Factor Receptor expression within Nucleus Ambiguus of the adult brainstem and E14, E16, E18, and E20 embryos. (−) negative signal; (+) positive signal.

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solution for 2 h at room temperature, as summarized in Table I Slides were mounted in Flouroshield mounting medium with DAPI.

Analysis

Slides were observed and imaged at 20× (NA = 0.8) and 40× (NA = 1.4) using Zeiss LSM Confocal and Zeiss Axio Imager M2 epi-fluorescences microscopes (Zeiss, Oberkochen, Germany). Images taken were then analyzed using Fiji software to evaluate the timing and level of expression of receptors in the motoneurons of the nucleus ambiguus and other nuclei of the brainstem. Motoneurons were defined as immunoreactive if green Islet-1 immunoreactivity was observed within a cell with blue DAPI labeled puncta. Up to 25 consecutive sections per animal (within 2–4 slides) were quantified through manual counting under the microscope. In total, 36 animals were analyzed. Descriptive statistics such as mean and standard deviation were presented as percentages; total number of identified motor neurons per GDNF receptor in the NAmb/total number of Islet-1 or ChAT immunoreactive cells identified in the NAmb.

RESULTS

Ret exhibited similar expression throughout the entirety of the medulla oblongata at all embryonic timepoints (Fig. 1). Motoneuron pools within the brainstem were identified with Islet-1 (Fig. 1A,E,I) or ChAT (Fig. 1D,H,L). Ret had similar expression in all three nuclei (Fig. 1B,F,J); however, Islet-1 immunoreactivity differed among them (Fig. 1A,E,I). Although cells exhibited mild Islet-1 immunoreactivity in the NAmb (Fig. 1A), Ret expression was much stronger (Fig. 1B). In contrast, immunoreactivity of Ret and Islet-1 in the facial and hypoglossal nuclei is relatively equal (Fig. 1C,E,F,G, I,J,K).

GFRα-1 was identified in motoneurons of the NAmb at E20.5 and adulthood, though nerve fiber immunoreactivity was noted at earlier time points (Table II). From E14.5 to E18.5 GFRα-1 immunoreactivity in the NAmb was diffuse with no apparent selectivity for Islet-1+ motoneurons (Fig. 2A–I). At E20.5, GFRα-1 expression was identified in a membranous pattern around Islet-1+ nuclei (Fig. 2J–L). Expression of GFRα-1 was quantified within the NAmb and found in 44.18% ± 8.57% of cells were GFRα-1 positive (Table III). In contrast, GFRα-1 expression was observed in the hypoglossal nucleus as early as E16.5 (Fig. 3A). GFRα-1 immunoreactivity was observed in the nerve fibers running along a ventromedial course that was consistent in pattern and direction as observed in the E16.5 brainstem (Fig. 3A). Immunoreactive nerve fibers were also noted in the cuneatus, fragilis, and spinal trigeminal tracts (Fig. 3A). In addition, an immunoreactive nodose ganglion was identified in the ventrolateral aspect of the CNS (Fig. 3A). In adults, GFRα-1 immunoreactivity was maintained in NAmb motoneurons with expression in 58.05% ± 43.1% of motoneurons (Fig. 2M–O, Table III).

GFRα-2 expression was not observed within the NAmb at any stage of development nor during adulthood (Figs. 2AA–OO and 3B, Table II). In a global view, at E16.5, the hypoglossal nuclei and NAmb were identifiable (Fig. 3B), yet GFRα-2 was not expressed in these neuronal populations. However, expression of GFRα-2 was present within other anatomic regions of the medulla

