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BIDIRECTIONAL MODULATION OF DEEP CEREBELLAR NUCLEAR CELLS REVEALED BY OPTOGENETIC MANIPULATION OF INHIBITORY INPUTS FROM PURKINJE CELLS

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Abstract—In the mammalian cerebellum, deep cerebellar nuclear (DCN) cells convey all information from cortical Purkinje cells (PCs) to premotor nuclei and other brain regions. However, how DCN cells integrate inhibitory input from PCs with excitatory inputs from other sources has been difficult to assess, in part due to the large spatial separation between cortical PCs and their target cells in the nuclei. To circumvent this problem we have used a Cre-mediated genetic approach to generate mice in which channelrhodopsin-2 (ChR2), fused with a fluorescent reporter, is selectively expressed by GABAergic neurons, including PCs. In recordings from brain slice preparations from this model, mammalian PCs can be robustly depolarized and discharged by brief photostimulation. In recordings of postsynaptic DCN cells, photostimulation of PC axons induces a strong inhibition that resembles these cells' responses to focal electrical stimulation, but without a requirement for the glutamate receptor blockers typically applied in such experiments. In this optogenetic model, laser pulses as brief as 1 ms can reliably induce an inhibition that shuts down the spontaneous spiking of a DCN cell for ~50 ms. If bursts of such brief light pulses are delivered, a fixed pattern of bistable bursting emerges. If these pulses are delivered continuously to a spontaneously bistable cell, the immediate response to such photostimulation is inhibitory in the cell's depolarized state and excitatory when the membrane has repolarized; a less regular burst pattern then persists after stimulation has been terminated. These results indicate that the spiking activity of DCN cells can be bidirectionally modulated by the optically activated synaptic inhibition of cortical PCs.

Key words: Purkinje cell, deep nuclear cell, synaptic inhibition, photostimulation, optogenetic model.

INTRODUCTION

The mammalian cerebellum plays an important role in motor control and motor learning, and is also involved in such non-motor functions such as arousal, emotion, and cognition (Leiner et al., 1993; Raymond et al., 1996; Ito, 2001; Stoodley et al., 2012). How such functions are accomplished, either directly by the cerebellum or through its interactions with other brain structures, is not well understood. The fact that its circuitry is remarkably uniform and well-defined in all its subregions and across many species has suggested that the cerebellum performs a single characteristic computation (Ito, 1984; Voogd and Glickstein, 1998; Apps and Garwicz, 2005). Despite great variation in its inputs, one constant is that the sole output projection of the cerebellar cortex is from the inhibitory Purkinje cells (PCs) to neurons of the deep cerebellar nuclei (DCN) and the vestibular nuclei (VN), where this inhibition is integrated with excitation transmitted by collaterals of mossy and climbing fibers (CFs). As DCN cells convey the final cerebellar output signals, how they process synaptic inhibition from PCs is a key factor in determining the result of the cerebellar computation that is sent to premotor and non-motor nuclei in the brainstem and thalamus (Eccles et al., 1967; Welsh et al., 1995; Teune et al., 2000; Bell et al., 2008; Sugihara, 2011).

Despite decades of effort, exactly how PC–DCN neuron interactions occur is far from clear. An outstanding question is how individual DCN cells integrate inhibitory inputs from PCs and excitatory inputs from the brainstem to generate their functional output signals. Such characterization has proved difficult to obtain because PCs and DCN neurons are too far apart in the mammalian cerebellum to allow monosynaptically-connected pairs of neurons to be

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Abbreviations: ACSF, artificial cerebrospinal fluid; AP5, (2R)-amino-5-phosphonovaleric acid; CF, climbing fiber; ChR2, channelrhodopsin-2; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DCN, deep cerebellar nuclei; E_{Cl}, Cl^- equilibrium potential; EGTA, ethylene glycol tetraacetic acid; EPSP, excitatory postsynaptic potential; EYFP, yellow fluorescent protein; $GABA_a$, type a gamma-Aminobutyric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; I_h, hyperpolarization-activated current; IPSCs, inhibitory postsynaptic currents; IPSPs, inhibitory postsynaptic potentials; NMDA, N-methyl-D-aspartate; PC, Purkinje cell; PF, parallel fiber; TTL, transistor-transistor logic; VN, vestibular nuclei.

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recorded simultaneously (McDevitt et al., 1987). As an approximation, studies using dynamic clamp to model PC-triggered conductances in DCN neurons have indicated an important role for PC firing synchrony in determining both the responsiveness of DCN cells to input from excitatory terminals and the timing of these cells' post-inhibitory spiking (Jaeger, 2011; Person and Raman, 2011). That the modulation of PC ensemble activity is likely to play a major role in DCN neuronal computation is reinforced by quantitative anatomy, which has demonstrated that each DCN neuron receives synaptic inputs from ~860 PCs (Palkovits et al., 1977), although there is more recent evidence that this convergence ratio may be more than an order of magnitude less (Person and Raman, 2011). Such real-time synaptic integration within the DCN is believed to be essential to movement coordination. Although an earlier model holds that the activity-dependent modification of synaptic strength, including long-term potentiation (LTP) and depression (LTD), in cortical PCs underlies associative eyeblink conditioning and vestibuloocular reflex (VOR) adaptation (Hansel et al., 2001; Ito, 2001; Jörntell and Hansel, 2006; Pugh and Raman, 2006; Gao et al., 2012), real-time interactions between cortical PCs and DCN cells during movement are also believed to be critically important for motor control and motor learning, as indicated by experimental studies and computational modeling (Mauk and Donegan, 1997; Ohyama et al., 2006; Shutoh et al., 2006).

