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SYNAPTIC VESICLE POOLS AT DIAPHRAGM NEUROMUSCULAR JUNCTIONS VARY WITH MOTONEURON SOMA, NOT AXON TERMINAL, INACTIVITY

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Abstract—Both spinal hemisection (SH) at C_2 and tetrodotoxin (TTX) phrenic nerve blockade result in diaphragm muscle paralysis and inactivity of the phrenic axon terminals. However, phrenic motoneuron somata are inactive with SH but remain active with TTX phrenic nerve blockade. Neuromuscular transmission failure with repeated activation decreases following SH and increases following TTX phrenic nerve blockade, suggesting that matching (or mismatching) of somal and synaptic inactivities of phrenic motoneurons differentially regulates synaptic vesicle pools at diaphragm neuromuscular junctions. At individual type-identified rat diaphragm presynaptic terminals, the size of the releasable pool of synaptic vesicles was analyzed by fluorescence confocal microscopy of *N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl) pyridinium dibromide (FM4-64) uptake and synaptic vesicle density at active zones was determined using transmission electron microscopy. After 14 days of SH and TTX-induced diaphragm muscle inactivity, neuromuscular junction size was not different at type I or IIa fibers, but increased at type IIx and/or IIb fibers (by 51% in SH and 35% in TTX) compared with control. With SH, synaptic vesicle pool size and density increased at presynaptic terminals innervating type I or IIa fibers (17 and 63%, respectively; $P<0.001$) and type IIx and/or IIb fibers (41 and 31%, respectively; $P<0.001$) when compared with controls. Following TTX, synaptic vesicle pool size and density decreased by 64 and 17%, respectively, at presynaptic terminals innervating type I or IIa fibers, and by 50 and 36%, respectively, at type IIx and/or IIb fibers ($P<0.001$, for all comparisons). Thus, matching motoneuron soma and axon terminal inactivity (SH) increases the size and density of releasable synaptic vesicle pools at adult rat diaphragm neuromuscular junctions. Mismatching motoneuron soma and axon terminal inactivities (TTX) results in converse presynaptic adaptations. Inactivity-induced neuromuscular plasticity reflects specific adaptations in the size and density of synaptic vesicle pools that depend on motoneuron soma rather than axon terminal (or muscle fiber) inactivity.

Key words: fiber type, inactivation, motor unit, phrenic, plasticity, transmission.

Activity-dependent plasticity greatly influences development and function in the nervous system. Although several studies have examined plasticity in response to inactivity in cultured synapses (Turrigiano et al., 1998; Wan and Poo, 1999; Murthy et al., 2001), less information is available regarding inactivity-dependent plasticity at the mammalian neuromuscular junction. Neuroplasticity may include morphological adaptations of individual neuromuscular junctions and/or changes in synaptic vesicle pools, neurotransmitter release or postsynaptic excitability, all of which have important functional implications.

Conflicting evidence is available regarding inactivity-induced synaptic plasticity. For example, in cultured rat visual cortical neurons, tetrodotoxin (TTX) blockade of neural activity increases excitatory postsynaptic currents after 7–9 days (Turrigiano et al., 1998). Similarly, in hippocampal cultures, inactivity caused by TTX increases synaptic strength, the number of docked vesicles and the total number of vesicles per synapse (Murthy et al., 2001). In contrast, there is little change in quantal content rundown with repetitive stimulation following 3–4 weeks of TTX blockade of the sciatic nerve (which inactivates the soleus muscle and presynaptic axon terminal of lumbar motoneurons without affecting the motoneuron soma or dendrites) (Reid et al., 2003). All components of the synapse, including the pre- and postsynaptic neurons, are inactivated by TTX treatment in neuronal cultures. Thus, the inconsistent findings regarding inactivity-induced neuroplasticity may relate to persistent activity at the soma of the presynaptic neuron following TTX-induced inactivation of axon terminals and postsynaptic cells.