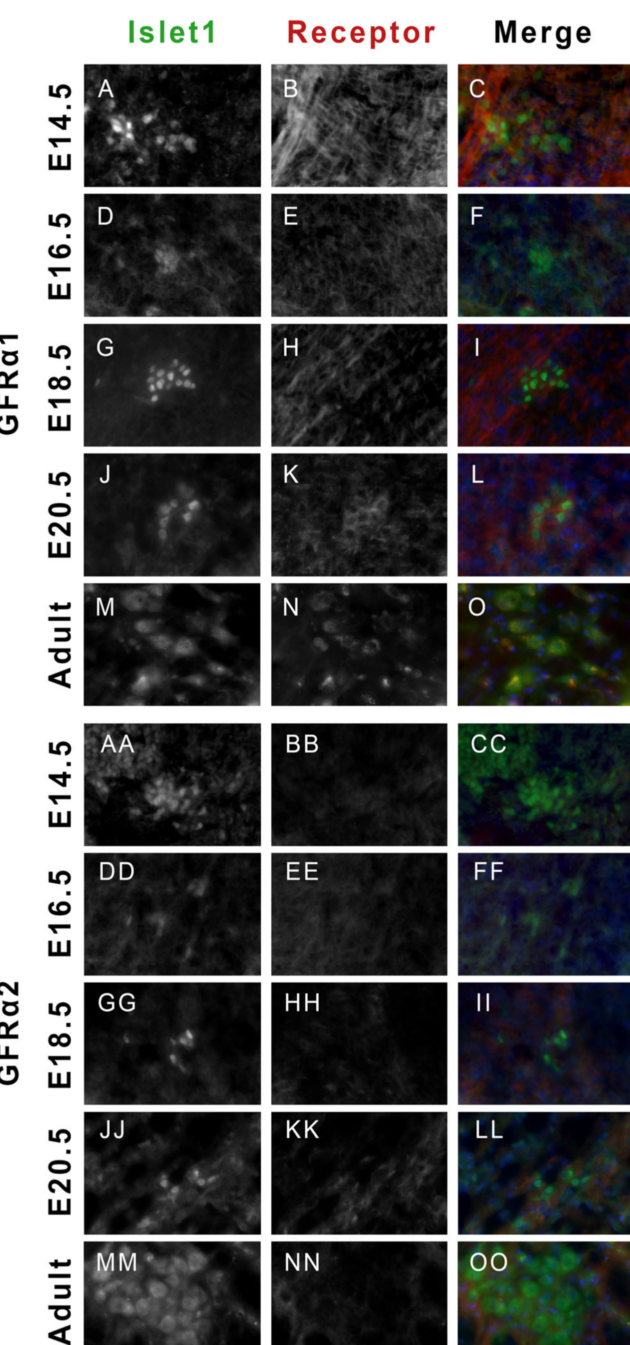

Fig. 2. GFRα-1 immunoreactivity was identified in the Namb at E18 whereas GFRα-2 immunoreactivity was not observed during development. Labeling of motoneurons in adult and E14-E20 embryos with Islet1 (A/AA, D/DD, G/GG, J/JJ, M/MM). Labeling of nucleus ambiguus motoneurons and/or nerve fibers with GFRα1 (B, E, H, K, N). Labeling of nucleus ambiguus motoneurons and/or nerve fibers with GFRα2 (BB, EE, HH, KK, NN). Merged image of Islet1 and GFRα1 (C, F, I, L, O). Merged image of Islet1 and GFRα2 (CC, FF, II, LL, OO). DAPI expression observed as blue nuclei in merged images (C/CC, F/FF, I/II, L/LL). Scale bar = 50 μm.

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	E16.5 ($N = 4$)		E18.5 ($N = 4$)		E20.5 ($N = 4$)		Adult ($N = 4$)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
GFRα1	0	0	0	0	44.18%	±8.57%	58.05%	±43.1%
GFRα2	0	0	0	0	0	0	0	0
GFRα3	63.8%	±5.29%	70.05%	±17.83%	68.4%	±6.8%	84%	±4.29%
Ret	0	0	0	0	0	0	89.63%	±2.21%

TABLE III.
Cell Counts.

Quantification of nucleus ambiguus motoneurons within the adult brainstem.
SD = standard deviation.

(Fig. 3B). Notably, receptor expression is observed in the cuneatus, gracilis, spinal trigeminal tract, and nodose ganglion (Fig. 3B). This pattern of GFRα-2 expression is maintained in the adult brainstem (Fig. 4).

