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Expression of Trophic Factors Receptors During Reinnervation After Recurrent Laryngeal Nerve Injury

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Objective: An injury of the recurrent laryngeal nerve (RLN) triggers axonal regeneration but results in a poor functional recovery. Netrin-1 and glial cell-derived neurotrophic factor (GDNF) expression are up-regulated in laryngeal muscles during RLN regeneration, but the role of their receptors produced in the nucleus ambiguus is unknown. The aim of this work was to determine the timing of the production of Netrin-1 and GDNF receptors during RLN regeneration and correlate this with the previously identified timing of up-regulation of their trophic factors in the laryngeal muscles.

Study Design: Laboratory experiment with rat model.

Methods: The right RLN was transected and dextran amine tracer applied. At 7, 14, and 21 days postinjury (DPI), brainstems were removed and harvested. Immunostaining was performed for Netrin-1 (deleted in colorectal carcinoma [DCC], UNC5A) and GDNF receptors (rearranged during transfection [Ret], glycosylphosphatidylinositol-linked cell surface receptors [GFRα1, GFRα2, GFRα3]). The timing and type of receptor production relative to injury as well as their position in the nucleus ambiguus was analyzed.

Results: Netrin-1 UNC5A receptors were minimal in the nucleus ambiguus during RLN regeneration. DCC, the receptor that plays an attract role, was immunopositive from 7 to 21 DPI. All GDNF receptors, except GFRα2, were clearly positive from 7 to 14 DPI. No differences of production were observed according to the position of the motor neurons in the nucleus ambiguus.

Conclusion: An injury of the RLN leads to a higher production of Netrin-1 DCC and GDNF receptors in the nucleus ambiguus. The timing of receptor production is similar to up-regulation of their trophic factors in the laryngeal muscles.

Key Words: Netrin-1, recurrent laryngeal nerve, GDNF, reinnervation, trophic factors receptors.

Level of Evidence: NA.

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INTRODUCTION

Acute injury to the recurrent laryngeal nerve (RLN) may lead to vocal fold paralysis that results in dramatic loss of the voice and inability to protect the airway. In the long term, vocal fold paralysis affects the quality of life.[1,2] From a biological perspective, an injury of a peripheral nerve triggers a molecular response that leads regeneration of the motor axons toward the denervated muscles. Although there is considerable potential of the proximal stump nerve fibers of the RLN to reach the larynx, vocal fold motion is never fully restored because reinnervation of the adductor and abductor laryngeal muscles is nonselective.[1–3]

Following the description of nerve growth factor,[4] several trophic factors have been shown to participate in axonal guidance during development and following peripheral nerve injury.[5–11] Previous studies establish that an acute injury of the RLN triggers overexpression of several trophic factors, such as glial cell-derived neurotrophic factor (GDNF) and Netrin-1, as a response to denervation of the ipsilateral intrinsic laryngeal musculature.[5,6,12] This upregulation of trophic factor expression is observed to vary within the muscles according to the days postinjury (DPI). They guide newly formed motor axons sprouting from proximal stump of the injured RLN toward the denervated larynx.[5,6] In the rat model following RLN injury, GDNF and Netrin-1 are upregulated first in the posterior cricoarytenoid muscles (PCA) around 7 DPI and then in the thyroarytenoid muscle (TA) at 14 to 21 DPI. This observed pattern of expression fails to guide the new axons back to their original target.[3,5,6,13–16] Attempts to alter the concentration of GDNF and Netrin-1 proteins available within laryngeal muscles resulted in differences in the reinnervation timing when compared to controls but still produced nonselective reinnervation of the larynx. An injection of exogenous GDNF into the PCA led axons to reinnervate the lateral thyroarytenoid muscle first at 7 DPI. Similarly, an injection of an anti-Netrin-1 antibody into the PCA also led to reinnervation of TA before the PCA. However, nonselective reinnervation was still observed.[17,18] To better understand the

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critical role that trophic factors play during RLN regeneration, one also needs to investigate the trophic factors receptors, which are produced in the nucleus ambiguus following RLN injury. Expressions of both trophic factors and receptors are synchronized in specific patterns during nerve regeneration.[5,17,19–21]

