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Characterization of activity-dependent changes in flavoprotein fluorescence in cerebellar slices from juvenile rats

Karick Jotty, C. William Shuttleworth, C. Fernando Valenzuela*

Department of Neurosciences, University of New Mexico Health Sciences Center, Albuquerque, NM 87131, USA

HIGHLIGHTS

- We characterized flavoprotein autofluorescence responses in rat cerebellar slices.
- Molecular layer stimulation initially increased fluorescence, which then decreased.
- Responses were abolished by Na^+ channel or AMPA receptor antagonists.
- Neither a $GABA_A$ receptor antagonist nor ethanol affected the responses.
- This technique could be used to study cerebellar effects of pharmacological agents.

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ABSTRACT

Flavoprotein autofluorescence signals attributed to neuronal metabolism have been used to assess synaptic function. Here, we characterized flavoprotein autofluorescence responses in the molecular layer of rat cerebellar slices. High frequency stimulation elicited a transient fluorescence increase (peak phase) that was followed by a longer-lasting fluorescence decrease (valley phase). The peak phase was restricted to the molecular layer, whereas the valley phase extended into the Purkinje cell layer and a portion of the granule cell layer. Responses were abolished by either the Na^+ channel antagonist, tetrodotoxin, or a combination of the AMPA receptor antagonists, NBQX and GIKI-53655, and were also reduced by a flavoprotein inhibitor (diphenyleneiodonium). These findings are consistent with responses being mediated by an increase in mitochondrial activity triggered by increased energy demands evoked by AMPA receptor-mediated synaptic transmission. The $GABA_A$ receptor antagonist picrotoxin did not significantly influence evoked responses. Likewise, exogenous application of ethanol, at concentrations known to increase $GABA_A$ receptor-mediated synaptic transmission at Purkinje cells, did not modify peak responses. These observations indicate that flavoprotein autofluorescence imaging could be useful to assess the coupling between glutamatergic synaptic transmission and neuronal metabolism in cerebellar slices.

1. Introduction

Autofluorescence imaging has been used to investigate regional activation in rodent brain tissues [15,17]. While much prior work has focused on nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate autofluorescence transients seen following ultraviolet excitation [1], a complementary approach with longer wavelengths has proven very useful for *in vivo* brain imaging studies. In a number of brain regions, excitation with blue light (420–480 nm) generates fluorescence transients following synaptic activation that are mainly due to changes in redox potential of flavin adenine mononucleotide- and dinucleotide-linked enzymes involved in the mitochondrial electron transport chain [15]. Mechanical skin stimulation evokes a flavoprotein autofluorescence signal in the primary somatosensory cortex of anesthetized rats [14]. Odor-evoked activity in the olfactory bulb [2] and nociceptive responses in the spinal cord [6] were also visualized in anesthetized rodents using this technique. Electrical stimulation of the cerebellar cortex evoked a biphasic beam-like flavoprotein autofluorescence signal in anesthetized mice consisting of a brief increase in fluorescence, followed by a longer lasting reduction in fluorescence [11–13].

Flavoprotein autofluorescence imaging studies have also been obtained using acute brain slices [17]. Electrical stimulation of

* Corresponding author at: Department of Neurosciences, School of Medicine, MSC08 4740, 1 University of New Mexico, Albuquerque, NM 87131-0001, USA. Tel.: +1 505 272 3128; fax: +1 505 272 8082.
E-mail address: fvalenzuela@salud.unm.edu (C.F. Valenzuela).

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the Schaffer collaterals evokes biphasic flavoprotein autofluorescence responses in the hippocampal CA1 pyramidal region of coronal brain slices from mice [16]. This technique was also used to characterize hippocampal spreading depression induced by hypoxia in brain slices [4]. Tetanic stimulation of layer V evokes stable flavoprotein autofluorescence responses in layer II/III of slices from the rat auditory cortex [14]. These responses were abolished by tetrodotoxin (TTX) and partially blocked by 6-cyano-7-nitroquinoxaline-2,3-dione, indicating that both presynaptic and postsynaptic activity contributes to the responses. Using thick slices from the cerebellar cortex of mice, Coutinho et al. [3] showed that electrical stimulation of the molecular layer (ML) elicited biphasic responses that followed the beam-like path of the parallel fibers. Stimulation of these fibers triggered activity of multiple units in the Purkinje cell (PC) layer with presynaptic and postsynaptic components, suggesting that fluorescence signals are correlated with PC firing. These studies demonstrate the utility of flavoprotein autofluorescence imaging with brain slices to map the activity of neuronal ensembles with good spatial and temporal resolution.

Several laboratories, including our own, have demonstrated that the cerebellum is an important target of ethanol [5,10,18]. Acute ethanol exposure has been shown to have significant effects on PC synaptic transmission, including increased GABA release onto these neurons and potentiation of $GABA_A$ receptor function [7–9].

In this study, we characterized the flavoprotein autofluorescence responses mediated by synaptic transmission between granule cell axons (both ascending segments and parallel fibers) and PCs in parasagittal slices from the cerebellar vermis of juvenile rats. Having established these as robust and reproducible, we tested their sensitivity to pharmacological agents that affect synaptic transmission, including ethanol.

