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※ 貓迷走-舌下神經雜接後其雜接神經的超微構造變化 ※

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行政院國家科學委員會專題研究計畫成果報告

貓迷走-舌下神經雜接後其雜接神經的超微構造變化

Ultrastructural Changes in the Heteroconnected Nerve Following Vagal-Hypoglossal Nerve Anastomosis in Cats

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一、中英文摘要

在迷走-舌下神經雜接後，本研究以山葵過氧化氫腌之逆向標誌技術來追蹤此雜接神經的細胞源頭--疑核與迷走神經背側運動核的時序性神經元再生情形，並觀察其雜接神經近心端的超微構造變化以及軸突出芽現象。本研究的最重大發現是：神經元再生的時序性變化完全吻合軸突出芽與回縮的過程，而且因為改換了新目標器官--舌頭的緣故，雜接神經及其細胞源頭均顯現了高度重塑性。疑核神經元的樹突有顯著的出芽現象，而其發出的有髓鞘軸突也展現了最佳的神經再生，此應與新目標器官的合適性有關，因為疑核類似舌下神經核，兩者原先都是支配骨骼肌的。迷走神經背側運動核原先支配的是內臟平滑肌，因此與新目標器官較不相容，導致神經元以及無髓鞘軸突有神經再生不良現象。

關鍵詞：迷走神經、舌下神經、神經雜接、軸突發芽、電子顯微鏡術

Abstract

Purpose: To investigate the relationships between the axonal sprouting and target neurotization by central neurons after nerve heteroconnection.

Methods: Unilateral (right) vagal-hypoglossal nerve anastomosis (VHA) was performed in adult cats. Following 3-315 days postoperation (dpo), quantitative analyses and ultrastructural changes in the proximal portion of the vagal-hypoglossal heteroconnected nerve as well as the time course of neuronal regeneration were studied. Along with this, horseradish peroxidase (HRP) retrograde tracing technique was used to label the neurons of dorsal motor vagal nucleus (DMV) and nucleus ambiguus (NA) to ascertain if target neurotization was established.

Results: The contralateral (left) intact vagus nerve proximal to the level of ansa cervicalis showed an average of 33±1 myelinated and 74±4 unmyelinated axons in 727 μm^2 sectional area of the nerve. In the heteroconnected nerve at the corresponding level just proximal to the anastomosis site, there was a marked increase in the number of small axons sprouting from the unmyelinated nerve fibers between 18 and 25 dpo. The number of these axonal sprouts appeared to decline at 32 dpo but its increase of 131% was sustained until the late regeneration stage at 315 dpo when compared with the contralateral nerve serving as a control. The mean number of myelinated axons per area unit (727 μm^2) was reduced to 18 at 3 dpo but was immediately restored to the normal range at 7 dpo. The retrograde labelling of neurons in both the DMV and NA was first detected at 22 dpo and was progressively increased peaking by about 67 dpo.

Conclusions: We conclude that compared with the unmyelinated axons, the myelinated axons may acquire a superior interaction with the new target. Furthermore, the postoperative neurotization of tongue muscles may initiate and facilitate the retraction of the redundant axonal sprouts.

Keywords: Vagus nerve; Hypoglossal nerve;

Nerve heteroconnection; Axonal sprouting; Electron microscopy

二、計畫緣由與目的

Proximal-to-distal joining of two different peripheral nerves, termed nerve heteroconnection, has offered a substantial promise in nerve regeneration or even in functional reinnervation. Some functional restorations had been achieved by motor-to-motor [2, 3, 6, 19, 31], visceral sensory-to-postganglionic sympathetic motor [28], visceral sensory-to-somatic motor [25] and preganglionic parasympathetic motor-to-somatic motor [9, 21, 25] nerve heteroconnections. In the latter, experiments of vagal-hypoglossal nerve anastomosis (VHA) have been carried out in rats by Flumerfelt et al. [9] and McWilliam et al. [21]. The studies which focused on the dorsal motor nucleus of the vagus (DMV) had demonstrated that the efferent neurons could reinnervate the tongue musculature by sending their axon terminals to the old motor endplates. Following VHA, the neuronal phenotype in the DMV was also altered from CGRP-negative to CGRP-positive [21]. A profound dendritic sprouting from the regenerating neurons in the nucleus ambiguus (NA), but not in the DMV, has recently been reported in cats after VHA [5]. It was suggested that the dendritic sprouting was induced by some special signals retrogradely transported from the new target, viz. tongue skeletal musculature, to the NA peripherally projecting neurons, most of them being somatic motoneurons innervating the skeletal muscles of larynx and pharynx [5].

