Axonal measurements of calcium implicate $GABA_A$ receptors in mediating parallel fiber excitation

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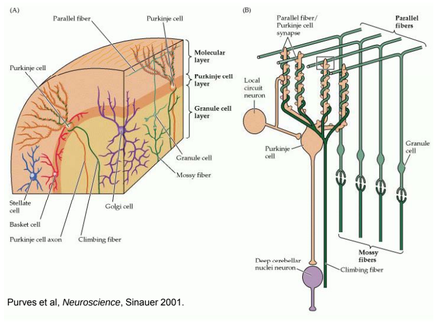
Introduction.

GABA has been found to be excitatory in various contexts [1]. In the cerebellar molecular layer, previous studies [2] have shown that the $GABA_A$ receptor agonist muscimol causes an increase in spontaneous EPSC frequencies in Purkinje cells and interneurons. Parallel fibers are axons of granule cells that excite Purkinje cells and interneurons. We propose that this excitation is caused by the activation of $GABA_A$Rs located on parallel fibers. The NKCC1 transporter accumulates chloride, allowing activation of $GABA_A$Rs to be excitatory during development [3]. The chloride extruding transporter KCC2 is also found in mature neurons [4]. We suspect that KCC2 is localized to granule cell bodies, while NKCC1 are found at their axons, so that excitation of parallel fibers by muscimol can be extinguished by blocking NKCC1 transport. Furthermore, we're interested in characterizing these $GABA_A$Rs pharmacologically to determine their subunit composition. To investigate these issues, we loaded parallel fibers with a calcium indicator using a local perfusion technique [5].

Cerebellar Circuit.



Purves et al, *Neuroscience*, Sinauer 2001.

Methods.

Transverse cerebellar slices (275-300 μm) were cut from 20 to 37 day old Sprague-Dawley rats and C57BL6 mice. The external aCSF contained (in mM) 119 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 25 glucose. Parallel fibers were labeled [5] using a puffer pipette (5-10 μm, less than 10 psi) containing 75 μM Oregon Green BAPTA 1 and a trace amount of Alexa 568, and a suction pipette (20 μm) to form a plume. Single pulses of 250 μsec stimuli were given via a bipolar theta electrode to elicit calcium transients measured by a photodiode.

References.

1. Marty and Llano. (2005). Trends in Neuroscience 28(6):284-289.
2. Stell et al. (2007). J Neuroscience 27(34):9022-9031.
3. Dzhala et al. (2005). Nature Medicine 11(11):1205-1213.
4. Rivera et al. (1999). Nature 397(6716):251-255.
5. Regehr and Atluri. (1995). Biophysical Journal 68:2156-2170.

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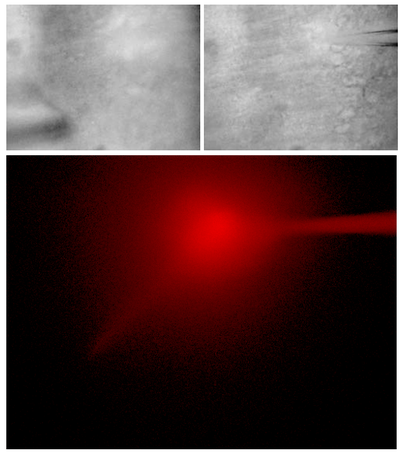
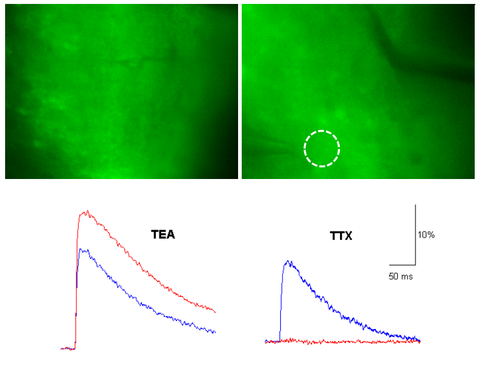
Localized loading of Oregon Green calcium indicator into molecular layer.



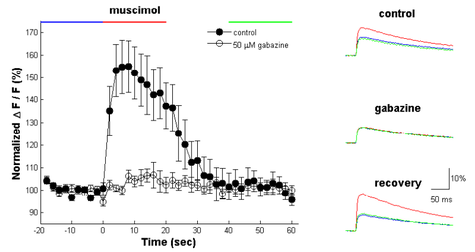
DIC image of injection pipette a third of the way into the molecular layer (top right), and DIC image of suction pipette above the slice diagonal from injection site (top left), forming a fluorescent plume visualized in red using Alexa 568. Plume is maintained for 40 minutes or longer before the experiment.

Stimulation of labeled parallel fibers elicit sodium channel dependent calcium transients.