To further explore and characterize the critical inhibitory synapse between cortical PCs and DCN cells, we have created a mouse model, in which the conditional expression of channelrhodopsin-2 (ChR2), fused with a fluorescent reporter protein, is induced by a $Gad2^{Cre}$ transgene, such that GABAergic neurons, including PCs, can be depolarized by blue light (Nagel et al., 2002; Taniguchi et al., 2011; Madisen et al., 2012; Tye and Deisseroth, 2012). Using cerebellar slices from this model, we have found that brief photostimulation can robustly depolarize PCs and induce a strong inhibition in DCN cells that is very similar to the inhibitory postsynaptic potentials (IPSPs) induced by conventional focal electrical stimulation under pharmacological isolation (Aizenman et al., 1998). We have further shown that the spontaneous firing activity of at least some DCN cells can be effectively modulated by the optical activation of PC axons through the voltage-dependent inhibition, shunting or excitation of the postsynaptic membrane current.

EXPERIMENTAL PROCEDURES

Generation of optogenetic mice for studies of GABAergic PCs

Two parental mouse strains allowing Cre-induced conditional expression of ChR2 from the mouse *Gt(ROSA)26Sor* locus were employed: Ai27, which expresses a ChR2(H134R)-tdTomato fusion protein, and Ai32, which expresses a ChR2(H134R)-yellow fluorescent protein (EYFP) fusion protein (Madisen et al., 2012). These were obtained from Dr. H. Zeng at the Allen Institute for Brain Science. The Cre-driver line $Gad2^{tm2(cre)Zjh/J}$ ($Gad2^{Cre}$) was obtained from The Jackson Laboratory (Taniguchi et al., 2011). The Cre-driver and optogenetic effector transgenic lines were maintained separately on a C57BL/6 genetic background, and were interbred to generate $Gad2^{Cre}$/Ai27 and $Gad2^{Cre}$/Ai32 double-heterozygotes for the experiments described below. It has been well documented that ChR2 is a light-gated nonspecific cation channel expressed in the plasma membranes of target neurons and that it opens on a millisecond timescale upon exposure to blue laser light, leading to the influx of Na^+, K^+, Ca^{2+} and H^+ (Nagel et al., 2002; Madisen et al., 2012). These basic channel properties are also present in the target cells in our model (see below).

Slice preparation

Mice of either sex between P14 and P30 were deeply anesthetized with isoflurane and decapitated. The brain was quickly removed and left in ice-cold oxygenated saline for ~1 min to harden the tissue. After trimming, the cerebellum (with the brainstem attached) was glued to a cutting stage with the back support of an agar block. The cutting tray was filled with oxygenated cold saline (bubbled with 95% O_2 and 5% CO_2) that included (in mM): sucrose 252, KCl 2, $MgCl_2$ 2, $CaCl_2$ 2.6, NaH_2PO_4 1.2, $NaHCO_3$ 26, and glucose 20, with the pH adjusted to 7.4 ± 0.5 and the osmolarity to 315 ± 5 mOsm. After cutting, typically at 200 μm in either the parasagittal or transverse plane, slices were immediately returned to the same solution and maintained in a warm bath (28 ± 0.5 °C) for recovery. After 30–60 min, they were transferred into normal oxygenated artificial cerebrospinal fluid (ACSF) with the same contents as before except for the replacement of sucrose by 126 mM NaCl. Slices were kept at room temperature until recording.

Whole-cell patch recording

Individual slices were placed in a submerged recording chamber and continuously perfused with oxygenated ACSF at a rate of 1–2 ml/min. Recording was done at 31 ± 1 °C. The glass pipettes for patch recording had resistances of 4–8 MΩ after being filled with an internal solution containing (in mM): K-gluconate 132, HEPES 10, $MgCl_2$ 2, EGTA 5, $CaCl_2$ 0.5, ATP 4, GTP 0.5 and phosphocreatine 5, with the pH is adjusted to 7.4 ± 0.5 and the osmolarity to 285 ± 5 mOsm. The internal solution was aliquoted and stored at −20 °C, and filtered before use. To perform perforated patch recordings, gramicidin, an antibiotic that forms pores in the patched membrane that are permeable to cations without disturbing the intracellular Cl^- concentration (Kyrozis and Reichling, 1995), was added to the internal solution (20 μg/ml) which was then filtered before filling the recording pipettes. In some cases, 40 mM K-gluconate in the internal saline was replaced by a molar equivalent of KCl to facilitate the detection of IPSPs and inhibitory postsynaptic currents (IPSCs), as noted.