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Research article

Nicotinic receptors modulate the onset of reactive oxygen species production and mitochondrial dysfunction evoked by glutamate uptake block in the rat hypoglossal nucleus

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HIGHLIGHTS

- Glutamate uptake block evokes hypoglossal motoneuron damage due to excitotoxicity.
- Reactive oxygen species are the earliest excitotoxic players for oxidative damage.
- Mitochondrial energy deficit is manifested later.
- Intrinsic cholinergic transmission is inadequate to arrest these events.
- Nicotinic receptor activation by nicotine provides strong neuroprotection.

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ABSTRACT

In several neurodegenerative diseases, glutamate-mediated excitotoxicity is considered to be a major process to initiate cell degeneration. Indeed, subsequent to excessive glutamate receptor stimulation, reactive oxygen species (ROS) generation and mitochondrial dysfunction are regarded as two major gateways leading to neuron death. These processes are mimicked in an *in vitro* model of rat brainstem slice when excitotoxicity is induced by DL-*threo*-β-benzyloxyaspartate (TBOA), a specific glutamate-uptake blocker that increases extracellular glutamate. Our recent study has demonstrated that brainstem hypoglossal motoneurons, which are very vulnerable to this damage, were neuroprotected from excitotoxicity with nicotine application through the activation of nicotinic acetylcholine receptors (nAChRs) and subsequent inhibition of ROS and mitochondrial dysfunction. The present study examined if endogenous cholinergic activity exerted any protective effect in this pathophysiological model and how ROS production (estimated with rhodamine fluorescence) and mitochondrial dysfunction (measured as methyltetrazolium reduction) were time-related during the early phase of excitotoxicity (0–4 h). nAChR antagonists did not modify TBOA-evoked ROS production (that was nearly doubled over control) or mitochondrial impairment (25% decline), suggesting that intrinsic nAChR activity was insufficient to contrast excitotoxicity and needed further stimulation with nicotine to become effective. ROS production always preceded mitochondrial dysfunction by about 2 h. Nicotine prevented both ROS production and mitochondrial metabolic depression with a delayed action that alluded to a complex chain of events targeting these two lesional processes. The present data indicate a relatively wide time frame during which strong nAChR activation can arrest a runaway neurotoxic process leading to cell death.

1. Introduction

In physiological conditions astrocytes and neurons tightly regulate the extracellular concentration of glutamate by membrane transporters [1] whose disruption causes excitotoxicity reported to occur in neurodegenerative diseases [2]. A significant example of increased extracellular glutamate related to neurodegeneration onset is amyotrophic lateral sclerosis (ALS) since the discovery of high levels of glutamate in the cerebrospinal fluid of ALS patients

Abbreviations: ACh, acetylcholine; ALS, amyotrophic lateral sclerosis; DHβE, dihydro-beta-erythroidine; DHR 123, dihydrorhodamine; EAATs, excitatory amino acid transporters; HMs, hypoglossal motoneurons; MLA, methyllycaconitine; MTT, mitochondrial toxicity test; nAChR, nicotinic acetylcholine receptor; Rho 123, rhodamine 123; ROS, reactive oxygen species; TBOA, DL-*threo*-beta-benzyloxyaspartate.

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[3–5]. Glutamate-excitotoxicity may evoke oxidative stress [6–8], due to accumulation of reactive oxygen species (ROS), and subsequent cell death [9]. In this scenario, an important role is also played by the mitochondrial respiratory chain whose collapse produces energy dysfunction including imbalanced Ca^{2+} homeostasis [10,11]. Since experimental block of excitatory aminoacid transporters (EAATs) reflects the pathological condition often present in ALS [12,13], we devised a model to mimic the early presymptomatic stage by pharmacological inhibition of glutamate uptake with DL-*threo*-beta-benzyloxyaspartate (TBOA). Thus, we used hypoglossal motoneurons (HMs) as an *in vitro* test system because these cells are very vulnerable to the ALS bulbar type [14].

It is well documented that nicotine can provide neuroprotection in certain neurodegenerative diseases [15]. Cholinergic transmission, mediated by nicotinic acetylcholine receptors (nAChRs), is widely distributed through the brain to regulate different processes contributing to synaptic transmission as well as to neuronal protection and cognitive performance efficiency [16,17]. In the brainstem cholinergic projections from pontine nuclei to the hypoglossal nucleus activate mainly α7 and α4β2 nAChRs that may confer neuroprotection after excitotoxic insult [18,19] even in neonatal animal models [15]. We have recently discovered that, in the nucleus hypoglossus, nicotine could prevent TBOA-induced motoneuron loss via inhibition of complex effects that include pathological bursting, ROS generation and mitochondrial dysfunction [20]. In this chain of events, while bursting emerges rapidly and induces irreversible increase in intracellular free Ca^{2+} within 30 min [21], the time course of excitotoxicity-evoked ROS production and mitochondrial damage remains unknown, a result potentially useful to develop future neuroprotective strategies. This issue was investigated in the present report. Furthermore, a role of endogenous acetylcholine (ACh) activity on downstream ROS production and mitochondrial energy metabolism has not been determined during the early phase of excitotoxicity. Thus, the present study explored these questions by applying dihydro-beta-erythroidine (DHβE) and methyllycaconitine (MLA; antagonists against the neuronal α4 and α7 receptor subunits, respectively) during the time frame of 4 h that ensures optimal viability of the brainstem slice preparation in physiological conditions [22].

