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Research Paper

Facilitation of distinct inhibitory synaptic inputs by chemical anoxia in neurons in the oculomotor, facial and hypoglossal motor nuclei of the rat

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by the selective loss of motor neurons in the brainstem and spinal cord. Clinical studies have indicated that there is a distinct region-dependent difference in the vulnerability of motor neurons. For example, the motor neurons in the facial and hypoglossal nuclei are more susceptible to neuronal death than those in the oculomotor nucleus. To understand the mechanism underlying the differential susceptibility to cell death of the neurons in different motor nuclei, we compared the effects of chemical anoxia on the membrane currents and postsynaptic currents in different motor nuclei. The membrane currents were recorded from neurons in the oculomotor, facial and hypoglossal nuclei in brain slices of juvenile Wistar rats by using whole-cell recording in the presence of tetrodotoxin that prevents action potential-dependent synaptic transmission. NaCN consistently induced an inward current and a significant increase in the frequency of spontaneous synaptic inputs in neurons from these three nuclei. However, this increase in the synaptic input frequency was abolished by strychnine, a glycine receptor antagonist, but not by picrotoxin in neurons from the hypoglossal and facial nuclei, whereas that in neurons from the oculomotor nucleus was abolished by picrotoxin, but not by strychnine. Blocking ionotropic glutamate receptors did not significantly affect the NaCN-induced release facilitation in any of the three motor nuclei. These results suggest that anoxia selectively facilitates glycine release in the hypoglossal and facial nuclei and GABA release in the oculomotor nucleus. The region-dependent differences in the neurotransmitters involved in the anoxia-triggered release facilitation might provide a basis for the selective vulnerability of motor neurons in the neurodegeneration associated with ALS.

1. Introduction

Motor neurons innervating the skeletal muscles are more vulnerable to energy deprivation and metabolic dysfunction than the other types of neurons (Ballanyi, 2004; von Lewinski and Keller, 2005), providing an aetiological basis for the motor neuron diseases (MND), including amyotrophic lateral sclerosis (ALS). A deficiency or failure in the supply and production of the cellular energy in the brain, such as those that observed during hypoxia, anoxia, metabolic stress and mitochondrial dysfunctions (Dupuis et al., 2004), induces various types of responses in the neurons, including depolarization (Tanaka et al., 2001; Thompson et al., 2006), hyperpolarization (Trapp and Ballanyi, 1995) and an increase in the synaptic inputs (Allen and Attwell, 2004; Kono et al., 2007).

Despite recent studies that have examined the mechanisms involved in motor neuron death in these neurodegenerative diseases, why only specific groups of motor neurons are selectively vulnerable remains poorly understood. For example, in ALS patients, the oculomotor, abducens, trochlear motor neurons and the Onuf"'s nucleus are more resistant to cell death, whereas other cranial and spinal motor neurons are highly affected. These differences have been attributed to changes in calcium homeostasis between cranial motor neurons (Reiner et al., 1995; Vanselow and Keller, 2000), expression patterns of postsynaptic NMDA receptor subunits in spinocranial motor neurons (Fukaya et al., 2005; Matsuda et al., 2003; Oshima et al., 2002) and the expression ratio of glycine and $GABA_A$ receptors in different cranial motor nuclei (Lorenzo et al., 2006). We previously demonstrated that anoxia using NaCN or 95% N_2 facilitates glycine release in hypoglossal motor neurons in rat brainstem slice preparations (Kono et al., 2007). This facilitation was absent in the neurons of the neighboring dorsal nucleus of the vagus nerve that are also cholinergic neurons innervating the non-skeletal muscles, but entirely resistant in these motor neuron diseases, unlike the hypoglossal nucleus (Kono et al., 2007). In addition, the facilitation of glycine release by anoxia resulted in an increase in the NMDA receptor-mediated currents that are sensitive to the

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pharmacological occlusion of the glycine binding sites (Kono et al., 2007). Therefore, we examined whether the selective facilitation of glycine release in response to anoxia is also present in other motor nuclei because previous studies had suggested that the predominance of glycine receptors compared to GABA receptors is related to the vulnerability of the motor neurons to cell death (Lorenzo et al., 2006).

Therefore, we analysed the dynamic effects of anoxia on the spontaneous synaptic inputs in the facial and oculomotor neurons, in comparison to the hypoglossal motor neurons, in the brainstem slices of rats with identical age ranges and examined whether the anoxia-induced synaptic responses correlate with the ALS-vulnerability of motor neurons.

2. Material and methods

2.1. Slice preparation

The manipulation of the animals was approved by the Animal Experiment Committee at the Jikei University School of Medicine and conformed to the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences of the Physiological Society of Japan (1998). Transverse brainstem slices from Wistar rats (10–17 days postnatal, P) were prepared as described by Shigetomi and Kato (2004) and Kono et al. (2007). Briefly, the brainstem was dissected out under anesthesia with 5% isoflurane (in 100% O_2) and secured on the cutting stage of a vibrating blade slicer (Linear Slicer PRO 7, Dosaka EM) with the caudal end facing upwards. One to two coronal slices of 400-μm thickness containing either of the bilateral oculomotor, facial or hypoglossal nucleus were cut in ice-cold "cutting" artificial cerebrospinal fluid (ACSF) composed of (in mM): 250 glycerol, 3 KCl, 0.1 $CaCl_2$, 5 $MgCl_2$, 1.25 NaH_2PO_4, 10 D-glucose, 0.4 L-ascorbic acid and 25 $NaHCO_3$ (pH = 7.4 when bubbled with 95% O_2 + 5% CO_2; osmolarity, ~310 mOsm/kg). The use of glycerol-containing medium increased the ability to obtain healthy slices with many highly viable motor neurons (Ye et al., 2006). The slices were first incubated in a holding chamber with a constant flow of "standard" ACSF composed of (in mM): 118 NaCl, 3 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 1.2 NaH_2PO_4, 10 D-glucose and 25 $NaHCO_3$, at 37 °C for 30 to 45 min. Next, the slices were kept at room temperature (20–25 °C) in the same chamber for 0.5–5 h until recording. Each 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 standard ACSF and continuously superfused at a rate of 3–4 ml/min.

