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EARLY AND TRANSIENT INCREASE IN SPONTANEOUS SYNAPTIC INPUTS TO THE RAT FACIAL MOTONEURONS AFTER AXOTOMY IN ISOLATED BRAINSTEM SLICES OF RATS

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Abstract—Section of motor nerve fibers (axotomy) elicits a variety of morphofunctional responses in the motoneurons in the motor nuclei. Later than the fifth post-operational day after section of the facial nerve, synapse elimination occurs in the facial motoneuron pool, leading to gradual abolishment of synaptic input-driven activities of the axotomized motoneurons. However, it remains unknown how the amount of synaptic input changes during this period between the axotomy and the synaptic elimination. Here we examined a hypothesis that axotomy of the motoneurons itself modifies the synaptic inputs to the motoneurons. One day after axotomy, the postsynaptic currents, mostly mediated by non-N-methyl-D-aspartic acid (non-NMDA) receptors, recorded from the axotomized facial motoneurons in the acute slice preparations of the rats were of higher frequency and larger amplitude than those in the intact motoneurons. This difference was not observed after the third post-operational day and appeared earlier than the changes in the electrophysiological properties and increase in the number of dead neurons in the axotomized motor nucleus. The larger postsynaptic current frequency of the axotomized motoneurons was observed both in the absence and in the presence of tetrodotoxin citrate, suggesting that increased excitability and facilitated release underlie the postsynaptic current frequency increase. These results suggest that synaptic re-organization occurs in the synapses of motoneurons at an early stage following axotomy. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: postsynaptic current, patch-clamp, motoneuron death, synaptophysin, synaptic re-organization.

Section of motor nerve fibers (axotomy) elicits a variety of morphofunctional responses in the motoneurons (reviewed in Moran and Graeber, 2004). The facial nerve axotomy has been successfully used as a model for studying the subsequent neurochemical events occurring at diverse latencies following axotomy. These events include changes in the various factors affecting the motoneuron excitability. For example, the membrane capacitance and the A-current-like K^+ conductance density decrease, the input resistance becomes higher and the after-hyperpolarization becomes longer at the fourth to sixth post-operational day after facial axotomy (Umemiya et al., 1993). Also, the expressions of GLT-1 glutamate transporter (Lopez-Redondo et al., 2000), K^+/Cl^- cotransporter (KCC2) (Toyoda et al., 2003) and GluR2 and GluR2/3 subunits (Popratiloff et al., 1996; Nagano et al., 2003) decrease on the third post-operational day after axotomy.

Motoneurons receive a large amount of synaptic inputs, which are the principal determinants of their excitability (Rekling et al., 2000). For example, the facial nerves of neonate animals show strong rhythmic activities related to sucking and respiratory movements (Kato et al., 1996; Nakamura et al., 1999); such rhythmic activities are generated by the central pattern generators upstream to the facial motoneurons in the en bloc preparations. It has been demonstrated that axotomy results in a drastic loss of synaptophysin-containing boutons in the motoneuron pool at later than the fifth post-operational day after axotomy (Gehlert et al., 1997), which might lead to the elimination of synaptic input-driven activities in the damaged motoneurons. However, it remains unknown how the synaptic inputs to the axotomized motoneurons change during this critical period between the motor nerve section (i.e. loss of innervated muscle contraction) and synapse elimination. In this study we examined whether the axonal injury affects the amount of synaptic inputs to the axotomized motoneurons (Gehlert et al., 1997; Schiefer et al., 1999). This issue is important because changes in the motoneuronal excitability in response to the excitatory synaptic inputs are considered to affect the survival of motoneurons, mostly through increased Ca^{2+} entry through voltage-dependent Ca^{2+} channels and NMDA receptor channels activated by synaptically released glutamate, which triggers sequences leading to apoptotic cell death. Indeed, in newborn and young animals, these changes are followed by apoptotic cell death of a large number of facial motoneurons (de Bilbao and Dubois-Dauphin, 1996; Moran and Graeber, 2004). To address this issue, we recorded postsynaptic currents (PSCs) of the axotomized motoneurons in the acute thick brainstem slices including the facial nucleus prepared following unilateral facial axotomy. The PSCs were compared with those recorded in the facial nucleus contralateral to the axotomy. Here we show that, following axonal injury, the amount of synaptic inputs converg-