2. Material and methods

2.1. Ethical approval

The Scuola Internazionale Superiore di Studi Avanzati (SISSA) ethics committee (prot. 3599, 28 May 2012) approved all experiments and treatment protocols carried out in accordance with the European Union rules for animal experimentation. The number of animals used for the present experiments and their suffering were minimized. Experiments were performed with an *in vitro* model of brainstem slices removed from neonatal Wistar rats (postnatal days 2–5; P2-P5) under i.p. urethane anesthesia (10% solution, 0.1 mL injection).

2.2. Slice preparation

Brainstems were cut in ice-cold, oxygenated (95% O2/5% CO2) Krebs solution, containing (in mM): 130 NaCl; 3 KCl; NaH2PO4, 1 CaCl2, 1.5 NaH2PO4, 5 MgCl2, 25 NaHCO3, and 18.5 glucose (pH 7.4; 300–320 mos ml l^{-1}) inside a Vibratome chamber (Leica 1000S, Wetzlar, Germany) [20,23]. Slices (250–450 μm thick) containing the hypoglossal nucleus were independently treated with TBOA (50 μM), TBOA + DHβE (5 μM) + MLA (5 nM), TBOA + nicotine, or nicotine (10 μM) for 0.5, 2, or 4 h at room temperature under continuous oxygenation. These concentrations were selected on the basis of our former neuroprotection experiments [20,21]. As control, untreated slices were processed as described below immediately after cutting procedures (t = 0 h) or after 0.5, 2, or 4 h of incubation in Krebs solution (sham). Measurements of ROS and mitochondrial metabolism were performed at 0.5, 2 and 4 h, i.e. from the earliest time of electrophysiological dysfunction [21] to the maximum slice damage time [20]. Quantification of HM loss after exposure to TBOA for 4 h was performed as described in our former studies [20,23] using an analogous region of interest (ROI).

2.3. Intracellular measurements of ROS generation

To investigate the generation of intracellular free oxygen species, dihydrorhodamine 123 (DHR 123; Molecular Probes, Invitrogen, Carlsbad, CA, USA) was used as a membrane permeable dye that, by oxidation, yields the fluorescence probe rhodamine 123 (Rho 123) [22]. After rapid rinsing in Krebs solution, slices (250 μm thick) were treated with DHR123 (5 μM) and the nuclear dye Hoechst 33342 (10 mg/mL stock from Molecular Probes; dilution 1:1000) for 20 min at room temperature. Hoechst 33342 was used for counterstaining as it emits blue fluorescence once bound to double-stranded DNA. Finally, slices were washed and transferred into a Petri dish (containing Krebs solution) to be examined with a TCS SP2 Leica confocal microscope (20X objective and 2X magnification). For fluorescence imaging of rhodamine 123 (Rho 123, the oxidized form of DHR 123) staining, slices were visualized by excitation at 514 nm and emission at 530–610 nm, while Hoechst 33342 was excited by ultraviolet light (blue fluorescence emission at 460–490 nm). For each slice side and for both hypoglossal nuclei, a 40 μm z- stack (corresponding to one HM plane) was acquired (5 μm step size) to reconstruct an average fluorescence signal that was independently processed by the experimenter (blind to the treatment) with ImageJ software (version 1.44p, W. Rasband, National Institutes of Health, Bethesda, MD, USA). Rho 123 fluorescence was not evaluated for TBOA + nicotine at 0.5 h, and nicotine at 0.5 and 2 h.

2.4. MTT mitochondrial toxicity test

Mitochondrial toxicity test (MTT) is a standard colorimetric assay for assessing cell viability. As described by Mosmann [24] and reported earlier in our laboratory [20], NADPH-dependent cellular oxido-reductase enzymes may be considered a direct index of cell viability and mitochondrial energy metabolism. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which has purple color whose intensity can be quantified. Under continuous oxygenation in Krebs solution, two slices (450 μm thick) were incubated as described above. For this experiment, nicotine (10 μM) or rotenone (1 μM) treatments lasted 4 h. Rotenone was used as a control for its capacity to interfere with the electron transport respiratory chain in mitochondria by inhibiting the transfer of electrons from complex I to ubiquinone [25]. After treatment, at the above indicated time points, slices were incubated with MTT (0.5 mg/mL; Sigma-Aldrich, Saint Louis, MO, USA) for 2 h at room temperature under oxygenation. MTT was firstly dissolved (5 mg/mL) in phosphate buffer (pH 7.4) and then diluted to 0.5 mg/mL in Krebs solution. Later, slices were treated with 0.5 mL hydrochloric acid plus 0.04 M isopropanol and shaken in a roller drum overnight at room temperature. Lysates were then centrifuged at 10,000*g* for 5 min and their absorbance values (wave length = 550 nm) were evaluated with a Bio-Rad microplate reader (model 550, Bio-Rad Laboratories, Poole, UK). Values were normalized with respect to the lysate protein content assayed with the bicinchoninic acid method (Sigma).