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Voltage-gated calcium channel types in cultured *C. elegans* CEPsh glial cells

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ABSTRACT

The four cephalic sensilla sheath (CEPsh) glial cells are important for development of the nervous system of *Caenorhabditis elegans*. Whether these invertebrate glia can generate intracellular Ca^{2+} increases, a hallmark of mammalian glial cell excitability, is not known. To address this issue, we developed a transgenic worm with the specific co-expression of genetically encoded red fluorescent protein and green Ca^{2+} sensor in CEPsh glial cells. This allowed us to identify CEPsh cells in culture and monitor their Ca^{2+} dynamics. We show that CEPsh glial cells, in response to depolarization, generate various intracellular Ca^{2+} increases mediated by voltage-gated Ca^{2+} channels (VGCCs). Using a pharmacological approach, we find that the L-type is the preponderant VGCC type mediating Ca^{2+} dynamics. Additionally, using a genetic approach we demonstrate that mutations in three known VGCC α_1-subunit genes, *cca-1*, *egl-19* and *unc-2*, can affect Ca^{2+} dynamics of CEPsh glial cells. We suggest that VGCC-mediated Ca^{2+} dynamics in the CEPsh glial cells are complex and display heterogeneity. These findings will aid understanding of how CEPsh glial cells contribute to the operation of the *C. elegans* nervous system.

1. Introduction

With only 302 neurons and 50 glial cells in its nervous system, *Caenorhabditis elegans* has served as a major model organism for neurobiological research. The simplicity of the *C. elegans* nervous system, along with its almost morphologically identical appearance between individual worms, allowed reconstruction at the ultrastructural level with mapping locations of individual synapses [1–3]. Only recently, however, *C. elegans* has emerged as an invertebrate model for studying glial cells (reviewed in [4]). A subset of glial cells in *C. elegans*, the sheath glia of the cephalic sensilla (CEPsh glia), possess some anatomical and functional characteristics that parallel those of astrocyte and oligodendrocyte lineages in the mammalian nervous system (reviewed in [4]). These invertebrate glia may represent in phylogeny an ancestral glial type, perhaps performing more ordinary rather than specialized macroglial functions [5].

Protoplasmic astrocytes occupy distinct domains within the mammalian central nervous system (CNS), parceling the grey matter, through a process referred to as "tiling", into more or less independent structural units [6,7]. Within their individual domains astrocytes have extensive morphological interactions with neurons. In rodents, astrocytes may contact 4–8 neurons and surround ~20,000–120,000 synapses [6,8], while a human astrocyte, due to its territorial expansion, integrates over ~2 million synapses. At synapses, astrocytes bi-directionally communicate to neurons leading to the concept of a functional tripartite synapse [9]. This communication requires increases in astrocytic intracellular Ca^{2+} concentration $[Ca^{2+}]_i$, which can have various spatiotemporal patterns, including oscillatory, intermediate and sustained temporal changes (reviewed in [10,11]).

Oligodendrocytes, the second major class of mammalian neuroglia, are pivotal for the establishment and maintenance of structure and function of white matter. Oligodendrocytes actively and bi-directionally communicate with neurons to achieve a precise information transfer via long axonal processes (reviewed in [12,13]). Such oligodendroglia–neuron interactions are developmentally regulated. Intracellular Ca^{2+} signaling has been implicated in oligodendrocyte membrane sheet retractions [14], and process extension [15] of these glial cells and their precursor cells (OPC) [16].

