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Measuring Feedforward Inhibition and its Impact on Local Circuit Function

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Abstract

This protocol describes a series of approaches to measure feedforward inhibition in acute brain slices from the cerebellar cortex. Using whole cell voltage and current clamp recordings from Purkinje cells, along with electrical stimulation of the parallel fibers, these approaches will demonstrate how to measure the relationship between excitation and inhibition in a feedforward circuit. In addition, this protocol will describe how to measure the impact of feedforward inhibition on Purkinje cell excitability, with an emphasis on spike timing.

Materials

Reagents

Artificial cerebrospinal fluid (ACSF)

Carbogen (95% O₂, 5% CO₂)

Cesium methanesulfonate and potassium gluconate recording solutions

Cs-methanesulfonate (Sigma, C1426)

K-gluconate (Sigma, P1847)

HEPES (Sigma, H4034)

EGTA (Sigma, 03777)

TEA-Cl (Sigma, 86616)

MgATP (Sigma, A9187)

NaGTP (Sigma, G8877)

Phosphocreatine-tris2 (Sigma, P1937)

QX314-Cl (Tocris, 2313)

GABAzine (SR95531) (25 mM stock solution) (Tocris, 1262)

NBQX (50 mM stock solution) (Tocris, 1044)

Equipment

Air table with Faraday cage

Amplifier, for voltage and current clamp recording

Data acquisition system

Dissection tools (scissors, forceps, scalpel and spatula) and petri dish with sylgard bottom

In-line solution heater, for recording at near physiological temperature

Microelectrode puller, for glass capillary recording and stimulating electrodes

Micromanipulators, for holding recording and stimulating electrodes

Osmometer, for calibrating solutions

Peristaltic pump, for delivering ACSF and pharmacological reagents to the slice chamber

Slice Recovery chamber

Stimulus isolator, for isolating stimulating electrode from electrical noise sources

Upright microscope with water-immersion 40X objective

Vibrating slicer, for cutting brain slices

Method

1. Follow the protocol entitled “Preparing Brain Slices to Study Basic Synaptic Properties” to produce ACSF and acute rodent cerebellar slices.
2. When dissecting the cerebellum, the initial cut should occur below the cerebellum at the spinal cord level, then continue around the skull until the bone is loose enough to remove with forceps. Gently lift the brain with a flat spatula, block off the cerebellum, and proceed as described in the slicing protocol. For transverse sections of cerebellar cortex, arrange the tissue such that the blade cuts in the direction of the parallel fibers (mediolateral). Glue a small piece of agar behind the tissue to act as a backstop of support during cutting.

Experiment 1: Evoking feedforward inhibition under voltage-clamp

1. These experiments require a cesium-based internal solution with Qx-Cl to allow voltage clamp at depolarized potentials. Follow “Preparing Brain Slices to Study Basic Synaptic Properties” protocol to obtain a whole-cell recording from a Purkinje cell.
2. Place a stimulating electrode in the parallel fibers 200–500 μm away from the recorded Purkinje cell. Stimulating electrode can be another glass micropipette, the same size as was used for Purkinje cell recording. This stimulating electrode should be filled with ASCF, and contain a bare wire attached to one pole of the

stimulus isolation unit. A second wire from the other pole of the isolation unit should be attached to the air table, or can be placed in the bath to minimize stimulus artifacts. In the latter case, the immersed wire should be silver so as not to introduce metal ions that could interfere with recordings.

3. The stimulus isolation unit should be set to “current” mode, and reside within the faraday cage. Care should be taken to avoid any power cables in its vicinity or near the wires traveling to the stimulus electrode, as these will carry electrical noise into the recording chamber.
4. Begin by voltage-clamping the Purkinje cell at -70 mV. Stimulate the parallel fiber pathway with single pulses at 0.1 Hz, gradually increasing stimulation intensity until a small (100 – 300 pA) EPSC appears. Stimulation in the range of a few to 10 s of μ A at 0.2 ms pulse width is usually sufficient to drive parallel fiber activity.
5. To measure feedforward excitation, adjust the membrane potential to find the reversal potential for inhibition (typically -70 to -80 mV with a standard low chloride (3 – 5 mM) internal solution. When adjusted properly, the EPSC should have a mono-exponential decay. Collect 15 – 20 sweeps at the reversal potential for inhibition.
6. For instructive purposes, adjust the membrane potential to a somewhat depolarized value, around -50 mV, where an outward current should develop with a 1 – 2 ms delay following the onset of excitation. At the end of the experiment, pharmacology can be used to isolate a pure EPSC by blocking GABA_A receptors to check that the measured reversal potential was accurate (described below).
7. To isolate the feedforward inhibition, slowly adjust the membrane potential toward 0 mV. Purkinje cells have many active conductances and are notoriously difficult to voltage-clamp well, and an outward holding current will develop as the neuron is depolarized. To identify the EPSC reversal potential, continue to depolarize the neuron while stimulating the parallel fibers until only an outward evoked current remains. Because of the liquid junction potential, this will typically occur around $+10$ mV. Importantly, this outward IPSC should occur with a 1 – 2 ms delay as compared to the previously evoked EPSC.
8. Purkinje cells receive input from molecular layer interneurons, which are spontaneously active. At depolarized potentials, a high rate of spontaneous IPSCs will also be present. These will not contaminate your measurement of evoked IPSCs as long as multiple trials are averaged.
9. After 15 – 20 sweeps have been collected at the reversal potential for excitation, there are two possible options: 1) Apply a selective GABA_A receptor antagonist (SR95531, 5 μ M) to block synaptic inhibition and pharmacologically verify (and correct for errors in) the accuracy of the chosen reversal potentials, or 2) Apply a selective ionotropic glutamate receptor antagonist (NBQX, 5 – 10 μ M) to block