GFRα-3 exhibited a nuclear staining pattern in motoneurons which was observed from E16.5 into adulthood (Fig. 5A–O, Table II). Diffuse immunoreactivity was observed throughout the medulla oblongata, and was present in both motoneurons and interneurons at E16.5 (Fig. 3C). In NAmb, GFRα-3 immunoreactivity was observed in 63.8% ± 5.29% of all cells Islet-1 positive (Table III). At E18.5, GFRα-3 immunoreactivity was observed in 70.05% ± 17.38% of Islet-I immunoreactive cells in the NAmb, which is similar to the quantification at E20.5, 68.4% ± 6.8% (Table III). In adults, the proportion of motoneurons in NAmb that exhibited GFRα-3 immunoreactivity increased to 84% ± 4.29% (Table III).

The Ret receptor was expressed solely within nerve fibers of developing brainstems (Fig. 5AA–LL). Expression of Ret within cell bodies of the NAmb was only

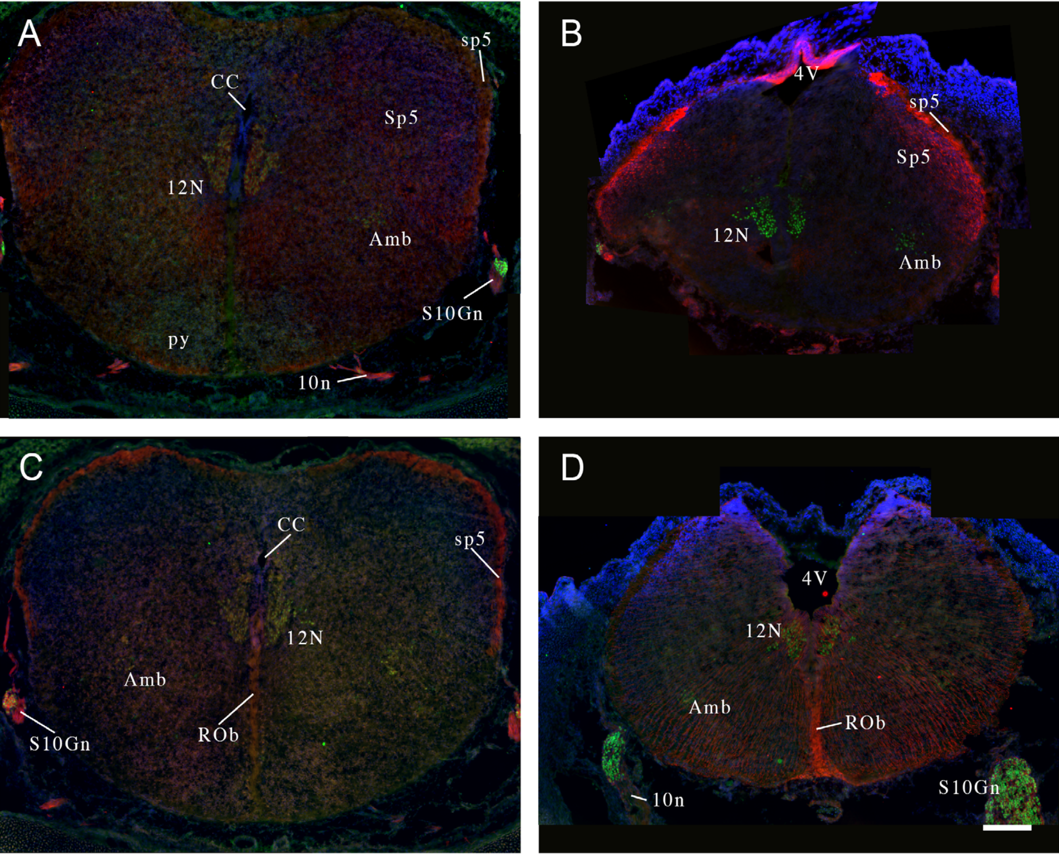

Fig. 3. Global images of the E16 Rat medulla oblongata display somatotopic variation in Glial Cell-Derived Neurotrophic Factor (GDNF) Receptor Expression. Labeling of motoneurons of the nucleus ambiguus and hypoglossal nucleus in green and GDNF receptors in red (A–D). Labeling of nuclei of the nucleus ambiguus, hypoglossal, and interneurons with GFRα-1 in red (A); GFRα-2 in red (B); GFRα-3 in red (C); Ret in red (D). 4V = Fourth Ventricle; 10N = Vagus nerve; 12N = Hypoglossal Nucleus; Amb = Nucleus Ambiguus; CC = Central Canal; py: Pyramids; Rob = Raphe Obsurus Nucleus; Sp5 = Spinal Trigeminal Nucleus; S10Gn: Superior Vagal Jugular Ganglion. Scale bar = 200 μm.

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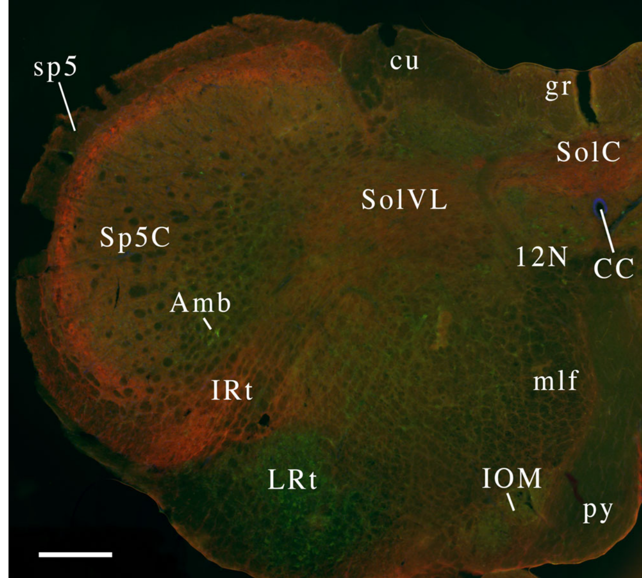