Since the vagus nerve originates from two brainstem nuclei, namely DMV and NA, and contains at least four functional component of fibers, i.e. general visceral efferent (GVE), special visceral efferent (SVE), general visceral afferent (GVA) and special visceral afferent (SVA) [15], it was considered to be a very good model for our ongoing studies designed to elucidate how a mixed nerve may rebuild its new pathway after transection and heteroconnection. This study was aimed to examine the process and changes of degeneration and regeneration in the peripheral heteroconnected nerve at the ultrastructural level, and to determine if this could be correlated with the central neuronal rearrangement following VHA as demonstrated by Chang et al. [5]. The information obtained will help to better clarify the possible governing factors essential for eventual and successful nerve regeneration.

三、結果與討論

Results

1. Light microscopic observations

Following simultaneous HRP injections into the left intact vagus nerve as well as into the intrinsic tongue musculature , HRP-positive cells were consistently localized in the hypoglossal nucleus (Fig. 1A), DMV (Fig. 1C) and NA (Fig. 1E) on the left side contralateral to the VHA. However, the hypoglossal nucleus on the VHA side was devoid of HRP-labelled cells at various time points after operation (Fig. 1B). On the left (control) side, results of total cell count from the 3 cats killed at 3, 18 and 315 dpo showed an average of 3275±74 (M±S.D.) and 936±23 HRP-labelled neurons in the DMV and NA, respectively (Group A in Table 1; n = 3).

HRP-labelled neurons were absent in the two vagal nuclei immediately following VHA (Group B in Table 1; n = 3). At about 22 dpo, some labelled cells were detected in the DMV (Fig. 1D) and NA (Fig. 1F). Between 18 and 25 dpo, the mean number of labelled DMV neurons was 467±410 and that of labelled NA neurons, 146±130 (Group C in Table 1; n = 3). Beyond the critical time point at 22 dpo, the number of HRP-labelled neurons was progressively increased peaking by about 67 dpo. From 32 to 315 dpo, the mean numbers of the HRP-labelled neurons in the DMV and NA were 3373±1355 and 1052±403, respectively (Group D in Table 1, n = 6).

In the two cats subjected to VHA

followed by HRP injections into the tongue only, HRP-labelled neurons were not detected in the left DMV and NA at 32 or 123 dpo, indicating that axons from these nuclei did not sprout across the midline to share with the right heteroconnected nerve in the reinnervation of tongue musculature.

2. Ultrastructure of the left intact vagus nerve

The left intact vagus nerve proximal to the level of ansa cervicalis was composed of a single nerve fascicle bounded by a well-defined multi-layered perineurium (arrowheads in Fig. 2), which was continuous with the outlying epineurium. Within the nerve fascicle, axonal groupings were separated by the endoneurial septa (arrow in Fig. 3A). Each axonal grouping was further surrounded by the endoneurium composed of the basal lamina and its associated Schwann cell (S in Figs. 3A, 3B), collagen and fibroblasts (F in Figs. 3A, 3B). In the interstitial spaces there were occasional macrophages (M in Fig. 3B) and profiles of capillary endothelial cells (E in Fig. 3B). Frequently, one or more vagal axons were grouped together within the so-called Schwann cell unit [1] (arrowheads in Figs. 3A, 3B) in which individual axons were separated by thin neurolemma cell processes. Often, one to six unmyelinated vagal axons were enveloped by a neurolemma cell (arrowheads in Fig. 3B) while a single myelinated vagal axon resided in a Schwann cell unit (arrowheads in Fig. 3A).