Similarly to astrocytes, the four CEPsh glial cells of *C. elegans* engulf the nerve ring with non-overlapping membrane extensions. The nerve ring is densely populated with synaptic contacts established between sensory neurons, interneurons and motor neurons. Additionally, each CEPsh glial cell sends a long anterior process that closely interacts with the dendritic extension of a nearby CEP neuron. Indeed, the ablation of CEPsh glial cells results

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in axon guidance defects [17]. CEPsh glial cell development has similarities with some aspects of mammalian oligodendrocyte lineage differentiation. The *C. elegans hlh-17* gene, which encodes a basic helix–loop–helix transcription factor, has homology to the human *Olig1* and *Olig2* genes expressed in oligodendrocytes [17,18]. HLH-17 is most highly expressed in the four CEPsh glial cells. Although CEPsh glial cells possess morphology and functional attributes reminiscent of their mammalian "cousins", whether CEPsh glial cells can exhibit a hallmark of mammalian glial cell excitability, intracellular Ca^{2+} dynamics/changes, has not yet been determined.

Immature astroglia and OPCs/immature oligodendroglia can respond to depolarization with Ca^{2+}-mediated currents that require activity of voltage-gated Ca^{2+} channels (VGCCs) ([19–21]; also reviewed in [22,23]). *C. elegans* possess three known genes encoding VGCC α_1 subunits, *egl-19*, *cca-1*, and *unc-2*, expression of which contributes to VGCC properties corresponding to L-, T-, and N, P/Q, R-type, respectively [24–26]. We sought to understand whether the CEPsh glial cells respond to depolarization with intracellular Ca^{2+} changes mediated by VGCCs. To address this issue, we generated transgenic worms expressing the genetically encoded fluorescent cytosolic Ca^{2+} indicator GCaMP2.0 solely in the four CEPsh glial cells from the embryo through adulthood. We prepared transgenic *C. elegans* cell culture, which allowed us to apply a depolarizing stimulus to CEPsh glial cells apart from the tangle of neurons, and muscle cells in which they exist *in vivo*. Using a combination of acute pharmacological and chronic genetic approaches, we present evidence for a functional role of all three known VGCC types in various aspects of CEPsh glial cell intracellular Ca^{2+} dynamics. Future work will need to investigate whether such intracellular Ca^{2+} dynamics in CEPsh glial cells can affect neuronal activity and exhibit influences on behavior.

2. Materials and methods

2.1. C. elegans strains

All worms used in this study were derived from the parental transgenic strain VPR108 co-expressing the genetically encoded intracellular red fluorescent marker mCherry and the green fluorescent cytosolic Ca^{2+} indicator GCaMP2.0 solely in the four CEPsh glial cells, from the embryo through adulthood. We described the production of this transgenic strain in detail elsewhere [27]. Briefly, plasmids used to generate the VPR108 strain contained the 2.5 kbps 5′ of the translation start site of the *hlh-17* gene (as originally described in [18]) copied from genomic DNA. This promoter (*hlh-17*p) was used for construction of *hlh-17*p::mCherry and *hlh-17*p::GCaMP2.0 plasmids to drive transcription of genes encoding mCherry (gift from K. Oegema, University of California, San Diego; [28]) or GCaMP2.0 (kindly provided by J. Nakai, Saitama University Brain Science Institute, Saitama, Japan; [29]). We microinjected the wild-type *C. elegans* variety Bristol, strain N2 worms with these plasmids and the resulting transgene array (*vprEx108*) was integrated with gamma irradiation. Worms with the integrated array (*vprIs108)* were back-crossed to N2 worms four times producing the VPR108 strain, which was then used as a background line to cross with the following mutant strains received from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN, www.cbs.umn.edu/CGC): (i) L-type voltage-gated Ca^{2+} channel (VGCC) α_1 subunit reduction-of-function (*rf*) mutant DA1006 *egl-19(ad1006) IV*, (ii) T-type VGCC α_1 subunit partial deletion knock-out (KO) VC39 *cca-1* (*gk30*) X, and (iii) N, P/Q, R-type VGCC α_1 subunit truncation KO CB55 *unc-2 (e55) X*. All worms were grown on nematode growth medium (NGM) plates [30] at room temperature (∼20 °C) and fed with OP-50 *E. coli*.