Fig. 4. Hemi section of adult medulla oblongata with motoneuron labeling with Islet1 in green and labeling of nerve fibers with GFRα-2 in red. Amb = Nucleus Ambiguus; CC = Central Canal; cu = Cuneate Fasciculus; gr = gracilis fasciculus; IOM = Inferior Olive, Medial Nucleus; IRt = Intermediate Reticular Nucleus; LRt = Lateral Reticular Nucleus; mlf = Medial Longitudinal Fasciculus; py = Pyramids; SolVL = Nucleus of the solitary tract, ventrolateral part; Sp5 = Spinal Trigeminal Nucleus; Sp5C = Spinal Trigeminal Nucleus, caudal part. Scale bar = 200 μm.

observed in the adult medulla oblongata (Fig. 5MM–OO). Ret expression was noted in 89.63% ± 2.21% of adult NAmb motoneurons in the rat (Table III). In contrast, Ret was clearly present in the nerve fibers of the brainstem from E14.5 (Figs. 3D and 5BB,EE,HH,KK).

DISCUSSION

Results of this study showed GDNF receptor production during development within the hindbrain. GDNF is a trophic factor whose expression was originally found to promote the survival of ventral midbrain dopaminergic neurons, such as the degenerating cell populations involved in Parkinson's disease.[6] We observed immunoreactivity of GFRα-1, GFRα-3, and Ret within the developing rat brainstems. Similarly, our previous work demonstrated the adult rat NAmb upregulates GFRα-1, GFRα-3, and Ret from 7 to 14 days post RLN injury (DPI), with a decline in GFRα-1/3 expression and sustained Ret expression at 21 DPI (Table S1 and S2).[11] GDNF mRNA is upregulated in the lateral thyroarytenoid, medial thyroarytenoid, and posterior cricoarytenoid muscles at 1 DPI, followed by steep down-regulation at 3 DPI, subsequent upregulation at 7 DPI with a return to basal expression at 14 DPI, and a down-regulation at 21 DPI (Table S1 and S2).[5] This pattern of post RLN injury up/down-regulation within the ILM and NAmb coincided with stages of reinnervation, axonal growth, guidance and NMJ maturation.[5,11] Given that