GDNF is a member of the transforming growth factor-β superfamily, which plays several roles during development.[22–26] The expression of GDNF enhances neural survival in the early stages of brain formation.[27,28] During development, GNDF expression within muscles is essential for the formation of neuromuscular junction formation and maintenance. An upregulation of the GNDF expression triggers polyinnervation of motor end plates before birth. Supernumerary axons are then removed, and the neuromuscular junction matures when GNDF expression decreases.[24–26] In the rat model of RLN injury, GDNF overexpression reaches its peak within PCA and TA as newly formed axons reach denervated muscles.[5,17] GDNF has three glycosylphosphatidylinositol-linked cell surface receptors (GFRα1, 2, 3) and a required tyrosine kinase, called *rearranged during transfection* (Ret), which triggers intracellular signaling pathways for neural survival and axonal outgrowth.[20,29,30] They play a critical role during development. GFRα1, GFRα2, GFRα3, and Ret are involved in sensory and motor innervation.[19,29,31] However, very little is known regarding the expression of these receptors in motoneurons in the central nervous system (CNS) following peripheral nerve injury in mature animals.[20,32]

Netrin-1 is a member of the extracellular laminin-related protein family.[33,34] During development, Netrin-1 is expressed in the floor plate of the neural tube, acting as guiding factor within the CNS.[9,33,35–37] When Netrin-1 is expressed in the floor plate, axons from the motor nuclei are repelled and projected dorsally. In contrast, as axons near the exit points of the CNS Netrin-1 can be chemoattractive.[11,35] This bidirectional nature of Netrin-1 is due to the presence of different Netrin-1 receptors in the axon membrane. Deleted in colorectal carcinoma (DCC) in the axonal membrane binds to the Netrin-1, triggering attraction through several pathways for the axonal extension, whereas Netrin-1 linked to both DCC and UNC5 acts as a repulsive factor.[11,38–40] Netrin-1 appears to be important to RLN axon guidance following injury in a rat model because the upregulation of Netrin-1 coincides with intrinsic laryngeal muscles reinnervation. Despite this, the role of Netrin-1 in these denervated muscles is not known.[6,18] The aim of this study was to evaluate the production of GDNF and Netrin-1 receptors within nucleus ambiguus following RLN injury.

MATERIALS AND METHODS

Experimental Animals

The present study was performed in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the Animal Welfare Act (7 U.S.C. et seq.). The handling of animals in research and the protocols were approved by the Columbia University's Institutional Animal Care and Use Committee. Sixteen adult female Sprague Dawley rats (250–300 g) were used in this study. There were four rats in the control group in which the RLN was uninjured. In the experimental groups, the nerve was transected and dextran amine conjugated to Texas Red was applied to the proximal stump (Molecular Probes, Eugene, OR), with rats subsequently sacrified at 7, 14, and 21 days postinjury.

Surgical Protocol: RLN Transection and Texas Red Application

Animals were anesthetized with an intraperitoneal injection of 75 mg/kg of ketamine and 5 mg/kg of xylazine. A midline incision was made in the ventral neck, and the right RLN was identified. A piece of gelfoam (Pharmacia & Upjohn, New York, NY) was placed beneath the RLN near the seventh tracheal ring, and the nerve then transected with an iridectomy scissor. Lyophilised dextran amine conjugated to Texas Red was applied to the proximal stump of the nerve, as described by Pascual-Font et al. in 2011.[41] The distal and proximal stumps were then aligned with a gap of less than 1 mm, and another piece of gelfoam was placed on top of the both stumps to secure the positions. In order to avoid collateral innervation to the denervated laryngeal muscles, the right superior laryngeal nerve was transected close to the larynx, and both ends were ligated.[42] The skin was then sutured, and animals were returned to their facility with supply of food and water *ad libitum*.

