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Abnormal cytoplasmic calcium dynamics in central neurons of a dystonia mouse model

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HIGHLIGHTS

- Hippocampal neurons were cultured from a knock-in mouse model of DYT1 dystonia.
- Changes in cytoplasmic calcium concentration were assessed by fluorescence imaging.
- Stimulus-induced transients decayed slowly in neurons of heterozygous-mutant mice.
- This effect was dependent on activation of ionotropic glutamate receptors.

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ABSTRACT

Increased activities of cytoplasmic calcium and the excitatory neurotransmitter glutamate have been independently implicated in dystonia pathophysiology. However, cellular-level evidence linking these two features is not available. Here we show that glutamate-dependent changes in neuronal calcium dynamics occur in a knock-in mouse model of DYT1 dystonia, the most common hereditary form of this disorder. Fluorescence-based analysis of the dynamics of cytoplasmic calcium concentration ($[Ca^{2+}]_c$) in cultured hippocampal neurons shows that electrical stimulation depolarizes the neurons and increases the dendritic $[Ca^{2+}]_c$, which then decays slowly to the pre-stimulus level. Whereas the peak amplitude of $[Ca^{2+}]_c$ was not affected, the decay period was prolonged in neurons of heterozygous mice whose genotype reflects the human condition. We found that this effect was blocked by the antagonists of ionotropic glutamate receptors, and confirmed that glutamate receptors are present in these neurons. As the $[Ca^{2+}]_c$ is readout and regulator of neuronal excitability, its abnormality represents an important cellular phenotype of dystonia

1. Introduction

Dystonia is characterized by sustained or repetitive involuntary muscle contractions that cause twisting movements. Despite the fact that it is a debilitating and relatively common movement disorder [9], its pathophysiology remains elusive [4,24,38]. Brains of patients with primary dystonia do not show signs of neuronal death [14,32,37], suggesting that the abnormalities underlying the disease pathophysiology are partly at the functional level [5].

Certain indirect lines of evidence have indicated that dystonia may be caused by dysregulation of the cytoplasmic concentration of the fundamental second messenger Ca^{2+} ($[Ca^{2+}]_c$). In wild-type neurons, the $[Ca^{2+}]_c$ increases following activation of voltage-dependent Ca^{2+} channels (VDCCs) [27,41] and ionotropic glutamate receptors [23]. In mice of both wild-type and "tottering" (harboring a missense mutation in P/Q-type VDCCs) genotypes, dystonia-like motor features can be induced by applying either agonists of the L-type VDCCs [6,17] or caffeine (known to increase $[Ca^{2+}]_c$ by stimulating Ca^{2+}-release from intracellular Ca^{2+} stores [31]). Notably, similar features can be triggered by applying glutamate-receptor agonists [10,29], suggesting a link between Ca^{2+}- and glutamate-mediated signaling in this disorder. However, the $[Ca^{2+}]_c$ has not been studied directly within brain neurons of dystonia models, leaving unclear how the $[Ca^{2+}]_c$ and glutamate receptors contribute to the disease pathophysiology.

We have tested the hypothesis that activation of the glutamatergic system leads to abnormal $[Ca^{2+}]_c$ dynamics in neurons of a mouse model of DYT1 dystonia. This is the most common hereditary form of dystonia, inherited in autosomal-dominant fashion, and caused by a deletion mutation that affects a glutamic-acid residue in torsinA (ΔE-torsinA) [25], a protein whose functions are poorly understood [5,12,16]. Using cultured hippocampal neurons from ΔE-torsinA knock-in mice [11,18,19], we evaluated transient changes in $[Ca^{2+}]_c$ when the neurons were depolarized by electrical field stimulation. We also tested the involvement of glutamate receptors in $[Ca^{2+}]_c$ dynamics by treating the neurons with

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competitive antagonists of AMPA and NMDA subtypes of ionotropic glutamate receptors.

2. Materials and methods

2.1. Neuronal preparation

Animal care and procedures were approved by the University of Iowa Animal Care and Use Committee. Hippocampal neurons were cultured from wild-type, heterozygous and homozygous ΔE-torsinA knock-in mice [11], on postnatal days 0–1, after genotyping (EZ BioResearch, St. Louis, MO) as described previously [18,19].

2.2. Ca^{2+} imaging

On 14–16 days *in vitro*, the neurons were loaded with $[Ca^{2+}]_c$ indicator Fluo-5F-AM (1 μM, Invitrogen, Carlsbad, CA) for 10 min at 37 °C, rinsed with dye-free Tyrode solution (in mM: 125 NaCl, 2 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 30 glucose, 25 HEPES, 310 mOsm, pH 7.4) for 10 min at room temperature, and imaged in the same solution in an imaging chamber equipped with stimulation electrodes (RC-21BRFS, Warner Instruments, Hamden, CT) at room temperature.

Neurons were imaged at 100 frames/s on an inverted microscope (Eclipse-TiE, Nikon, Melville, NY), using a 40× objective lens (NA1.30), a filter cube (490/20-nm excitation, 510-nm dichroic-long-pass, 520-nm-long-pass emission), and an EMCCD camera (iXon^{EM}+ DU-860, Andor Technology, Belfast, UK). Fluo-5F was excited using a 490-nm light-emitting diode (LED, CoolLED-Custom Interconnect, Hampshire, UK) at 5% of maximal intensity. Exposure time was minimized to 9.67-ms by limiting illumination to the period of image capture (LED was triggered by digital output from camera). Neurons were imaged for a total of 2 s, i.e. 1 s before and 1 s after stimulation.

