Spontaneous calcium waves in granule cells in cerebellar slice cultures

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HIGHLIGHTS

- Slice cultures of the postnatal cerebellum show calcium waves.
- Calcium waves pass through NeuN-positive granule-like cells.
- Calcium waves are blocked by TTX and NBQX, but persist in MK-801.
- Local putative transglial ATP-driven calcium waves are also present.
- Waves may be related to processes that ensure correct postnatal wiring of the cerebellar circuits.

ARTICLE INFO

ABSTRACT

Multiple regions in the CNS display propagating correlated activity during embryonic and postnatal development. This activity can be recorded as waves of increased calcium concentrations in spiking neurons or glia cells, and have been suggested to be involved in patterning, axonal guidance and establishment of synaptic transmission. Here, we used calcium imaging in slice cultures of the postnatal cerebellum, and observe spontaneous propagating calcium waves in NeuN-positive granule-like cells. Wave formation was blocked by TTX and the AMPA antagonist NBQX, but persisted after NMDA receptor blockade with MK-801. Whole-cell recordings during wave formation showed cyclic EPSP barrages with an amplitude of 10–20 mV concurrent with wave activity. Local non-propagating putative transglial waves were also present in the cultures, and could be reproduced by pressure application of ATP. We hypothesize, that the propagating wave activity is carried through the tissue by axonal collaterals formed by neighboring granule cells, and further suggest that the correlated activity may be related to processes that ensure correct postnatal wiring of the cerebellar circuits.

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1. Introduction

Waves of correlated activity in neighboring neurons is a hallmark of the developing brain in many CNS regions, and during the embryonic period and postnatally these waves can pass through several neighboring areas of the CNS [1,2]. The correlated activity can be evoked by stimulation of afferent input to the brain, but also occur as spontaneously repetitive events, suggesting that intrinsic oscillators are involved. The exact function of this activity is still unknown, but might relate to growth, synaptic refinement [3], synaptic strength [4], and axonal guidance [5]. Thus, for example large-scale oscillatory waves of correlated activity, recorded as waves of increases in intracellular calcium, are present in the neocortex [6]. These calcium waves propagate relatively slowly, depend on sodium action potentials and activation of glutamate receptors [6,7], and co-exist with other non-propagating spontaneous oscillatory activity in the cortex [8–10]. Several functions have been ascribed to this wave activity, such as involvement in long-distance wiring, synaptic maturation, regulation of intrinsic ion channel properties, but none of these functions have yet found direct experimental support.

Local coordinated multicellular increases in calcium have also been observed in the cerebellum, where they take the form of radially expanding calcium waves that pass through specialized Bergman glia cells [11]. These transglial calcium waves are dependent on activation of purinergic receptors, are likely induced by local release of ATP, and increase with age as well as low resting brain oxygen tension [11,12]. Motor behavior is associated with calcium increases in networks of Bergman glia, which suggest that the coordinated calcium activity may relate to brain dynamics or blood flow [13]. Propagating calcium waves are found in Purkinje neurons in the early postnatal period, are mediated a depolarizing action of GABA via local axonal collaterals, and has been suggested to be involved in proper development of mature cerebellar circuits [14].

At the cellular level individual granule cells in the cerebellum of postnatal mice show calcium oscillations with a frequency that is dependent on the position along the migratory pathway and positively correlated with the rate of cell movement [15]. Application of a Slit-2 gradient in front of the leading process of cultured cerebellar granule neurons induces a propagating calcium wave from the growth cone to the soma, the subsequent collapse of the growth cone, and redirection of growth direction [16]. Thus, calcium waves in individual granule cells may be important determinants in the directional control of growth, which is particularly prominent in the postnatal period where most of the patterning of cerebellar circuit take place [17]. However, large scale coordinated calcium waves in multiple granule cells in the developing cerebellum have not been reported. Here, we report that calcium waves are observed in cerebellar slice cultures, and these waves pass through thousands of granule cells mediated by AMPA receptor based synaptic transmission.

2. Materials and methods

2.1. Slice cultures, and Fluo-8,AM loading

All experiments and procedures were approved by the Department of Experimental Medicine, and according to procedures laid out by Danish Ministry of Justice and the Danish National Committee for Ethics in Animal Research. Neonate (P0.5–P8.5) NMRI mice were anaesthetized with isoflurane, and killed with a cut across the thorax. The neuraxis was removed by dissection in ice cold Gey's Balanced Salt Solution, and the brainstem and the cerebellum were isolated. 400 μm brainstem slices were cut in the transversal plane at the level of the inferior olive, and 400 μm cerebellar slices were cut in the saggital plane using a home built tissue chopper. One cerebellar slice (monoculture), or one brainstem slice and two cerebellar slices juxtaposed to the lateral borders of the brainstem slice (co-culture), was transferred to sterile porous membrane units (Millicell 30 mm CM PTFE 0.4 mm, Millipore, Bedford, USA). The membrane unit was placed in a 6-well plate (#3516, Corning Inc., NY, USA) containing 1 ml culture medium/well. The culture medium consisted of 50% Minimum Essential Medium with Earle's salts, 25% Hanks Balanced Salt Solution, 25% heat inactivated horse serum, 1 mM L-glutamine, 3.5 mM glucose, 10 mM HEPES, 200 U/ml penicillin, and 50 μg/ml streptomycin. pH was adjusted to 7.25 with HCl or NaOH. Cultures were exposed to 5 μM MK-801 for 24 h following explantation to reduce trauma-induced excitoxicity. Slices were cultured at the interface between the membrane units in a humidified atmosphere of 95% air/5% CO_2 at 35 °C (CO_2 incubator). The culture medium was changed three times a week.