Neuromuscular transmission failure in the rat diaphragm muscle increases following TTX blockade of the phrenic nerve, but decreases following a cervical spinal cord hemisection (SH) at C_2 (Miyata et al., 1995). Unilateral SH in rats results in ipsilateral loss of descending excitatory input to phrenic motoneurons and inactivity in motoneuron somata, axon terminals and diaphragm mus-

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Abbreviations: a.u., arbitrary unit; BDNF, brain-derived neurotrophic factor; EMG, electromyography/electromyographically/electromyographic; FM4-64, *N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl) pyridinium dibromide; mEPP, miniature end-plate potential; MHC, myosin heavy chain; NT-4, neurotrophin-4; PBS, phosphate-buffered saline; S.E., standard error; SH, spinal cord hemisection at C_2; TBS, Tris-buffered saline; TrkB, tropomyosin receptor kinase B; TTX, tetrodotoxin.

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cle fibers (Prakash et al., 1999; Goshgarian, 2003). We hypothesized that the matching (or mismatching) of phrenic motoneuron soma and axon terminal inactivities result in distinct changes in presynaptic synaptic vesicle pools. In previous studies (Prakash et al., 1996; Mantilla et al., 2004a), differences in neuromuscular junction morphology and synaptic vesicle pools were identified across diaphragm muscle fiber types which may be important when considering inactivity-induced synaptic plasticity. Accordingly, in the present study, the effect of matching (SH) or mismatching (TTX) phrenic motoneuron somal and axon terminal inactivities on the size and density of synaptic vesicle pools was examined at type-identified diaphragm neuromuscular junctions.

EXPERIMENTAL PROCEDURES

Adult male Sprague–Dawley rats (colony 236, Harlan, Indianapolis, IN, USA; initial body weight ~330 g) were randomly assigned to the following groups: unilateral C_2 spinal hemisection (SH; $n=10$), unilateral TTX-blockade of the phrenic nerve (TTX; $n=10$), and untreated control ($n=10$). All experimental procedures were approved by the Institutional Animal Care and Use Committee at Mayo Clinic, and they were conducted in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. Specific attention was paid to minimizing the number of animals used and their suffering.

Surgical procedures

Spinal hemisection. Details of animal management, the surgical procedure, and validation of the technique have been previously published (Miyata et al., 1995; Prakash et al., 1999). Briefly, anesthesia was achieved by i.m. injection of a combination of ketamine (90 mg/kg body weight) and xylazine (10 mg/kg). Using strict sterile technique, the cervical spinal cord was exposed via a bilateral dorsal laminectomy at C_2. The dura was then incised and retracted to facilitate transection of the right half of the spinal cord anteriorly to the dorsal fissure (i.e. involving only the lateral and ventral funiculi, while preserving the dorsal funiculus). Thus, the ipsilateral phrenic motoneuron pool became completely isolated from descending input. Inactivity of phrenic motoneurons following SH was verified visually by paralysis of the right hemidiaphragm observed via laparotomy and electromyographically (EMG) by absence of activity immediately following the transection and at the terminal experiment. All animals were observed daily after surgery and administered i.m. antibiotics (penicillin G) and oral analgesics (acetaminophen).

TTX blockade of the phrenic nerve. Details of this model have also been previously published (Miyata et al., 1995; Prakash et al., 1999). Briefly, adult rats were anesthetized with the mixture of ketamine and xylazine. The right phrenic nerve was exposed in the neck and a Silastic cuff (outer diameter: 2.0 mm; inner diameter: 1.5 mm, 2 mm long) was placed around the nerve. The cuff was fixed in place by suturing to surrounding muscles and it was connected via a tunneled polyethylene cannula to a miniosmotic pump (Alzet model 2002; Durect Corporation, Cupertino, CA, USA) implanted s.c. in the dorsum of the animal. The miniosmotic pump (225 μl total volume) delivered a solution of 0.0125% TTX (Sigma-Aldrich Corporation, St. Louis, MO, USA) at a constant rate of 0.5 μl/h over 14 days. Paralysis of the ipsilateral hemidiaphragm was verified in the TTX group by the absence of EMG activity at the terminal experiment. Complete blockade of axonal conduction following TTX was verified visually by the lack of diaphragm muscle contraction during stimulation of the phrenic nerve with electrodes placed proximal to the cuff. All animals were administered antibiotics and analgesics after surgery, and were observed daily.

