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ACTIVITY-DEPENDENT REGULATION OF CALCIUM AND RIBOSOMES IN THE CHICK COCHLEAR NUCLEUS

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Abstract—Cochlea removal results in the death of 20–30% of neurons in the chick cochlear nucleus, nucleus magnocellularis (NM). Two potentially cytotoxic events, a dramatic rise in intracellular calcium concentration ($[Ca^{2+}]_i$) and a decline in the integrity of ribosomes are observed within 1 h of deafferentation. Glutamatergic input from the auditory nerve has been shown to preserve NM neuron health by activating metabotropic glutamate receptors (mGluRs), maintaining both normal $[Ca^{2+}]_i$ and ribosomal integrity. One interpretation of these results is that a common mGluR-activated signaling cascade is required for the maintenance of both $[Ca^{2+}]_i$ and ribosomal integrity. This could happen if both responses are influenced directly by a common messenger, or if the loss of mGluR activation causes changes in one component that secondarily causes changes in the other. The present studies tested this common-mediator hypothesis in slice preparations by examining activity-dependent regulation of $[Ca^{2+}]_i$ and ribosomes in the same tissue after selectively blocking group I mGluRs (1-Aminoindan-1,5-dicarboxylic acid (AIDA)) or group II mGluRs (LY 341495) during unilateral auditory nerve stimulation. Changes in $[Ca^{2+}]_i$ of NM neurons were measured using fura-2 ratiometric calcium imaging and the tissue was subsequently processed for Y10B immunoreactivity (Y10B-ir), an antibody that recognizes a ribosomal epitope. The group I mGluR antagonist blocked the activity-dependent regulation of both $[Ca^{2+}]_i$ and Y10B-ir, but the group II antagonist blocked only the activity-dependent regulation of Y10B-ir. That is, even when group II receptors were blocked, stimulation continued to maintain low $[Ca^{2+}]_i$, but it did not maintain Y10B-ir. These results suggest a dissociation in how calcium and ribosomes are regulated in NM neurons and that ribosomes can be regulated through a mechanism that is independent of calcium regulation.

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Abbreviations: AIDA, 1-Aminoindan-1,5-dicarboxylic acid; aCSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; eEF-2, eukaryotic elongation factor-2; ERK-2, extracellular signal-regulated kinase 2; FMRP, fragile X mental retardation protein; mGluRs, metabotropic glutamate receptors; MCPG, α-Methyl-4-carboxyphenyl glycine; NM, nucleus magnocellularis; NGS, normal goat serum; PBS, phosphate-buffered saline; Y10B-ir, Y10B immunoreactivity.

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INTRODUCTION

Cochlea removal results in the death of 20–30% of neurons in the chick nucleus magnocellularis (NM) (Born and Rubel, 1985). How and why this subpopulation of neurons dies is not fully understood, though the steps these neurons undergo to meet this fate are reasonably well-documented. The initial stages, occurring within 1–3 h of deafferentation, are defined by increases in intracellular calcium concentration ($[Ca^{2+}]_i$) (Zirpel et al., 1995), as well as changes in ribosomes and ribosome-associated proteins that correspond to reduced protein synthesis (Garden et al., 1995a,b; Hyson and Rubel, 1995; McBride et al., 2013). By 6–12 h, protein synthesis ceases in the dying subpopulation, while the rest of the deafferented NM neurons synthesize proteins at low levels (Steward and Rubel, 1985).

It has been shown that metabotropic glutamate receptor (mGluR) activation by NM neurons' sole glutamatergic input, the auditory nerve, can provide trophic support to prevent modulation of calcium homeostasis and ribosome integrity as well as other apoptosis-associated responses (Zirpel and Rubel, 1996; Hyson, 1998; Nicholas and Hyson, 2004). There are three groups of mGluRs based on their protein sequence and signaling mechanisms. All these receptors activate G-proteins intracellularly to begin complex cascades of events upon binding of glutamate, although they initiate different downstream pathways (see Rondard and Pin, 2015 for a review). In NM neurons, mGluRs have physiological roles (Lu, 2014) and modulate changes in calcium homeostasis via release from intracellular stores or influx from the extracellular environment (Lachica et al., 1995; Kato and Rubel, 1999).

Similar to $[Ca^{2+}]_i$, ribosome structure and function in NM neurons are also regulated by mGluRs. Without afferent activity, NM neurons' protein synthesis fades within one hour of deafferentation (Steward and Rubel, 1985; Born and Rubel, 1988; Hyson and Rubel, 1989). Changes in the ribosomes can be assayed by immunolabeling with Y10B (Y10B-ir), an antibody that recognizes a ribosomal RNA epitope (Lerner et al., 1981; Garden et al., 1995a). Stimulation of group I and II mGluRs with 3, 5-Dihydroxyphenylglycine (DHPG) and LY 354740, respectively, both rescued the negative effect of deafferentation on ribosome structure, as assayed by Y10B-ir (Carzoli and Hyson, 2014), indicating a trophic

role of both groups of mGluRs in regulating ribosomes in NM neurons. Conversely, application of the group I and II mGluR antagonists 1-Aminoindan-1,5-dicarboxylic acid (AIDA) and LY 341495, respectively, reversed the positive effect of auditory nerve stimulation *in vitro* (Nicholas and Hyson, 2004) and also resulted in cell death of normally innervated NM neurons *in vivo* (Carzoli and Hyson, 2011).