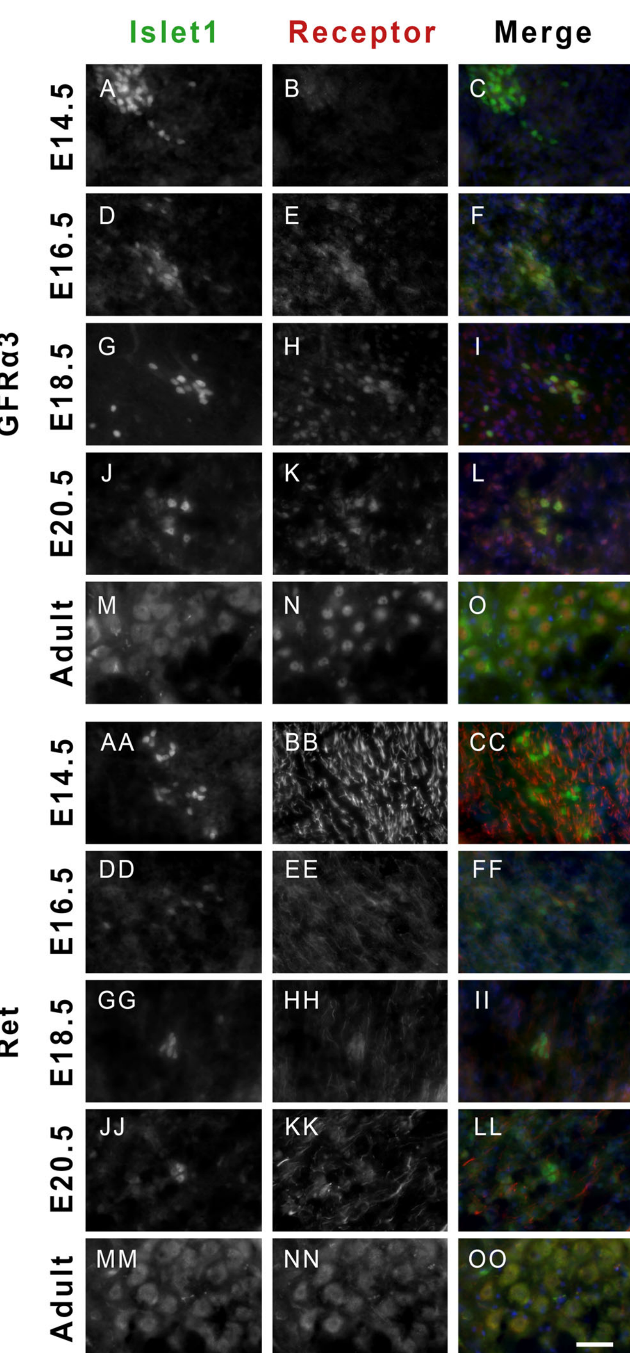

Fig. 5. GFRα-3 immunoreactivity was identified in the Namb at E16 whereas Ret immunoreactivity was observed only in nerve fibers during development. Labeling of nucleus ambiguus motoneurons in adult and E14-E20 embryos with Islet1 (A/AA, D/DD, G/GG, J/JJ, M/MM). Labeling of nucleus ambiguus motoneurons and/or nerve fibers with GFRα3 (B, E, H, K, N). Labeling of nucleus ambiguus motoneurons and/or nerve fibers with Ret (BB, EE, HH, KK, NN). Merged image of Islet1 and GFRα3 (C, F, I, L, O). Merged image of Islet1 and Ret (CC, FF, II, LL, OO). DAPI expression observed as blue nuclei in merged images (C/CC, F/FF, I/II, L/LL). Scale bar = 50 μm.

the expression of GDNF and its receptors is temporally correlated with RLN aberrant reinnervation in the adult rat, this study aimed to evaluate their expression in the

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embryo where primary laryngeal innervation is selective and results in normal vocal fold motion. Identifying similarities or differences in GDNF receptor expression in embryogenesis compared to RLN reinnervation in the adult rat may be of importance when considering further investigations aimed toward developing therapeutic treatments for RLN injury.