Functional Evaluation

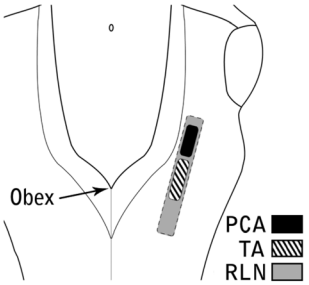
Injury of the right RLN was assessed with a transorally inserted 0° 4 mm endoscope (Karl Storz, Tuttlingen, Germany), as performed previously.[6,14,18,43] Vocal fold movement was recorded and evaluated after nerve injury and before sacrifice using a camera attached to the endoscope with a digital recording system (Richard Wolf Solid State Color Video Camera, Melville, NY). The videos were reviewed for blinded grading of motion of the right vocal fold: 0 for immobile, 1 for slight nonpurposeful motion, 2 for purposeful but paretic motion, and 3 for normal motion.[17,18,43]

Immunohistochemistry

Animals were euthanized by a lethal dose of ketamine (100 mg/kg) and xylazine (10 mg/kg). Animals were then transcardially perfused with sterile saline solution, followed by 4% paraformaldehyde in phosphate buffer saline (PBS) (pH 7.1). Brainstems were dissected out and postfixed for 2 hours before they were immersed in 15% sucrose in PBS. Once they sank, the pieces were immersed in 30% sucrose in PBS until they were cut into 40-μm coronal sections in a cryostat. Serial sections were placed in 24-multiwell plates with PBS.

Immunostaining was performed to assess the expression of trophic factors receptors. Twenty-four multiwell plates were divided into six columns. Each column was stained with goat anti-UNC5A (AF1405), goat anti-DCC (AF844), goat anti-Ret (AF482), goat anti-GFRα1 (AF560), goat anti-GFRα2 (AF429), and goat anti-GFRα3 (AF2645) (1:100, all from R&D Systems, Minneapolis, MN) in order to analyze the expression of each receptor in the 40-μm section every 240 μm. Sections were incubated with the primary antibody with 2% donkey serum in PBST (PBS with 0.2% triton) for 48 hours at 4°C. After washing, the sections were incubated in Alexa Fluor conjugated to rabbit anti-goat (1:200, MilliporeSigma, Burlington, MA) for 2 hours at room temperature. The secondary antibody was conjugated to AF488 (green). After the staining was performed, sections were captured sequentially and then mounted in Fluoroshield Mounting Medium with 4'6-diamidino-2-phenylindole dihydrochloride (DAPI)

A



B

	Control		Experiment		Other than
	PCA (left)	TA (left)	PCA (right)	TA (right)	n. ambiguus
DCC	-	-	-	+	No
UNC5A	-	-	-	-	No
GFRα1	-	-	+	+	No
GFRα2	-	-	-	-	No
GFRα3	+	+	+	+	Yes
Ret	+	+	+	+	Yes

Fig. 1. Diagram summarizing the position of RLN motoneurons in the medulla oblongata (A), and a table that summarizes the staining for DCC, UNC5A, GFRα1, GFRα2, GFRα3, and Ret in control and experimental animals (B). DCC = deleted colorectal cancer; GFRα1, GFRα2, GFRα3 = glycosylphosphatidylinositol-linked cell surface receptors 1, 2 of 3; UNC5A = UNC5 homolog A; PCA = posterior cricoarytenoid muscles; Ret = rearranged during transfection; RLN = recurrent laryngeal nerve; TA = thyroarytenoid muscle.

(blue) (Abcam, Cambridge, MA). Experiments were analyzed using a Zeiss Axio Imager M2 epi-fluorescence microscope (Zeiss, Oberkochen, Germany) in order to determine the presence and absence of trophic factor receptor immunoreactivity. The number and position of RLN motoneurons innervating the PCA and the TA within the nucleus ambiguus were also assessed.

RESULTS

Vocal Fold Motion Evaluation

Immediately following nerve injury, all experimental animals showed no vocal fold motion (grade 0). Before sacrifice, all animals except one displayed no vocal fold motion (grade 0). Only one rat (at 21 DPI) showed purposeful but paretic motion vocal fold motion (grade 2).

Motoneurons Tracing

Traced motoneurons observed in the ventrolateral column of the brainstem were identified as the loose formation of the nucleus ambiguus. Neither neurons nor fibers were found labeled in other locations of the medulla oblongata.