Verification of diaphragm muscle inactivity. During the surgical procedure in SH and at the terminal experiment in both SH and TTX animals, EMG activity was recorded using bipolar wire electrodes inserted into the right and left hemidiaphragm muscles. Efficacy of the surgical procedure was confirmed by the absence of spontaneous and inspiratory-related activity ipsilateral to the surgery in all cases (Fig. 1). In addition, we confirmed absence of inspiratory-related EMG activity in the right hemidiaphragm during hypercapnic/hypoxic conditions (5% CO_2 and 10% O_2) immediately and 14 days following SH. In all cases, EMG signals were amplified and band-pass filtered between 20 Hz and 2 kHz. In a subset of animals, histological evaluation of the nerve cuff site was also conducted to verify lack of gross nerve damage.

Fluorescence confocal microscopy of diaphragm neuromuscular junctions

Six rats in each experimental group (SH, TTX and control) were processed for fluorescence confocal microscopy.

Diaphragm muscle–phrenic nerve preparation. At the terminal experiment, following EMG verification of diaphragm muscle paralysis, the right midcostal hemidiaphragm and phrenic nerve were rapidly excised and placed in Rees-Simpson solution (of the following composition in mM: Na^+ 135, K^+ 5, Ca^{2+} 2, Mg^{2+} 1, Cl^- 120, HCO_3^- 25) while bubbled with 95% O_2/5% CO_2 at 26 °C. The PO_2 and PCO_2 in solution were previously determined to be ~60 kPa and 5 kPa, respectively, maintaining adequate O_2 delivery and CO_2 levels in this live preparation (Lattari et al., 1997). Subsequently, the excised hemidiaphragm muscle was stretched to ~1.5 times resting length and mounted on a silicon rubber (Sylgard; Dow Corning, Midland, MI, USA)–coated dish for labeling of neuromuscular junctions.

Fluorescent labeling of neuromuscular junctions. Synaptic vesicles were labeled by uptake of the styryl dye FM4-64 (*N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl)-hexatrienyl) pyridinium dibromide; excitation: 558 nm; emission: 734 nm; Invitrogen Corporation, Carlsbad, CA, USA). FM4-64, like FM1-43 (*N*-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl)pyridinium dibromide, Invitrogen), is taken up by synaptic vesicles as they undergo repeated cycles of exo- and endocytosis (Betz et al., 1992, 1996; Reid et al., 1999). Acetylcholine receptors present at motor end-plates were fluorescently labeled with Alexa Fluor 488-conjugated α-bungarotoxin (excitation: 495 nm, emission: 519 nm; Invitrogen) to facilitate visualization and morphological characterization of individual neuromuscular junctions according to muscle fiber type (Prakash et al., 1996; Mantilla et al., 2004a). Classification of diaphragm muscle fiber types was based on myosin heavy chain (MHC) isoform expression.

The diaphragm muscle–phrenic nerve preparation was preincubated for 30 min in Rees-Simpson solution containing 5 μM FM4-64 and 1 μg/ml Alexa Fluor 488-conjugated α-bungarotoxin. Following a thorough wash, the phrenic nerve was taken into a suction electrode and stimulated using an A-M Systems 2100 isolated pulse stimulator at 10 Hz (0.5 ms supramaximal pulses with a 67% duty cycle) for a 20-min period in the presence of 5 μM FM4-64. A visible (albeit weak) contraction of the diaphragm muscle was elicited in all cases at this time. In preliminary experiments, we confirmed that longer lasting stimulation (beyond 20 min) did not result in increased terminal FM4-64 staining. The diaphragm muscle–phrenic nerve preparation was then placed in oxygenated Rees-Simpson for confocal imaging.

Confocal imaging. Diaphragm neuromuscular junctions were visualized using an Olympus FluoView 200 laser scanning