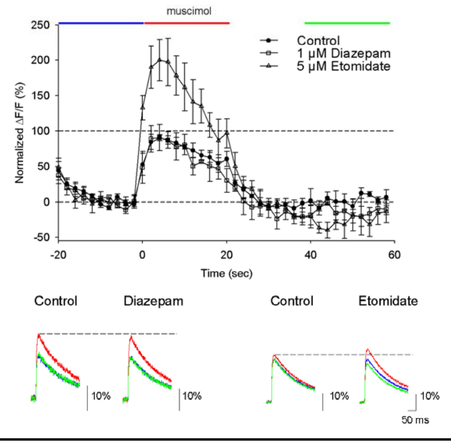
Fluorescent image of parallel fibers labeled by Oregon Green BAPTA 1 (top left), and puffer pipette next to the recording site with the shadow of the stimulation pipette farther away (top right). TTX (red trace) extinguishes calcium transients at the recording site evoked by bipolar stimulation of parallel fibers (lower right). TEA (red trace) enhances calcium transients evoked by bipolar stimulation (lower left). This indicates that the transients are driven by sodium action potentials. Traces are averages of ten successive stimulations. Pretreatment levels are in blue.

Presynaptic calcium transients are enhanced by $GABA_A$R agonist muscimol.



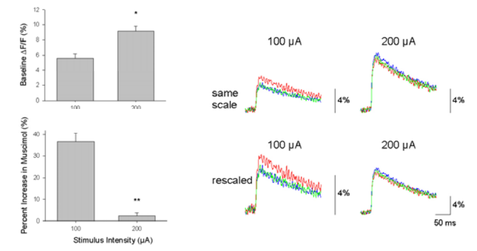
10 μM muscimol applied by pressure pipette to parallel fibers next to the recording site enhances calcium signals evoked by bipolar stimulation ($p<0.001$, $n=10$). This effect is prevented by bath application of $GABA_A$ receptor antagonist gabazine (50 μM). To the right are averaged example traces from a single spot before (blue), during (red), and after (green) muscimol application.

Etomidate but not diazepam, enhances the muscimol-induced potentiation of stimulus-evoked calcium transients.



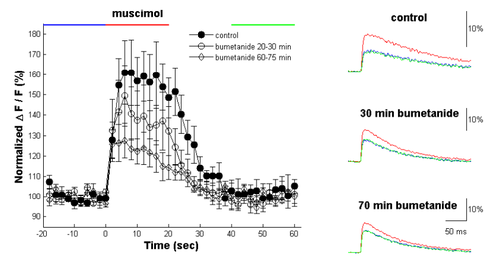
Etomidate but not diazepam enhance the muscimol potentiation of stimulus-evoked calcium transients, suggesting the $GABA_A$ receptor mediating this effect does not contain a gamma subunit. Recordings were made as described in Methods, before (Control) and after washin of either diazepam (1 μM) or etomidate (5 μM) (N=3 experiments). The data on top are plotted so that the muscimol potentiation in the control condition spans 100%. The diazepam and etomidate data are normalized to their respective controls. On the bottom are averaged example traces which are scaled in such a manner so that all baseline calcium transients span the same distance. Neither etomidate nor diazepam reached significance (Paired T-test: *Diazepam*, $p=0.79$; *Etomidate*, $p=0.076$).

Muscimol potentiation of stimulus-induced calcium transients is dependent on stimulus intensity.



Muscimol has little effect on stimulus-evoked calcium transients at high stimulation intensity. Recordings were made as described in methods except the stimulus intensity was set at either 100 or 200 μA. Data are from a single experiment (n=2-3). Both the baseline ΔF/F and the percent increases in muscimol were significantly different based on a Student's T-Test (*, $p<0.05$; **,$p<0.01$).

NKCC1 transporter blocker bumetanide reduces the muscimol-induced potentiation of stimulus-evoked calcium transients.



10 to 20 μM bath-applied bumetanide reduces muscimol induced potentiation of stimulus-evoked calcium signals (30 minutes $p=0.031$, 60 minutes $p=0.027$). Averaged example traces from a single spot before (blue), during (red), and after (green) muscimol application, scaled to the pre-muscimol calcium transient baseline (right).

Conclusions.

Parallel fiber axons can be excited by $GABA_A$ receptor agonists (e.g. muscimol), by bringing additional fibers closer to threshold for firing an action potential.

$GABA_A$ receptors mediating this phenomenon do not appear to contain a gamma subunit.

The excitatory effect of muscimol appears to arise from an altered Cl- gradient due to the activity of the NKCC1 Cl- transporter.

Future experiments will test whether depolarizing GABA on parallel fibers serves a physiological role and determine the subunit composition of the axonal $GABA_A$ receptors mediating this effect.