2.2. Patch-clamp recording

Whole-cell transmembrane currents were recorded from neurons in the oculomotor, facial and hypoglossal nuclei that were visually identified under an upright microscope (BX-50WI, Olympus) with infrared differential interference contrast (IR-DIC) optics. The locations of these nuclei were confirmed by using the adjacent myelinated structures, the ventricles and the midline (see Fig. 1 for details). The use of IR-DIC optics allowed us to record from the deep structures (>100 μm) in the slice. In addition to analysing the anoxic effects in the facial and oculomotor neurons in rats at P10–17, the responses from the hypoglossal neurons that we had previously reported in rats at P16–23 (Kono et al., 2007) were re-examined in younger animals. Patch-clamp electrodes were made from borosilicate glass pipettes (1B120F-4; World Precision Instruments). The pipette solution contained (in mM): 120 CsCl, 20 TEA, 1 NaCl, 0.5 $CaCl_2$, 1 $MgCl_2$, 1 Na_2ATP, 1 BAPTA and 10 HEPES (pH 7.2, as adjusted with CsOH; osmolarity; ~310 mOsm/kg). The tip resistance of the electrode when using these solutions was 3.2–8.5 MΩ. Use of this internal solution allowed us to record the EPSCs and ISPCs as inward postsynaptic currents. The neurons we recorded consistently had the following properties: large soma size as observed with IR-DIC (>~20 μm) and small input resistance (<100 MΩ; see Table 1). Though we cannot completely rule out the possibility that some of the recordings were made from interneuron within these motor nucleus, these criteria are supportive of the notion that most of the neurons recorded are likely to be motor neurons.

The membrane currents were recorded using a MultiClamp 700B amplifier (Molecular Devices), low-pass filtered at 2 kHz and sampled at 4 kHz using a PowerLab interface (AD Instruments, CO, USA). The series resistance was 12.5 ± 1.1 MΩ ($n = 26$), 12.6 ± 0.7 MΩ ($n = 27$) and 13.4 ± 1.2 MΩ ($n = 27$) for neurons in the oculomotor, facial and hypoglossal nucleus, respectively. Whole-cell capacitance was compensated for. The resting membrane potential and input resistance (break-in resistance) was measured immediately after the cell membrane was ruptured by measuring the current response to a 5 mV (5 ms duration) command pulse, which was controlled by the Pclamp 9.0 software (Axon Instruments) (Table 1). Cells showing no overshooting action potentials upon injection of depolarising current and those showing unstable or small resting potential were discarded. After verification of the action potentials, all recordings were made in the presence of 1 μM tetrodotoxin citrate (TTX; Alomone, Israel) to block action potential-dependent transmitter release. The membrane potential was held at −70 mV during the recording. All experiments were performed at room temperature (20–25 °C).

2.3. Metabolic disturbance and drug application

Slices were treated with two types of metabolic disturbance: (i) "anoxia", application of ACSF saturated with 95% N_2 + 5% CO_2 instead of 95% O_2 + 5% CO_2 and (ii) "chemical anoxia", addition of 1 mM NaCN to the ACSF. In each slice only one neuron was recorded, and one slice was treated only once with either of the two metabolic disturbances. In addition to 1 μM TTX, 1 μM strychnine, 100 μM picrotoxin or 3 mM kynurenic acid were dissolved in the ACSF and bath-applied to selectively block glycine, $GABA_{A/C}$ or ionotropic glutamate receptors, respectively. The blockers were perfused for a minimum of 15 min before the addition of NaCN. During these experiments, the ACSF with NaCN also contained the same concentration of each blocker. Both the hypoxic ACSF and NaCN solutions were applied via a separate pipette with the outflow located near the slice, and the application was controlled with electromagnetic valves. All other compounds were purchased from Sigma-Aldrich or Nacalai Tesque (Kyoto, Japan).

2.4. Data analysis

The recorded membrane currents, including the postsynaptic currents (PSCs), were analysed off-line with an Igor Pro (WaveMetrics, OR, USA) using procedures written by one of the authors (F. K). The details of the PSC analysis are described elsewhere (Kato and Shigetomi, 2001; Shigetomi and Kato, 2004). Briefly, the action-potential independent PSCs were semi-automatically detected by calculating the co-variance function between the original trace and the template PSC waveforms. All detected synaptic events were visually confirmed so that each event had a typical PSC waveform, i.e., a rapid rise and an exponential decay. The "unlikely" PSC events and the PSC events with the peak amplitude being smaller than the basal noise level (1.96 fold of the standard deviation of the background amplitude fluctuation) were carefully discarded by visual inspection of the time-extended traces. The PSC frequency was defined as the number of PSCs occurring within a fixed time window divided by its duration (10 s for each neuron). These durations were defined empirically so that the stable and smooth time-course of the PSC frequency could be described.

In the Results section and Figures, we defined the following parameters to quantify the changes in synaptic inputs and to make statistical comparisons. (1) "PSC frequency": as defined above, the number of detected PSC events within 10-s windows and divided by 10 to give a value for events/s. This was indicated as "IPSC frequency" only when the recording was made in the presence of kynurenic acid (Fig. 3A and