The above-mentioned segment of the intact vagus nerve contained a mixture of myelinated and unmyelinated axons. Myelinated axons which were fewer in number appeared to concentrate in the medial and ventral peripheral zones of the nerve fascicle (Fig. 2). Ultrastructurally, the unmyelinated axons resembled the myelinated fibers except for their smaller caliber and the lack of multi-layered myelin sheath (Figs. 3A, 3B). The axoplasm of both types of axons contained abundant neurofilaments, bundles of microtubules, mitochondria and endoplasmic reticulum. In the 3 cats killed at 3, 18 and 315 dpo and their intact vagus nerves served as Group A (Table 1), the size, ultrastructure, and number of axons per area unit of the nerves were comparable. The mean number of myelinated axons per 727 μm^2 sectional area of the intact vagus nerves was 33±1 and that of unmyelinated axons, 74±4 (Table 1).

3. Ultrastructural changes in the proximal portion of the VHA nerve

The proximal segment of the heteroconnected nerve was examined at 3 (Figs. 4A, 4B), 7, 14 (Figs. 5A, 5B), 18 (Figs. 6A, 6B), 22 (Figs. 7A, 7B), 25, 32, 46, 67 (Figs. 8A, 8B), 92, 123 (Figs. 9A, 9B), 131, 232, 272 and 315 (Figs. 10A, 10B) days after VHA. Signs of retrograde degeneration were observed during the first 2 weeks postoperation. Many axons were indented by adaxonal processes of the Schwann cells so that their outlines became relatively irregular (Figs. 4A, 4B) when compared with those in the intact vagus nerve (Figs. 3A, 3B). Some myelin sheaths showed lamellar splaying at the inner and outer mesaxons (Figs. 4A, 4B). Myelin debris was observed in gaps of the splayed myelin laminae. A few unmyelinated axons (asterisks in Fig. 4B) appeared dilated and they often contained aggregates of glycogen-like particles. The myelin splaying and separation as well as the axonal swelling, however, were less drastic at 14 days after VHA (Figs. 5A, 5B). In the degeneration stage in Group B (Table 1; n = 3), although the number of myelinated axons per area unit of the heteroconnected nerve was reduced at 3 dpo, it was quickly up to normal level at 7 dpo; the mean number was 25±6 during this stage (Group B in Table 1). The mean number of unmyelinated axons per area unit was noticeably increased in Group B (125±48) compared with that in the intact vagus nevers in Group A (74±4) (Table 1). Other structural alterations included accumulation of mitochondria [7] in some unmyelinated axons (asterisk in Fig. 8B) and Schwann cells (arrows in Figs. 6A, 9A). In addition, electron-dense debris, myelin-like figures and vacuoles

of variable sizes persisted in some myelinated and unmyelinated fibers as well as in the Schwann cell cytoplasm as late as 315 dpo (Figs. 4A-10B).

Axonal sprouting indicative of nerve regeneration was apparent at 14 days after VHA in the heteroconnected nerve proximal to the anastomosis site. This was manifested by the progressive increase in numbers of small nonmyelinated axon-like profiles, i.e. axonal sprouts (arrows in Figs. 5A, 5B). The axonal sprouts displayed all the ultrastructural features described previously by Blümcke et al. [4]. A noteworthy feature was the increase in size of the Schwann cell units associated with unmyelinated axons, i.e. increase in ratio of axons per Schwann cell unit. Each unit contained as many as up to a dozen of small axonal sprouts (Figs. 5A, 5B) compared with 1-6 unmyelinated axons of normal diameters in the intact vagus nerve (Figs. 3A, 3B). From 18 to 25 dpo representing the stage of profound axonal sprouting (Group C in Table 1, n = 3), the mean number of axonal sprouts was markedly increased to 408±72 (Figs. 6A, 6B; Table 1), thus giving the nerve fascicle an almost homogeneous appearance. In contrast, the mean number (28±1) of myelinated axons (Group C in Table 1) and the size of the Schwann cell units associated with these fibers were only moderately increased or virtually unchanged; occasional units were observed to contain axonal sprouts (arrowheads in Fig. 6B).