All traced motoneurons were multipolar, most of them in a stellate morphology. Their position formed a continuous rostrocaudal column from 2 mm above the obex to 1 mm below it (2.20 ± 0.250 mm to −1.07 ± 0.43 mm) (Fig. 1). The number of traced motoneurons ranged between 110 to 205 (mean 156 ± 23). No differences in the traced motoneurons were found in the brainstem at 7 and 14 DPI. The number of counted motoneurons dropped in 21 DPI animals.

Immunohistochemistry

Analyzed brainstems showed positive staining for some of the studied trophic factor receptors following nerve injury at different time points. Netrin-1 receptors (DCC and UNC5) showed weaker staining in comparison to GDNF receptors (GFRα1, GFRα2, GFRα3, and Ret).

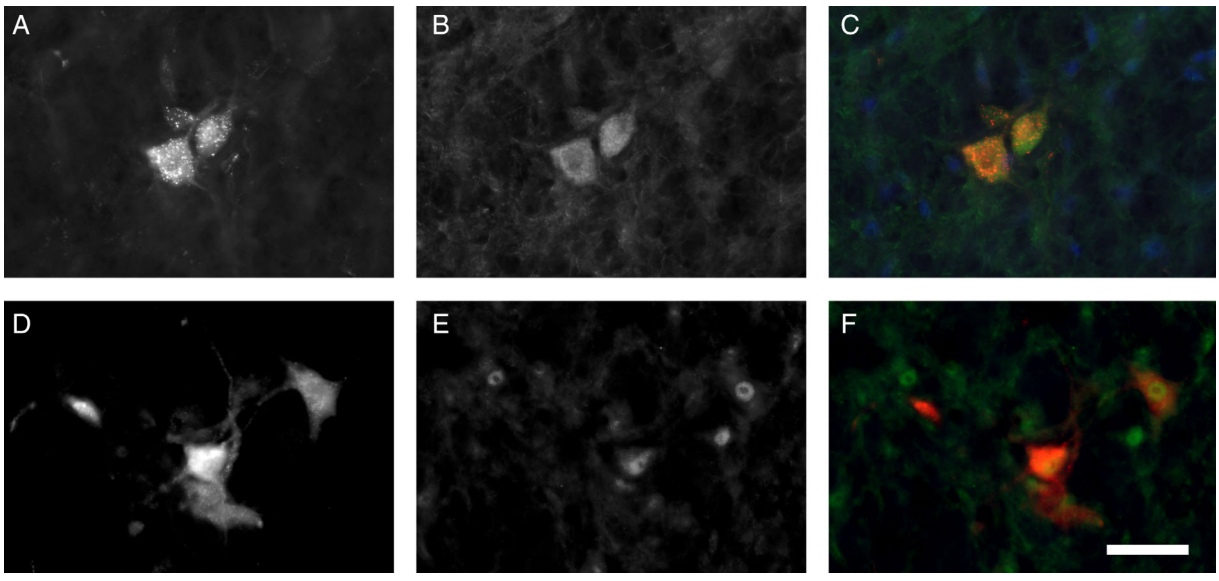


Fig. 2. Immunostaining evaluation of DCC and GFRα3 at 14 DPI in nucleus ambiguus. (A, B, C) Traced RLN motoneurons (B), staining for DCC (C), and merge (D). (D, E, F) Traced RLN motoneurons (E), staining for GFRα3 (F), and merge (G). Scale bar = 50 μm. DCC = deleted colorectal cancer; DPI = days postinjury; GFRα3 = glycosylphosphatidylinositol-linked cell surface receptor 3; RLN = recurrent laryngeal nerve. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