2.2. Cell culture

We prepared *C. elegans* cell culture using a modification [27] of the originally described procedure [31]. We used the cell-specific expression of fluorescent markers based on the *hlh-17* gene promoter for identification of CEPsh glial cells in culture. Briefly, NGM plates containing mainly gravid adult worms were rinsed with M9 solution containing (in mM): KH_2PO_4 (22), NaH_2PO_4 (40), NaCl (86) and $MgSO_4$ (1) in water. Collected worms were spun (bench-top mini-centrifuge, 5 s) and treated with hypochlorite solution (20% v/v household bleach and 80% v/v of 625 mM NaOH in water) on a rotisserie mixer to obtain eggs. Following centrifugation (bench-top mini-centrifuge, 5 s), the resulting egg mass was resuspended in egg buffer solution (EBS) containing (in mM): NaCl (118), KCl (48), $CaCl_2$ (2), $MgCl_2$ (2), and HEPES (25) in water; pH 7.3. The eggs were enzymatically treated with chitinase (40 U/mL; from *Onchocera volvulus*, New England Biosciences, Ipswich, MA) for ∼1 h to release the majority of embryos. At this juncture the embryos and the "uncracked" eggs were triturated to obtain a cellular suspension, which was filtered through a 5 μm pore size syringe filter (Millex®-SV Low Protein Binding Durapore®, Millipore Corp., Bedford, MA) to elute cells and remove larger debris. The resulting filtered cells in EBS were diluted with complete culturing media to a 1:1 ratio, and plated onto polyethyleneimine (PEI, 1 mg/mL)-coated round (12 mm in diameter) glass coverslips. The complete culturing media was composed of Leibovitz's L-15 media (without phenol red; Cat. No.: 21083-027, Invitrogen Corp., Carlsbad, CA), supplemented with fetal bovine serum (10% v/v; Thermo Scientific HyClone, Logan, UT), penicillin/streptomycin (100 IU/mL/100 μg/mL), D-glucose (20 mM) and L-glutamine (2 mM); osmolarity was adjusted to 335–345 mOsm/kg with sucrose. After incubation for 3 hours to allow cell attachment to the PEI-coated coverslips, they were rinsed with media, which was aspirated to remove loose cells, and then fresh media was applied. Cells were grown on coverslips in plastic Petri dishes (35 mm in diameter) each receiving 2–3 such coverslips seeded with *C. elegans* cells. Culture was maintained at room temperature in a humidified chamber/box in ambient air atmosphere, shielded from light and air currents. Cultured cells were used for experiments between 4 and 14 days after plating with their media being replaced every 4 days.

2.3. Imaging acquisition and processing

All experiments were done at room temperature (∼20 °C). Individual worms were immobilized using sodium azide solution (20 mM), deposited onto a glass coverslip and imaged. Cultured CEPsh glial cells on glass coverslips were imaged while bathed in external solution containing (in mM): NaCl (145), KCl (5), $MgCl_2$ (1) $CaCl_2$ (2), D-glucose (5) and HEPES (10) in water; osmolarity was adjusted to 340 mOsm/kg with sucrose, pH 7.3. CEPsh cells that were in direct contact with other cultured cells were excluded from experiments. We used an inverted microscope (IX71, Olympus) equipped with wide-field epifluorescence and differential interference contrast (DIC) illumination. Visualization of GCaMP2.0 was accomplished using a standard fluorescein isothiocyanate (FITC) filter set, while a standard Texas Red (TXR) filter set was used for imaging mCherry. A standard 4′,6-diamidino-2-phenylindole (DAPI) filter set was used to assess autofluorescence of cells. In a subset of experiments, we simultaneously visualized GCaMP2.0 and mCherry using a dual EGFP/DsRed filter set in conjunction with the dual-view DV2 emission splitting system (Photometrics, Tucson, AZ) enabling acquisition of two spatially identical but spectrally distinct images routed on its own half of the camera's imaging array. All filter sets were from Chroma Technology Corp., Bellow Falls, VT. We refer to them in the text as blue (DAPI), green (FITC or EGFP) and red (TXR or DsRed) channels, unless specifics are