Together, these previous lines of research are in agreement that the loss of mGluR activation leads to the dysregulation of both calcium homeostasis and ribosome integrity. One parsimonious interpretation of these parallel results is that a common mGluR-activated cascade controls both $[Ca^{2+}]_i$ and the ribosomal integrity. This common messenger might influence both components directly, or changes in one component, caused by the loss of mGluR activation, might secondarily cause changes in the other. It has been suggested, for example, that increased $[Ca^{2+}]_i$ following the loss of mGluR activation induces changes in the ribosomes' structure/function via various calcium-signaling mechanisms (Hyson and Rubel, 1995; Zirpel and Rubel, 1996; Zirpel et al., 1998; Nicholas and Hyson, 2004; Carzoli and Hyson, 2014). To test this common-mediator hypothesis, the present study investigates both calcium homeostasis and Y10B-ir in the same tissue following pharmacological manipulations of different groups of mGluRs. If a common mediator (or sequential action) is responsible for both calcium dysregulation and ribosomal dysfunction, then increases in $[Ca^{2+}]_i$ due to receptor antagonism should correlate with deficits in ribosome integrity. This was not always the case in the present series of studies.

EXPERIMENTAL PROCEDURES

Slice preparation

Coronal brainstem slices were prepared from chick (*Gallus gallus*) embryos aged E17–E19 ($N = 37$). Embryonic ages were used because loading of fura-2 dye and imaging of the fluorescent signals are better in the relatively unmyelinated embryonic tissue. Eggs were obtained from a commercial supplier (Charles River, North Franklin, CT, USA) and incubated at Florida State University until the age of preparation. All procedures were approved by the Florida State University IACUC. The procedure for obtaining slices was essentially the same as those used in previous publications (Hyson and Rubel, 1989; Stincic and Hyson, 2011; Carzoli and Hyson, 2014). Embryos were anesthetized by numbing while inside the egg by submersion in ice for 5–10 min. Eggs were then punctured and the embryo was swiftly decapitated with surgical scissors. A thick section of the brain and skull containing the entire brainstem was then removed using a razor blade. This section was transferred to a bath of artificial cerebrospinal fluid (aCSF) bubbled with a gas mixture containing 95% O_2 and 5% CO_2. aCSF consisted of (in mM) 130 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 26 $NaHCO_3$, 1.23 NaH_2PO_4, and 10 dextrose. The brainstem was dissected from the skull and the remainder of the cerebellum and forebrain was removed. The remaining brainstem was glued onto a plastic slide, which was glued onto a microtome stage using cyanoacrylate glue. This apparatus was subsequently placed into a second bath of oxygenated aCSF and 300-μm coronal slices containing the middle portion of NM were taken with a vibrating blade microtome (Campden Instruments, Lafayette, IN, USA).

Slices were then transferred to a third bath of oxygenated aCSF and allowed to incubate for 35–45 min before being transferred to an oxygenated bath of 2.5 ml aCSF, 2% fura-2 AM ester in DMSO (Biotium, Hayward, CA, USA), and 0.25% F-127 pluronic in DMSO for an additional 25–30 min to bulk load cells with fura-2.

Slices were then transferred to the imaging chamber of an Olympus IX51 inverted microscope, which was perfused with 50 ml aCSF, or LY 341495 or AIDA working solutions, which circulated between the chamber and an oxygenated reservoir. Slices incubated in this chamber for 15–25 min prior to data acquisition as setup occurred.

Stimulation and field potential recording

Each slice rested on the floor of a custom-made chamber—a 1-mm-thick glass coverslip—and was held down by a custom-made harp consisting of a bent bar of platinum with parallel braided nylon strings. The tip of a stimulation electrode made of twisted Teflon-coated platinum wire was gently rested on the dorsolateral aspect of the slice, where auditory nerve afferents to NM are thickest. 20-μs pulses were delivered at 5 Hz from a digital stimulator (PG 4000, Neuro Data Instruments Corp., New York, NY, USA) and amplitude and polarity were controlled by a constant current isolator (SIU 90, Neuro Data Instruments Corp., New York, NY, USA). Amplitude was maintained at 0.3 mA for all experiments. Slices were stimulated unilaterally for 1 h.

The recording electrode was a borosilicate glass micropipette filled with aCSF. The electrode was broken slightly at the tip to reduce resistance. After the stimulating electrode was in place, the tip of the recording electrode was placed into the center of the ipsilateral NM. Signals were amplified (P15 A.C. preamplifier, Grass Instrument Co., Quincy, MA, USA) and viewed on an oscilloscope (TDS 340, Tektronix, Beaverton, OR, USA). Positions of the stimulating and recording electrode were adjusted until clear, pre- and postsynaptic potentials were observed on the oscilloscope. Once adequate stimulation of NM neurons was confirmed, the recording electrode was removed from the bath and the stimulating electrode was left in position and remained on throughout the duration of the experiment.

Pharmacology

For mGluR antagonist experiments, stimulated slices were perfused with 50 ml solutions of either the group I antagonist AIDA (100 μM in aCSF, $n = 11$ slices) or the group II antagonist LY 341495 (10 nM in aCSF, $n = 8$