Biased acquisition of fluorescence images was avoided by preselecting neurons for imaging based on DIC optics, i.e., in the absence of prior information about fluorescence images. Neurons were selected based on the following indicators of good health: a clear cellular margin, extended dendrites, a uniform glial layer, a lack of clustered somata, and a lack of bundled neurites. Fluorescence images were acquired only afterward. In each image, a neuronal soma was placed near the center.

$[Ca^{2+}]_c$ transients were induced by applying one pulse of field stimulation with 1-ms constant current at 30 mA, 1 s after image acquisition was initiated, using a pulse generator (Master-8, AMPI, Jerusalem, Israel) and an isolated stimulator (DS3, Digitimer, Hertfordshire, UK). The timing of field stimulus was confirmed in a separate experiment in which illumination was triggered using the pulse that controls field stimulation, and measuring fluorescence intensity [42]. Images taken during 1 s before stimulation were averaged to determine the pre-stimulus baseline intensity (F_0). The change in $[Ca^{2+}]_c$ was expressed as the fold-change in fluorescence intensity (F) from baseline (F_0): $(F - F_0)/F_0 = \Delta F/F_0$. Statistical significance was assessed using the non-parametric Kolmogorov–Smirnov test [19] or paired Student's t-test, with two-tailed p values.

The following blockers were prepared in Tyrode solution: tetrodotoxin (TTX, 1 μM, Tocris Bioscience, Ellisville, MO); cadmium ion ($CdCl_2$, 200 μM); 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM, Tocris Bioscience) and DL-2-amino-5-phosphonopentanoic acid (DL-AP5, 50 μM, Tocris Bioscience).

In preparing Tyrode solutions of modified extracellular Ca^{2+} concentrations ($[Ca^{2+}]_o$), the Mg^{2+} concentration ($[Mg^{2+}]_o$) was adjusted such that $[Ca^{2+}]_o + [Mg^{2+}]_o = 4$ mM. Ca^{2+}-free solution was prepared by further adding ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA, 1 mM) to 0-mM Ca^{2+} solution.

2.3. Immunocytochemistry

Immunocytochemical detection and image analysis of GluA1 subunit of AMPA receptor and of vesicular glutamate transporter 1 (VGLUT1) were carried out according to our protocol [19]. Briefly, the cultured neurons were fixed, blocked and permeabilized. Thereafter they were treated with polyclonal, guinea-pig anti-VGLUT1 antibody (AB5905, Chemicon-Millipore, Billerica, MA) (1000×-dilution), and polyclonal, rabbit anti-glutamate receptor 1 antibody (AB1504, Chemicon-Millipore) (400×-dilution) overnight at 4 °C. Following rinsing, the neurons were incubated with goat anti-guinea-pig IgG antibody conjugated with Alexa Fluor 594 (Invitrogen) (1000×-dilution), and goat anti-rabbit IgG antibody conjugated with Alexa Fluor 405 (Invitrogen) (1000×-dilution) for 60 min at room temperature. They were imaged using a 40× objective lens and an interline-CCD camera (Clara, Andor Technology).

2.4. Chemical reagents

All chemical reagents were purchased from Sigma–Aldrich unless otherwise specified.

3. Results

3.1. Induction of $[Ca^{2+}]_c$ transients in hippocampal neurons

In order to visualize $[Ca^{2+}]_c$ dynamics, we depolarized the neurons by applying a pulse of electrical field-stimulation to neurons treated with Fluo-5F-AM. $[Ca^{2+}]_c$-representing fluorescence intensity increased in neuronal dendrites, reached a peak, and decayed slowly (Fig. 1A and B). Such a change was not observed in glial cells (Fig. 1B). The $[Ca^{2+}]_c$ transients were dependent on $[Ca^{2+}]_o$, increasing in amplitude as the $[Ca^{2+}]_o$ was increased (Fig. 1C).

We evaluated whether the mechanisms underlying induction of the transients were different in mutant and wild-type neurons, by measuring the peak amplitudes (Fig. 1D). In neurons of three genotypes, the $[Ca^{2+}]_c$ transients were blocked by: applying tetrodotoxin, a blocker of voltage-dependent Na^+ channels (Fig. 1E); applying cadmium, a blocker of VDCCs (Fig. 1F); or eliminating extracellular Ca^{2+} (Fig. 1G). The effects of these treatments were similar in neurons of all three genotypes ($p < 10^{-5}$, treatment vs. pre-treatment control, $n = 16$–23 dendrites/genotype, paired t-test) (Fig. 1E–G). These results indicate that these cells share the following sequential process for generating $[Ca^{2+}]_c$ transients: electrical stimulation → membrane depolarization → opening of voltage-dependent Na^+ channels → generation of an action potential → opening of VDCCs → Ca^{2+} influx from the extracellular solution into the cytoplasm → increased $[Ca^{2+}]_c$.

The amplitudes of $[Ca^{2+}]_c$ transients in heterozygous and homozygous neurons were also similar to those of wild-type neurons ($p > 0.3$, Kolmogorov–Smirnov test, Fig. 2A). This was the case even when AMPA and NMDA receptors were antagonized by CNQX and AP5, respectively ($p > 0.1$, Fig. 2B). These results suggest that glutamate receptors are only minimally involved in regulating the amplitude of Ca^{2+} signal in our system.

3.2. Changes in the $[Ca^{2+}]_c$ transients of mutant neurons

A genotypic difference is that, relative to the decay from the peak in wild-type neurons (Fig. 2C, black trace), that in heterozygous neurons was slower (red) and that in homozygous neurons was faster (green). The differences in the decay time constants (τ) were statistically significant (*in both cases, $p < 0.05$ when