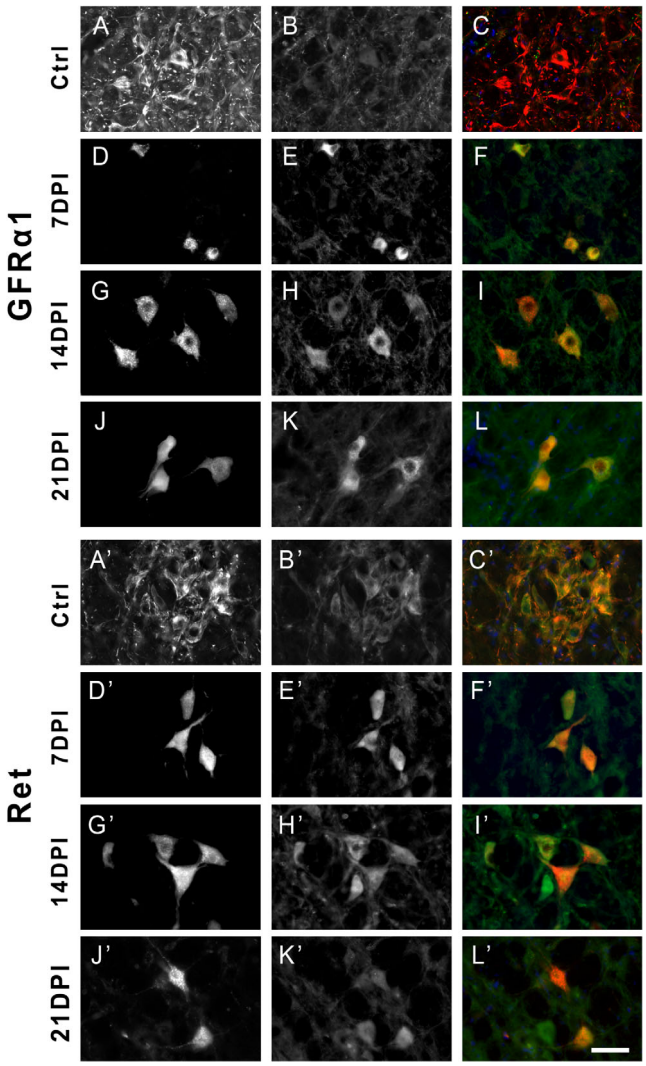


Fig. 3. Staining of traced RLN motoneurons for GFRα1 and Ret. (A–L) Staining for neurofilament in control (A), staining for GFRα1 (B), and merge (C). (D–L) Staining for GFRα1 at different time points. (D, G, J) Dextran amine tracing of motoneurons. (E, H, K) GFRα1 staining. (F, I, L) Merge. (A'–L') Staining for neurofilament in control (A'), staining for Ret (B'), and merge (C'). (D'–L') Staining for Ret at different time points. (D', G', J') Dextran amine tracing of motoneurons. (E', H', K') Ret staining. (F', I', L') merge. Ctrl = control; DPI = days postinjury; GFRα1 = glycosylphosphatidylinositol-linked cell surface receptor 1; Ret = rearranged during transfection; RLN = recurrent laryngeal nerve. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

In control animals, there was no significant staining at any time period for DCC, UNC5, GFRα1, and GFRα2. Ret and GFRα3 showed weak staining throughout the cells of the medulla oblongata.

In experimental animals, DCC was positively identified at 7 DPI. At 7 DPI, three of the four animals showed positive but weak staining in the caudal nucleus ambiguus, from 0.7 mm above to 0.5 mm below the obex (Figs. 1 and 2). This area is consistent with the TA motoneurons in the nucleus ambiguus. At 14 and 21 DPI, one of four animals stained positive for DCC. UNC5A staining was weakly and unreliably positive in all animals from 7 to 21 DPI. Positive staining was not found outside the nucleus ambiguus.

GFRα1 was positively identified at all time points within motoneurons of the right nucleus ambiguus (Figs. 1 and 3). All motoneurons from 0.5 mm below the obex to 2.2 mm above the obex were positive in right nucleus ambiguus. This correlates to the area of the RLN motoneurons. No differences were observed between rostral and caudal nucleus ambiguus, which corresponds with the position of the PCA and TA, respectively. No positive staining for GFRα2 was observed at any time point.

Positive staining for GFRα3 was observed at all time points studied (Figs. 1 and 2). Compared to controls, experimental animals showed a stronger immunoreactivity in all time periods. No differences between right and left medulla oblongata were noted. GFRα3 positive staining was observed not only in motoneurons but in all cells of the brainstem, colocalizing to the staining for the nucleus with DAPI.

Ret was positive at all time points (Fig. 3). Left nucleus ambiguus and other cells in the right brainstem, as well as neurons identified in the area of facial nucleus, showed a weaker immunoreactivity compared to right nucleus ambiguus. Positive staining fills cell bodies, as was observed in GFRα1-stained cells.