Along with the above changes, some myelinated and unmyelinated axons were labelled by HRP injected into the intrinsic tongue musculature. This was first detected at 22 days after VHA (Figs. 7A, 7B). They were readily identified by the presence of electron-dense rod-like reaction product in the axoplasm. With the HRP labelling, the small axonal sprouts were less frequently observed, but the axonal diameter was increased; furthermore, the myelin sheath of some axons was gradually thickened (Figs. 8A, 8B, 9A, 9B, 10A, 10B). The mean number (29±1) of myelinated axons per area unit was sustained in the heteroconnected nerves from 32 to 315 dpo (Group D in Table 1, n = 9). However, the size of the Schwann cell units associated with unmyelinated axons was concomitantly reduced, resulting in a progressive decrease in the mean number of unmyelinated axons per area unit from 32 to 315 dpo (Group D in Table 1), averaging 225±30 in this stage (Table 1). In spite of this, the increase in mean number of unmyelinated axons as much as 131% still maintained at 315 dpo compared with that of the intact vagus nerve (Table 1), giving a rather homogeneous appearance of the heteroconnected nerve at this time point (Fig. 11).

Discussion

Present ultrastructural study has shown that axonal sprouting in the heteroconnected nerve began as early as 14 days after VHA, was markedly evident from 18 to 25 dpo, but appeared to "decline" by 32 dpo as manifested by the changes in the number of nonmyelinated fibers. The target neurotization by the DMV and NA as indicated by the HRP retrograde labelling of some of the vagal motoneurons was established at around 22 dpo, i.e. between 18 and 25 days after VHA, and it was gradually strengthened after 32 dpo as shown by the increase in neuronal labelling. The axonal sprouting and its decline thus appear to follow closely the same time course as the postoperative neurotization of tongue muscles by vagal motoneurons. Present results have shown that the majority of axonal sprouts in the proximal portion of the heteroconnected nerve originated from the unmyelinated axons. This is evidenced by the increase in the ratio of axons per Schwann cell unit associated with the unmyelinated axons. However, the number of myelinated axons as well as the size of their associated Schwann cell units, remained relatively unaltered.

No attempt was made to count all myelinated or unmyelinated axons within the entire nerve fascicle. Instead, the mean numbers of the two axonal profiles were obtained by sampling of 727 μm^2 sectional area of the nerve in electron micrographs

(magnified at X3,000 plus 1.85 times). This method was adopted to obviate the following problems. *Firstly,* counting of nerve fibers under the light microscope was unreliable as the identification of axonal sprouts was difficult. *Secondly,* the vagal and vagal-hypoglossal heteroconnected nerves of the cat were too large to allow a full transverse section of the nerves to be included in a single electron microscopic grid square. This would inevitably lead to an underestimation of count as some axons were obscured by grid bars. Present results of cell count have revealed that the mean numbers of HRP-labelled neurons in the DMV and NA after VHA in longer surviving animals (Group D) were obviously greater than those on the left control side, i.e. Group A. This does not seem to reconcile with the general consensus that some cells of origin may not be able to survive the traumatic nerve anastomosis or may not be traced because of the unsuccessful establishment of contact with the target musculature after VHA, leading to a reduced neuronal labelling. The discrepancy may be attributed to different methodology used. In the present study, HRP was injected intraneurally for tracing of the intact vagal neurons, while intramuscular injection of the tracer was used for labelling of the DMV and NA neurons on the VHA side. One possible explanation for the lower incidence of labelled intact vagal neurons may be that the HRP injected intraneurally was not readily taken up by all the intact vagus nerve fibers.

It has been reported that the ruptured cell membranes of the proximal stump of the severed nerve are resealed 5-30 min after nerve transection [30], and soon afterward a terminal swelling or *"end-bulb"* is formed [10]. Within about a day after injury, numerous fine axonal branches begin to emerge from the end-bulb and start to elongate [8, 26]. The regenerating sprouts are guided by the Schwann cell processes [27] and grow down the endoneurial tubes towards their final destination probably under the influence of local guidance cues [17]. The elongation of the regenerating axonal sprouts to reach the target appears to depend on certain factors secreted from the distal stump of the transected nerve together with the denervated target cells. These factors include laminin, fibronectin, fibrin, heparan sulfate proteoglycan, nerve growth factor, insulin-like growth factors, ciliary neurotrophic factor, fibroblast growth factors, interleukins, transforming growth factor-beta, epidermal growth factors, platelet-derived growth factors and apolipoprotein-E [8]. Our quantitative study has shown a marked increase in the number of sprouts from the unmyelinated axons in the proximal portion of the heteroconnected nerve following VHA. Remarkably, the number of myelinated axons in the same regenerating nerve segment remained relatively unchanged. It is speculated that this may be linked to the compatibility of different axons with the available synaptic sites in the tongue musculature. The present demonstration of the lack of the axonal sprouts in Schwann cell units associated with myelinated axons suggests that most of the injured myelinated axons need but only send out one terminal sprout which somehow could regenerate successfully. This may be due to the stronger affinity of the neurite-promoting/neurotrophic factors secreted from the heteroconnected nerve and the new target, tongue skeletal muscle, to the myelinated axons. This would then explain the exuberant dendritic sprouting in the NA [5] since the majority, if not all, of myelinated axons in the original vagus nerve are derived from this nucleus [19, 23].