excitation and verify that all evoked inhibition was disynaptic, and not the result of direct activation of interneurons.

- a. Option 1: Apply SR95531 via bath perfusion while the Purkinje cell is still voltage clamped at 0 mV. All evoked and spontaneous inhibition should be blocked. Any small remaining evoked current is the result of mis-estimation of the EPSC reversal potential. Collect several sweeps under these conditions to correct the initially recorded IPSC by subtracting the remaining current. While still in the presence of SR95531, gradually change the membrane potential back to the reversal potential of inhibition measured previously. Again, collect several sweeps for averaging, as this measurement represents the pure, uncontaminated EPSC, which can be compared to what was obtained prior to blockade of GABA_A receptors. Finally, apply NBQX to the bath in addition to SR95531. This will block all remaining current, and provide an average trace with only the stimulation artifact. This can also be subtracted from previous recordings to cosmetically eliminate the electrical artifact.
- b. Option 2: Apply NBQX while recording at the EPSC reversal potential. This manipulation should block all evoked inhibition, but leave spontaneous inhibition intact. This measurement is important to confirm that all evoked inhibition resulted from recruitment of molecular layer interneurons by the parallel fibers and not by direct electrical depolarization of the interneurons via the stimulus electrode.

Experiment 2: Testing the impact of feedforward inhibition on spike timing

1. These experiments require a potassium-based internal solution to allow spiking and perform current-clamp measurements.
2. As before, achieve a whole-cell recording from a Purkinje cell and place a stimulating electrode in the parallel fibers. Then, switch to current clamp mode, but do not inject any current. The Purkinje cell will spike spontaneously, and this should occur at a rate of 20–50 Hz. This spiking should be stable; if the rate continues to increase the recording should be abandoned due to poor quality. A poor quality recording can be confirmed by switching back to voltage-clamp, and checking whether significant holding current (>100 pA) is present at –60 mV.
3. Apply just enough negative holding current to stop the cell from spiking, which should result in a membrane potential near –60 mV. Stimulate the parallel fibers at 0.1 Hz, adjusting the stimulus intensity until action potentials are generated on approximately 30–60% of trials.
4. Apply SR95531 (5 μ M) via bath perfusion while continuing to stimulate at the same frequency and intensity. By blocking feedforward inhibition, the probability of action potential generation should increase. Furthermore, the jitter in action potential timing should increase as the integration time window broadens in the absence of inhibition. A more advanced version of this

experiment would require a second stimulation electrode to activate an independent set of parallel fibers. In this configuration, it is possible to stimulate the second pathway with variable delay with respect to the first pathway, and thus measure the duration of the integration time window in the presence and absence of feedforward inhibition.

Recipes

Cs methanesulfonate recording solution (in mM):

140 Cs-methanesulfonate, 15 HEPES, 0.5 EGTA, 2 TEA-Cl, 2 MgATP, 0.3 NaGTP, 10 phosphocreatine-tris₂, 2 QX 314-Cl. pH adjusted to 7.2 with CsOH.

K gluconate recording solutions (in mM):

150 K-gluconate, 3 KCl, 10 HEPES, 0.5 EGTA, 3 MgATP, 0.5 GTP, 5 phosphocreatine-tris₂, and 5 phosphocreatine-Na₂. pH adjusted to 7.2 with NaOH.

In 100 ml volumetric flask, add about 70 mL of distilled water, and combine above listed chemicals. Remove stir bar, and add water to fill to exactly 100 mL. Stir again, and adjust pH to 7.25. Osmolarity should be ~305 mOsm, and can be adjusted by adding water. Aliquot solutions into 1–1.5 mL Eppendorf tubes, and store at –80 degrees until use (within 1 year).

Figure 1.

Schematic of recording setup and example of evoked feedforward excitation and inhibition.

A) Recording configuration showing voltage-clamp of Purkinje cell (PC), electrical stimulation of parallel fibers (stim) emanating from granule cells (GrC), as well as molecular layer interneurons (MLI) providing feedforward inhibition. B). Feedforward IPSC recorded at 0 mV, and EPSC, and direct parallel fiber (PF) EPSC recorded at -75 mV. C) Higher temporal resolution from B showing average 1.8 ms latency between direct EPSC and feedforward IPSC ($n=12$). Adapted from Hull and Regehr, 2012.