In the developing brainstem, Ret displays global expression at all timepoints with no local NAmb expression. Thus, it is possible that the Ret expression we are appreciating in the nerve fibers of the embryos is not related to the NAmb at all and is instead contributing to other processes entirely. Conversely, in the adult brainstem, we have seen global Ret expression in the adult at 7 DPI, with local NAmb upregulation at 14 DPI (Table S3). This may suggest that Ret plays a role in cell maintenance, survival, and other GDNF-related processes, contrasting with its function in embryologic development. In development, it is possible GDNF is functioning through Ret-independent pathways.[24]

GFRα-1 and GFRα-3 are positioned to be involved in RLN axonal guidance, muscle targeting, and NMJ innervation. Ventral and dorsal projections of the RLN branch toward the ILM around E16.5/E17.5 with selection of their muscle targets occurring at E19.5 and complete NMJ maturation of synapses at E21.5.[25,26] GFRα-1 immunoreactivity is seen at E20.5 and begins one or possibly 2 days before NMJ maturation is completed (expression at E19.5 was not tested). Being that it is expressed late, it is unlikely to be involved in axon guidance but in NMJ formation. GFRα-3 expression was noted to begin in the E16.5 rat brainstem and be maintained through embryogenesis. Considering RLN branching toward the ILM occurs at E16.5/E17.5, this suggests that GFRα-3 expression is timed to play a role in axonal guidance as well as having implications for NMJ formation. Similar to our previous study, we observed a lack of GFRα-2 in the NAmb at all timepoints, though it was present in other nuclei throughout development.[11]

Limitations of this study include that analysis and counting of receptor immunoreactive cells was constrained by technical capabilities. Namely, complete capture of the NAmb from its caudal to rostral segment would allow for more precise quantification of all NAmb cells instead of reporting a percentage. Precise angling during cryosectioning, although technically challenging, could minimize error in correct identification of nuclei that intersect (e.g., NAmb and facial nucleus at the pontomedullary junction). Confocal imaging instead of microscopic analysis could facilitate the identification of immunoreactive motor neurons.

Future direction studies should aim to quantify levels of GDNF receptor expression within each somatotopically distinct, abductor versus adductor, region of the NAmb within a broader range of developmental timepoints, starting at NAmb formation at E12.5. In addition, comparisons of GDNF receptor knockout rats may shed light into their individual roles. Especially since, in development, there are known Ret-independent GDNF pathways.[24] Although this study provides a thorough qualitative analysis of GDNF receptor expression, further quantification with intensity analysis and in-situ hybridization may reveal dynamic changes in expression levels of receptors across timepoints.

CONCLUSION

This study investigated the temporal relationship of GDNF receptor expression within the NAmb of the developing rat brainstem compared to that of an adult rat post RLN injury. GDNF receptor expression differed from that of the adult laryngeal muscles and adult NAmb. Adult RLN transected rats experienced patterns of up and down regulation throughout nerve regeneration. In contrast, the developing brainstem experiences upregulation of GFRα-1 and GFRα-3 at discrete timepoints with signaling sustained through adulthood. GFRα-1 expression is seen at E20.5 and may be present at E19.5. This is after initial RLN laryngeal innervation (E16.5/E17.5) and just prior to the completion of muscle targeting at E19.5 and NMJ maturation at E21.5, suggesting a GFRα-1 contribution to these activities is possible. GFRα-2 expression was not observed within the NAmb during development nor adulthood. GFRα-3 expression begins at E16.5 and thus may play a role in axon guidance, muscle targeting, and NMJ maturation. It is likely that GDNF is functioning at these embryologic timepoints via a Ret-independent pathway.

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