2. Methods

All chemicals were from Sigma (St. Louis, MO) or Tocris Bioscience (Ellisville, MO). All experiments were approved by the University of New Mexico Health Sciences Center Institutional Animal Care and Use Committee. Male Sprague Dawley rats (21–25 day-old) from Harlan Laboratories (Indianapolis, IN) were used for this study. Animals were euthanized by rapid decapitation under deep anesthesia with ketamine (250 mg/kg, I.P.). Brains were rapidly removed and held for 2 min in an ice-cold solution containing (in mM): 220 sucrose, 2 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 12 $MgSO_4$, 10 glucose, 0.2 $CaCl_2$ and 0.43 ketamine pre-equilibrated with 95% O_2/5% CO_2. Cerebellar vermis parasagittal slices (200 μm) were cut in the same sucrose-containing solution using a vibrating tissue slicer (Leica Microsystems, Bannockburn, IL, USA). Slices were then transferred into artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 2 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, 1 $MgSO_4$, 2 $CaCl_2$ and 0.4 ascorbic acid, equilibrated with 95% O_2/5% CO_2, and held for 30 min at 34–35 °C. Subsequently, slices were allowed to recover at 21 °C for at least 30 min before being transferred to a recording chamber perfused with ACSF (2 ml/min) at 32 °C.

Concentric bipolar electrodes (25 μm inner pole diameter and 125 μm outer pole diameter; FHC, Bowdoin, ME) connected to an ISO-Flex stimulus isolator and a Master 8 stimulator (A.M.P.I., Jerusalem, Israel) were used to stimulate granule cell axons in the outer third of the ML. Stimulus trains (50 Hz, 100 μs duration, 0.1–1 mA) were applied at 5 min intervals. Flavoprotein autofluorescence was monitored following excitation at 470 nm using a Polychrome V system (Till Photonics, Grafeling, Germany), as previously described [16]. The fluorescence emission was monitored using a 520–540 nm band pass filter and a 10× water-immersion objective (0.3 NA, Olympus, Center Valley, PA). Optical responses were acquired using a cooled interline transfer charged-coupled device camera (IMAGO Camera; Till Photonics). The frame acquisition rate of the optical response was 3 Hz with an exposure time of 100 ms. Fluorescence data were collected after 4 × 4 binning of the 344 × 260 line image. Individual pixels after binning correspond to an area of 0.27 μm^2. To quantify optical responses, a series of images consisting of 300 sequential frames was acquired. The first 49 pre-stimulus frames of each response were fitted to a double exponential decay phase curve ($R^2 = 0.89 \pm 0.03$; $n = 7$ slices from 4 animals), which was then used to correct evoked flavoprotein autofluorescence responses for bleaching effects. Results are presented

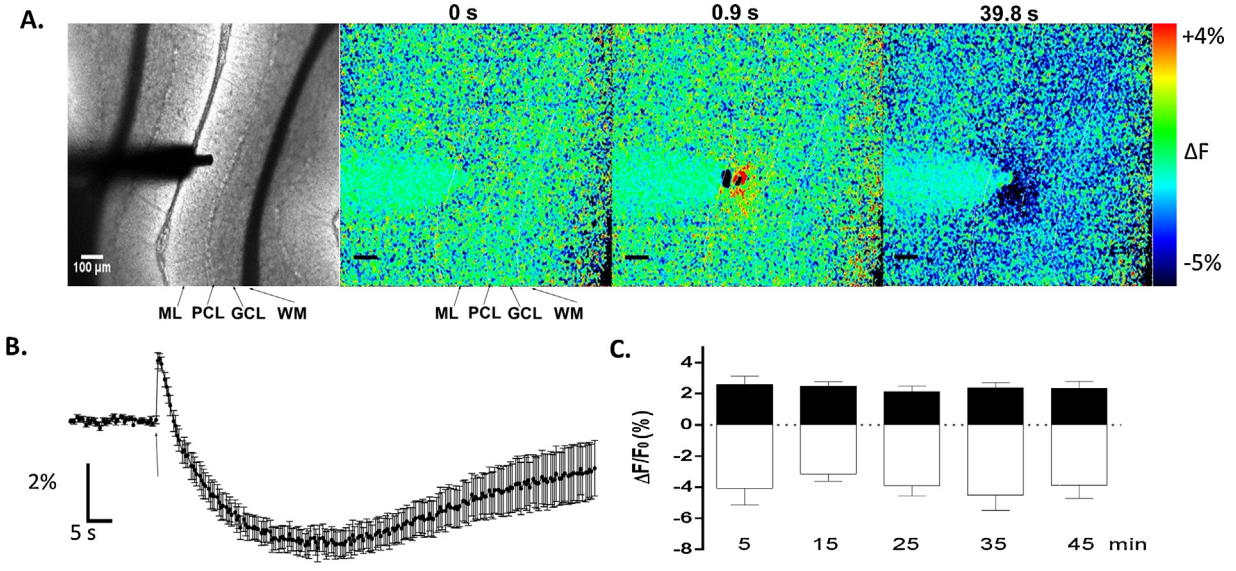

Fig. 1. Characterization of flavoprotein autofluorescence responses in cerebellar slices. (A) Shown in the left panel is a bright-field image of a cerebellar slice showing the molecular layer (ML), granule cell layer (GCL), Purkinje cell layer (PCL), white matter bundle (WM) and the stimulating electrode placement (scale bar = 100 μm). The panels on the right show fluorescence images obtained from the same slice before stimulation ($t = 0$ s) and $t = 0.92$ s corresponding to the peak phase and $t = 39.8$ s corresponding to the valley phase. (B) Average flavoprotein autofluorescence responses illustrating the peak and valley phases ($n = 11$ slices from six animals). The arrow indicates time of stimulation. Data are presented as the mean $\Delta F/F_0$ (%) ± SEM. (C) Average amplitude of the peak (black bars) and valley (white bars) phases as a function of time ($n = 5$ slices from 3 animals). The responses shown in all panels were evoked by a train of 10 pulses (pulse duration = 100 μs) at 50 Hz (stimulation intensity = 600 μA).