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Abbreviations: ACSF, artificial cerebrospinal fluid; AMPA, (*S*)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; D1, D3, D5 and D7, the first, third, fifth and seventh, respectively, post-operational day; IPSP, inhibitory postsynaptic potential; IR-DIC, infrared differential interference contrast; LY, Lucifer Yellow; NMDA, N-methyl-D-aspartic acid; PBS, phosphate-buffered saline; PSC, postsynaptic current; TTX, tetrodotoxin citrate.

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ing to the axotomized motoneurons is markedly increased to about three-fold of that of the intact motoneurons as early as 24 h after axotomy, which is much earlier than the changes in the membrane properties of the axotomized motoneurons and than the global motoneuronal death.

EXPERIMENTAL PROCEDURES

Unilateral facial nerve axotomy

Our manipulation of animals conformed to the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences of the Physiological Society of Japan (1988) and International guiding principles for biomedical research involving animals by the Council for International Organizations of Medical Sciences (1984). Experimental protocol was approved by the Animal Experiments Committee of the Jikei University School of Medicine. All possible efforts were made to minimize the number of animals used and their suffering. Wistar rats of either sex aged 1-to-14 days postnatal (P) were anesthetized with diethyl ether. The skin behind the right ear was cut open using a sterile surgical blade. The whole trunk of the facial nerve was exposed and isolated carefully, then transected with sharp microscissors at its emergence from the stylomastoid foramen (axotomized; ipsilateral to the axotomy). The left facial nerve was exposed and isolated but remained unsectioned (intact; contralateral to axotomy). In the "sham" operation group, left and right facial nerves were exposed but no transection was made. The wounds were sutured with 4/0 Ethicon. The rat was returned to the mother after recovery from the anesthesia, during which the rat was kept warm. In a subset of animals, hydroxystilbamidine (methansulfonate salt, Molecular Probe, OR, USA; Fluoro-Gold) was dissolved in distilled sterile water (2.5–5%) and injected into the nasolabial and mentalis muscles 3 to 5 days before the axotomy, which resulted in Fluoro-Gold labeling in a number of facial motoneurons in the slices for the patch clamp recordings (see Results) (Roste, 1989; Angelov et al., 1995; Kamijo et al., 2003).

Preparation of brainstem slices

The rats that underwent the unilateral axotomy were decapitated under deep ketamine anesthesia (100–150 mg/kg, i.p.) on the first, third, fifth or seventh day after the operation, which are designated hereafter as D1, D3, D5 and D7, respectively, on the assumption that the day of axotomy is the 0th post-operational day. Brainstem slices containing the facial nuclei were made according to methods described by Kato and Shigetomi (2001) and Shigetomi and Kato (2004). Briefly, the upper brainstem was dissected out and secured on the cutting stage of vibrating blade slicer (DSK-1000, Dosaka EM, Kyoto, Japan) with the caudal end upwards. Two to three coronal slices of 400-μm thickness containing the bilateral facial nuclei were cut in ice-cold "cutting" artificial cerebrospinal fluid (ACSF) composed of (in mM) NaCl 125, KCl 3, $CaCl_2$ 0.1, $MgCl_2$ 5 or 3, NaH_2PO_4 1.25, D-glucose 12.5, L-ascorbic acid 0.4 and $NaHCO_3$ 25 (pH=7.4 when bubbled with 95% O_2+5% CO_2; osmolarity, ~330 mOsm kg^{-1}). The slices were first incubated in a holding chamber with a constant flow of "standard" ACSF, of which the concentrations of $CaCl_2$ and $MgCl_2$ were 2 mM and 1.3 mM, respectively, at 37 °C for 30–45 min. The slices were then kept at room temperature (20–25 °C) in the same chamber until the recording. A slice was transferred to a recording chamber (~0.4-ml volume) and fixed with nylon grids attached to a platinum frame. The slice was submerged in and continuously superfused at a rate of 1–2 ml/min with standard ACSF.

