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Two specific tongue regions receive bilateral hypoglossal innervation: A study in neonatal rat pups

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ABSTRACT

Objective: The purpose of this study was to investigate the functional role of bilateral hypoglossal (XII) nerve innervation of the tongue.
Materials and methods: The possibility of bilateral innervation of XII nerve in the tongue was examined using postmortem fibre tracing in normal neonatal rats. In addition, immunohistochemical testing for synaptophysin or vesicular acetylcholine transporter (VAChT) was carried out in unilaterally XII nerve-resected neonatal rats.
Results: Postmortem fibre tracing revealed constant distribution of the nerve fibres extending across the midline and existence of bilateral innervated area in the rostroventral and caudodorsal regions of the tongue. Synaptophysin-positive and VAChT-positive nerve terminals were also distributed continuously from the XII nerve-intact to the nerve-resected side across the midline of the tongue. The contralaterally projecting VAChT-positive nerve terminals were more numerous in suckling P2 rats (6.6 ± 0.5/section) than those in non-suckling P2 rats (4.9 ± 0.3/section) 24 h after nerve resection. Furthermore, the contralaterally projecting VAChT-positive nerve terminals were more numerous in P7 rats with nerve resection on P1 (6.3 ± 0.2/section) than those in P7 rats with nerve resection on P6 (3.1 ± 0.8/section).
Conclusion: We concluded that neonatal rats have two specific tongue regions receiving bilateral XII innervation, which allowed suckling in unilaterally XII nerve-resected neonatal rats.

1. Introduction

We have previously reported axonal sprouting across the midline of the tongue from the intact side to the hypoglossal (XII) nerve-resected side in neonatal rats.[1] Although the speed of axonal sprouting to the contralateral tongue remains uncertain, early onset of axonal sprouting within 24 h after a nerve insult still raises the possibility of residual nerve components of bilateral innervation of the XII nerve. Although

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the problem of postmortem fibre tracing comes from difficulties in constant visualization of finely arborized neural networks as judged from the relatively large number of unsuccessful cases, we re-examined XII innervation in neonatal rat tongues using a large number of normal newborn pups and many tongue preparations cut serially with shorter intervals. In addition, synaptophysin and vesicular acetylcholine transporter (VAChT), presynaptic terminal markers for the neuromuscular junction,[2–5] were immunohistochemically detected in the unilaterally XII nerve-resected tongues, because tongue movement is essential for suckling in developing rats.[6–8] We performed quantitative analyses of the nerve terminals across the midline of the tongue in neonatal rats with suckling disturbance induced by unilateral resection of the XII nerve. In this study, we will discuss the functional role of bilateral XII innervation of the tongue.

2. Methods

2.1. Animals

Newborn Wistar rats (Japan SLC Inc., Shizuoka, Japan) of both sexes were used in this study. Postnatal day (P) 0 refers to the first 24 h after birth. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and protocols were approved by our Institutional Animal Care and Use Committee. Every effort was made to minimize animal suffering and pain.

2.2. Postmortem neuronal tracing

Normal pups were euthanatized with sodium pentobarbital (100 mg/kg, intraperitoneally) and perfused through the heart with 20 mL of 4% paraformaldehyde in 0.1 M phosphate buffer on P0 (n = 18), P1 (n = 25), and P2 (n = 22). The left XII nerve was exposed and transected under a surgical microscope. Then 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Eugene, OR) crystals were placed on the distal stump of the nerve to trace the innervation of the tongue. Pups were incubated in 0.4% paraformaldehyde solution at 37 °C in the dark for 3 months for postmortem anterograde neuronal tracing to the tongue. After the incubation period, the tongues were removed and cut into 200-μm thick serial sections along the frontal plane using a vibratome. Sections were collected at 400-μm intervals (19–21 sections per animal), mounted in 50% glycerol, and examined for the extent of DiI-labelled nerve fibres under a fluorescence microscope. Furthermore, to reveal the bilateral innervation of the tongue, the left and right XII nerves were traced by DiI and 4-(4-(didecylamino)styryl)-N-methylpyridinium iodide (4-Di-10-ASP; Molecular Probes), respectively, on P1 (n = 18) in the same manner.

2.3. Unilateral resection of the XII nerve

Surgical procedures were performed after the induction of deep anaesthesia by hypothermia (−20 °C, 15 min). Individual pups were identified by making a small incision in an auricle or a toe tip. Under a surgical microscope, the right XII nerve was exposed and a length of about 1.5 mm was resected on P1 (n = 21). Then, the pups were subcutaneously injected with acetated Ringer's solution with 5% glucose (50 mL/kg) and they were returned to the dam.[1] The pups were housed with their dam in a single cage (26 cm × 42 cm × 18 cm) under standard laboratory conditions with a 12-h light/dark cycle and room temperature maintained at 22 °C. Food and water were supplied *ad libitum*. After 24 h, pups' suckling capabilities were estimated by their sucking behaviour and stomach milk.[9] To compare the differences of the time of nerve resection, the right XII nerve was resected on P1 (n = 7) and P6 (n = 4) and injected with acetated Ringer's solution in the same manner. The pups were housed with the dam until P7. Three pups receiving nerve resection on P1 showed continual decrease in body weight and died by P5.

2.4. Tissue preparation and immunohistochemistry for synaptophysin and VAChT

At P2 (n = 21) or P7 (n = 8), the right XII nerve-resected rats were euthanatized with sodium pentobarbital (100 mg/kg, intraperitoneally) and perfused through the heart with 20 mL of 4% paraformaldehyde in 0.1 M phosphate buffer. The tongues were removed, post-fixed overnight in the same fixative, and soaked in 30% phosphate-buffered sucrose for 2 days. Frontal sections of the tongue were cut serially into 50-μm slices on a freezing microtome, and collected at 100-μm intervals (P2, 47–57 sections per animal; P7, 63–75 sections per animal). The sections were immersed in 0.3% H_2O_2 for 30 min to suppress endogenous peroxidase activity, and for 2 h in phosphate-buffered saline containing 0.3% Triton X-100. They were then incubated overnight with rabbit polyclonal anti-synaptophysin antibody (1:200; Zymed Laboratories, South San Francisco, CA) or guinea pig polyclonal anti-VAChT antibody (1:2000; Chemicon, Temecula, CA). After washing, the sections were incubated with biotinylated anti-rabbit immunoglobulin (1:500; Dako, Glostrup, Denmark) or biotinylated anti-guinea pig immunoglobulin (1:500; Chemicon) for 2 h, and then with streptavidin-peroxidase (1:500; Dako) for 2 h. The peroxidase reaction product was visualized using the Metal Enhanced DAB Substrate Kit (Thermo Fisher Scientific Inc., Pierce Biotechnology, Rockford, IL) (diaminobenzidine: 0.01%; H_2O_2: 0.0015%). The sections were rinsed, mounted on coated slides, air-dried, dehydrated, and coverslipped with Entellan New (Merck, Darmstadt, Germany).

2.5. Quantification of VAChT-positive nerve terminals

VAChT-positive nerve terminals extending to the right side of the tongue were counted two times under a light microscope in the serial tongue sections taken from the right XII nerve-resected rats, and the means were calculated for each section. The mean values of the number of VAChT-positive nerve terminals per section in each animal were then obtained for each experimental group; the data are expressed as mean ± standard error of the mean (SEM). Statistical significance of the mean value obtained from the experimental groups was evaluated by Student's t-test. P values less than 0.05 were considered statistically significant.