It remains to be explained why numerous sprouts emerge from the unmyelinated axons thought to be sensory fibers [23], or preganglionic parasympathetic fibers which originate at least partially from the DMV [20]. It is known that after injury, the regenerating axons respond with axon branching mainly at the nodes of Ranvier, up to 6 mm proximal to the injury site [24], rather than from the axon terminal end-bulb [10]. Each parent axon may give rise to 25 daughter axons [14]. Under ideal conditions only one of the axonal sprouts should reach its original target. When forward growth is,

however, blocked, the sprouts are stunted forming a tangled terminal mass, termed *"neuroma"* [8]. Therefore, the sustained increase in unmyelinated axons after VHA could result from an impeded regeneration of these axons and formation of neuroma proximal to the suture site. As regeneration proceeds, some of the supernumerary axon branches are pruned off over a period of up to 12 months [18]. Those that are lost are presumed to be those that fail to make a connection with a peripheral target. There are, however, persistently higher numbers of axon branches in the regenerated peripheral nerves compared to the parent nerves [18]. From a speculative point of view, the axonal sprouts could have been elicited by VHA but misguided to their inappropriate target, i.e. tongue skeletal muscles. The misdirected axonal sprouts which failed to make terminal contact with the target inappropriate to them may have undergone retrograde atrophy to allow the maturation of other successfully regenerating unmyelinated axons. The aborted attempt in making contact with their target organ and the further retraction may explain the subsequent reduction in the number of the unmyelinated axons. In this connection, the first and subsequent increase in labelling of the regenerating neurons in the two vagal nuclei by HRP may be significant. It is speculated that the progressive neuronal regeneration after VHA may in fact facilitate the decline of the misdirected and/or aberrant axonal sprouts or vice versa as part of the overall recovery process.

A sustained increase in myelinated fibers sprouts, with comparatively less increase or even a decrease in unmyelinated axons sprouts after peripheral nerve lesions followed by successful regeneration has been reported by some authors [11, 13]. Since low power electron micrographs [13] and light microscopic images [11] were used to count regenerating nerve fibers in these studies, it is possible that the actual number of unmyelinated axons might have been underestimated. Indeed, in the present study, tiny axonal sprouts being extremely small in size could not be identified with certainty at low magnification. Giannini et al. [11] suggested that morphometric characterization of fiber regeneration in a distal nerve after focal proximal nerve injury may be related to the types of nerve lesion, misdirected regrowth of fibers and cellular alterations at the site of injury or in the distal nerve which inhibit neural outgrowth or elongation or do not inhibit outgrowth but retard or prevent maturation. In the light of this, it is suggested that the nerve heteroconnection used in the present study is probably the governing factor which appears to selectively facilitate the regeneration of the myelinated axons but trigger the aberrant sprouting of the unmyelinated axons. How this is effected may depend on the properties of the two axons and their interactions with the new target musculature.

四、計畫成果自評

本計畫承接上一研究計畫以時序性方式完整的觀測出：貓迷走-舌下神經雜接後，其神經元再生的時序性變化完全吻合軸突出芽與回縮的過程，而且因為改換了新目標器官--舌頭的緣故，雜接神經及其細胞源頭--疑核與迷走神經背側運動核均顯現了高度重塑性。這個發現可謂極具突破性，所撰寫論文已為國際知名雜誌 Restorative Neurology and Neuroscience 接受並刊印發表中。此實驗結果也啟發我們以後要更進一步探索其反應模式與誘發機制，俾便有朝一日運用於增進中樞神經系統的重塑，解決神經再生面臨的一些瓶頸與困境。

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