DISCUSSION

The results of this study suggest that following RLN injury Netrin-1 and GDNF receptor production in the nucleus ambiguus is chronologically correlated with GDNF and Netrin-1 trophic factor expression in the denervated laryngeal muscles.[5,6]

The retrograde-labeled motoneurons were of a similar number and position, as described in past studies evaluating RLN motoneurons. This confirms that the motoneurons studied here were correctly identified as RLN motoneurons.[3,41,44]

Experimental findings regarding the production of Netrin-1 receptors showed positive DCC staining in the nucleus ambiguus at 7 DPI. In contrast, no staining of UNC5A was observed at any time point. Following RLN injury, Netrin-1 is overexpressed in the denervated PCA and TA muscles at 7 and 14 DPI.[6] Similarly, this expression returns to the base line in both muscles at 21 DPI. It appears that the expression of Netrin-1 is chronologically correlated to the production of DCC in the nucleus ambiguus. Netrin-1 can have both an attractant and repellent role for the axonal pathfinding in the forming brain.[9,35,36,38,40,45] These properties are due to the interaction of Netrin-1 with two different receptors.[40] The presence of DCC receptor in the growth cone modulates an attraction of motor axons to Netrin-1, whereas the presence of UNC5 or both DCC and UNC5 receptors modulates a chemorepulsion of the newly formed axons to Netrin-1.[11,38,40] UNC5A was not identified in the nucleus ambiguus at any time point in this study. The presence of DCC and absence of UNC5A suggests that Netrin-1 upregulated in the laryngeal muscles after RLN injury plays only an attractive role for RLN axons approaching the laryngeal muscles. The absence of a UNC5A production may be one reason for nonselective reinnervation. Knowing

that Netrin-1 is upregulated in response to the nerve injury, further studies that evaluate the expression of Netrin-1 receptors within the nucleus ambiguus would enhance our understanding of the role of Netrin-1 in selective reinnervation of the larynx.

GFRα1, GFRα3, and Ret all show increased staining at 7 DPI. Although Ret and GFRα3 seem to be globally upregulated from controls in a nonspecific manner throughout the medulla, Ret staining had more immunoreactivity in the right nucleus ambiguus than elsewhere from 7 DPI to 21 DPI; however, at 21 DPI the immunoreactivity in the nucleus ambiguus was decreasing. In contrast, GFRα1 was positively identified at all time points within motoneurons of the right nucleus ambiguus, which is consistent with both the PCA and TA muscles. At 21 DPI immunostaining for GFRα1, the injured side was still present but decreasing in intensity. This change in GDNF receptors production chronologically correlates with GDNF expression in laryngeal muscles after RLN injury.[5] Following a RLN injury, GDNF is upregulated in the PCA at 7 DPI, and then its expression drops to basal level at 14 DPI. Meanwhile, GDNF upregulation in the TA is slightly delayed as it is upregulated at 14 DPI and then returns to basal level at 21 DPI. This temporal pattern correlates with the timing of axon reinnervation of the laryngeal muscles, with PCA occuring first followed by TA. The binding of GDNF to its GFRα receptors requires the presence of Ret that triggers intracellular signaling for axonal outgrowth. GDNF receptors presence were previously reported following nerve injury in the cell bodies and at the neuromuscular junction.[19,20,46] The simultaneous expression of GFRα1 and Ret in the nucleus ambiguus at a time similar to the upregulation of GDNF in the laryngeal muscles following RLN injury suggests a role of GDNF and its receptors in the guidance of axons toward denervated muscles. This is similar to observations during development.[47,48]

CONCLUSION

The expression of Netrin-1 and GNDF in the intrinsic laryngeal muscles is chronologically correlated to the production of DCC, GFRα1, and Ret in the ipsilateral nucleus ambiguus following RLN injury. This correlation suggests they play a role in reinnervation and may interact after RLN injury. DCC, GFRα1, and Ret would result in axon attraction to the laryngeal muscles producing GDNF and Netrin-1. The absence of UNC5A from the nucleus ambiguus after RLN injury may be just as important. The lack of this repulsive cue from UNC5A to repel improper axons may be one reason for nonselective reinnervation after RLN injury.

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