At different days in vitro (DIV) the slices cultures were loaded with the Ca^{2+}-sensitive dye Fluo-8, AM (AAT Bioquest, Sunnyvale, USA) by placing the preparation in a loading solution for 45 min–1 h at room temperature under bubbling with 95% O_2, 5% CO_2. The loading solution was prepared by dissolving 50 μg Fluo-8, AM in 50 μl DMSO, combining 20 μl of this solution with 2.5 μl cremophore EL (Fluka, St. Louis, USA) and 5 μl 20% Pluronic F-127 in DMSO (AAT Bioquest, Sunnyvale, USA), adding these 30 μl to 1 ml ACSF with 100 μM MK-571 (a multidrug resistance transport blocker, Sigma, St. Louis, USA). The final Fluo-8, AM concentration was 20 μM.

2.2. NeuN immunolabeling

In some preparations calcium imaging was followed by overnight fixation (4% paraformaldehyde in 0.1 M Sorensen Phosphate buffer). Preparations were then incubated under constant agitation in diluted NeuN antibody solution (Anti-NeuN Cat# MAB377B, Dilution 1:500, 24 h, +4 °C, Millipore, Billerica, USA).

NeuN immunoreactivity was revealed by a peroxidase reaction using the Vectastain ABC kit (peroxidase standard PK4000, Vector Laboratories, Burlingame, USA).

2.3. Calcium imaging

Optical recordings commenced 20 min after the preparation was placed in the recording chamber, which had a volume of 2 ml, a temperature of 29.0 °C and was constantly superfused at a rate of 2 ml/min with preheated oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid solution (ACSF). The ACSF solution contained (in mM) 129 NaCl, 3 KCl, 5 KH_2PO_4, 22 $NaHCO_3$, 30 D-(+)-glucose, 0.4 $MgSO_4$ and 0.7 $CaCl_2$. For detection of Fluo-8 fluorescence, a metal halide light source (PhotoFluor II, 89 North, Burlington, USA) was coupled to the stereo microscope via a liquid light guide, and appropriate optical filters (Leica GFP3: Excitation 470/40 nm. Barrier: 525/50) were used. Live image stacks were captured by an EMCCD camera (Andor Luca EM S DL-658M, Andor Technology, Belfast, United Kingdom), controlled by the SOLIS software (Andor Technology, Belfast, United Kingdom). Imaging was done using magnification ×40–115, at sampling rates 10–128 frames/s (10 frames/s unbinned, 70, 128 frames/s 2 × 2 binning), in image sessions lasting 6–45 s.

2.4. ATP application

ATP-Mg^{2+} (5 mM dissolved in ACSF, Sigma, St. Louis, USA) was applied by a 500 ms duration 1–5 PSI pressure pulse using a patch pipette placed just below the surface of the tissue. The pressure and timing was controlled by a Toohey Spritzer pressure system IIe (Toohey Company, Fairfield, NJ, USA), and a Master-8 (AMPI, Jerusalem, Israel).

2.5. Whole-cell patch clamp recordings

Putative granule cells were visualized using an Olympus BX51 with an x63 objective. Glass micropipettes were pulled from filamented glass tubes (outer diameter 1.5 mm, inner diameter 0.86 mm, Harvard Apparatus, Holliston, USA) using a PUL-100 micropipette puller (World Precision Instruments, Sarasota, USA) and filled with a solution containing (in mM): 145 potassium D-gluconate, 10 NaCl, 1 $MgCl_2$, 0.01 $CaCl_2$, 0.1 BAPTA, 10 HEPES, 3 ATP(Mg^{2+}), 0.3 GTP (Na^+), pH = 7.3. Recording pipettes were mounted in a HS-2 headstage (Gain 0.01) and current clamp recordings performed with an AxoClamp2B amplifier (Molecular Devices, Sunnyvale, USA). Input resistance was measured by applying a small amplitude (0.05–0.1 nA, 500 ms) hyperpolarizing current pulse from resting membrane potential giving rise to a membrane deflection of no more than 10 mV.

2.6. Analysis and statistics

Analysis of whole-cell patch clamp, and optical recordings was done offline using Igor Pro (Wavemetrics, Lake Oswego, USA), Clampex (Molecular Devices, Sunnyvale, USA), and ImageJ (Open Source, National Institutes of Health, Bethesda, USA, http://rsbweb.nih.gov/ij/). Raw image stacks were partially corrected for bleaching of the Fluo-8 dye, and turned into image stacks showing relative changes in fluorescence (ΔF) by subtracting the Image 1 s before each frame in the stack. Fluorescence output from Fluo-8, AM labeled tissue is dependent on loading time, age of the slice culture, light exposure time, and quenching – all variables that changed substantially from experiment to experiment. For these reasons amplitude calibration bars on ΔF images have little meaning, and have been omitted from the figures. However, the relative changes in the data within one experiment can be distinguished as