Patch-clamp recordings and cell visualization

The facial motoneurons were visually identified with an upright microscope (BX-50WI, Olympus, Tokyo, Japan) with infrared differential interference contrast (IR-DIC) optics. Recordings were made after pre-selection of "healthy-looking" neurons with smooth and clear soma surfaces. Especially in the facial nucleus ipsilateral to the axotomy at >D3, the number of motoneurons with "healthy-looking" cells was reduced. The IR-DIC and fluorescent images were captured with a chilled CCD camera (C5985-02, Hamamatsu Photonics, Hamamatsu, Japan) and stored digitally on the computer. Only brightness and contrast were modified using PhotoShop software (Adobe) for the reconstructed photomicrographs (Fig. 1).

The whole-cell transmembrane current was recorded from motoneurons in the facial nucleus either ipsilateral or contralateral

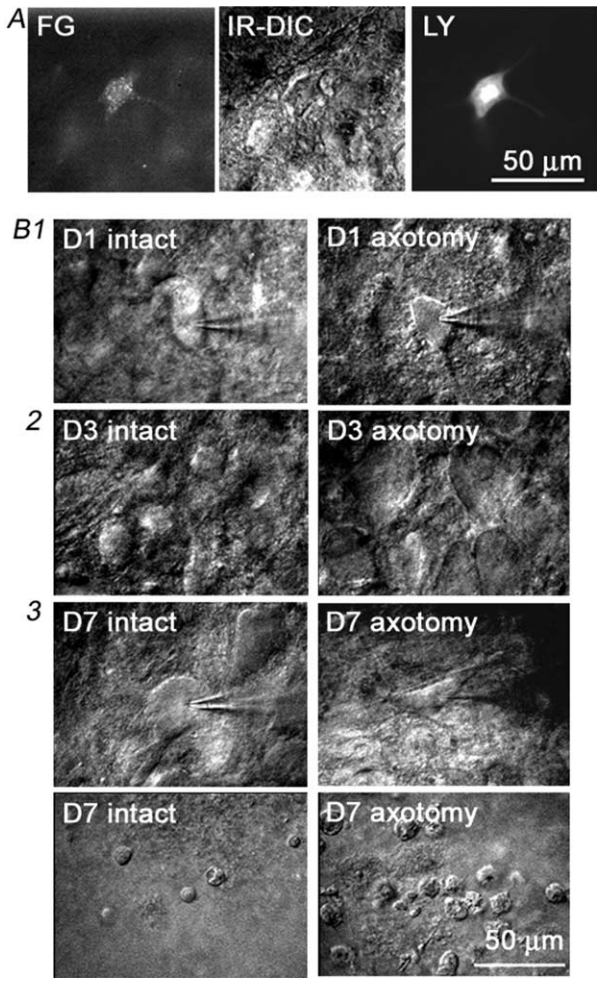

Fig. 1. Morphological properties of the facial motoneurons after axotomy. (A) Left, fluorescent image of the facial motoneurons stained with Fluoro-Gold (FG) injected to the nasolabial and mentalis muscles. Middle, an IR-DIC image of the same field as in the left. Right, a FG-positive neuron was filled with LY during whole-cell patch-clamp recording (LY). Note that the same shapes of the soma with FG and LY fluorescence can be clearly identified in the IR-DIC image. The slice was made at D1. (B) Left and right panels were the IR-DIC images of the facial nucleus contralateral (intact) and ipsilateral (axotomy) to the axotomy, respectively. 1, 2 and 3 were recorded on D1, D3 and D7, respectively. The upper panels and the lower panels in 3 show the images at different focus levels at the motoneurons and slice surfaces, respectively, of the same observation field.