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## Negative shift in the glycine reversal potential mediated by a $\text{Ca}^{2+}$ and pH-dependent mechanism in interneurons

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### Abstract

Cartwheel cells are glycinergic auditory interneurons which fire  $\text{Na}^+$  and  $\text{Ca}^{2+}$  dependent spike bursts, termed complex spikes, and which synapse on both principal cells and one another. The reversal potential for glycine ( $E_{\text{gly}}$ ) can be hyperpolarizing or depolarizing in cartwheel cells, and many cells are even excited by glycine. We explored the role of spike activity in determining  $E_{\text{gly}}$  in mouse cartwheel cells using gramicidin perforated-patch recording.  $E_{\text{gly}}$  was found to shift toward more negative potentials after a period of complex spiking or  $\text{Ca}^{2+}$  spiking induced by depolarization, thus enhancing glycine's inhibitory effect for about 30 s following cessation of spiking. Combined perforated patch electrophysiology and imaging studies showed that the negative  $E_{\text{gly}}$  shift was triggered by a  $\text{Ca}^{2+}$ -dependent intracellular acidification. The effect on  $E_{\text{gly}}$  was likely caused by bicarbonate- $\text{Cl}^-$  exchanger-mediated reduction in intracellular  $\text{Cl}^-$ , as  $\text{H}_2\text{DIDS}$  and removal of  $\text{HCO}_3^-/\text{CO}_2$  inhibited the negative  $E_{\text{gly}}$  shift. The outward  $\text{Cl}^-$  flux underlying the negative shift in  $E_{\text{gly}}$  opposed a positive shift triggered by passive  $\text{Cl}^-$  redistribution during the depolarization. Thus, a  $\text{Ca}^{2+}$  dependent mechanism serves to maintain or enhance the strength of inhibition in the face of increased excitatory activity.

### Keywords

cochlear nucleus; calcium channel; pH; glycine; auditory; Acid

### INTRODUCTION

Glycinergic and GABAergic synapses typically mediate neural inhibition. Nevertheless, while many produce hyperpolarizing IPSPs, it is well known that glycinergic or GABAergic inputs can be depolarizing, exerting shunting inhibition or excitation in developing or mature neurons (Marty and Llano, 2005; Ben-Ari et al., 2007). This range of effects arises from two characteristics. First, reversal potentials for glycine or GABA-mediated IPSPs ( $E_{\text{gly/GABA}}$ ) vary widely (e.g.  $-85$  mV in Purkinje neurons, Chavas and Marty (2003),  $-37$  mV in some adult hypothalamic neurons, DeFazio et al. (2002)). Second,  $E_{\text{gly/GABA}}$  is generally close to the resting potential, so that variation in resting potential can switch the polarity of glycine/GABA effects (Marty and Llano, 2005). Glycine/GABA<sub>A</sub> receptor channels are permeable to  $\text{Cl}^-$  and, to a lesser extent,  $\text{HCO}_3^-$  (Bormann et al., 1987). The heterogeneity of  $E_{\text{gly/GABA}}$  among neurons has thus been attributed to the difference in intracellular  $\text{Cl}^-$ , assuming that intracellular  $[\text{HCO}_3^-]$ , determined by intracellular pH, is constant.  $\text{Cl}^-$  imaging has revealed

that intracellular  $\text{Cl}^-$  concentration ( $[\text{Cl}^-]_i$ ) is correspondingly high in cells having depolarized  $E_{\text{GABA}}$  (Duebel et al., 2006; Rocha-Gonzalez et al., 2008), and that decrease in  $[\text{Cl}^-]_i$  may occur during developmental periods when  $E_{\text{GABA}}$  shifts negative (Kuner and Augustine, 2000; Berglund et al., 2006).

Neuronal  $\text{Cl}^-$  levels may be regulated by cation- $\text{Cl}^-$  cotransporters, KCC2 and NKCC1, and  $\text{Na}^+$ -independent and  $\text{Na}^+$ -driven  $\text{Cl}^-$ - $\text{HCO}_3^-$  exchangers, as well as by  $\text{Cl}^-$  channels (Farrant and Kaila, 2007). Therefore, steady-state  $E_{\text{gly/GABA}}$  is dependent on the balance of  $\text{Cl}^-$  extrusion and accumulation mechanisms. For example, KCC2 expression increases during the period of developmental negative  $E_{\text{GABA}}$  shift in pyramidal neurons (Rivera et al., 1999; Yamada et al., 2004). NKCC1, and not KCC2, is expressed in some neurons having depolarized  $E_{\text{GABA}}$  (DeFazio et al., 2002; Kim and Chung, 2007).  $E_{\text{gly/GABA}}$  may be modulated, by passive redistribution of  $\text{Cl}^-$  (Kaila and Voipio, 1987; Staley et al., 1995; Ehrlich et al., 1999; Billups and Attwell, 2002) or by KCC2 or NKCC1 (Fiumelli et al., 2005; Brumback and Staley, 2008). Interestingly, although  $\text{Cl}^-$ - $\text{HCO}_3^-$  exchangers have been identified in neurons (Kopito et al., 1989; Schwiening and Boron, 1994; Grichtchenko et al., 2001; Brett et al., 2002), their role in regulating  $[\text{Cl}^-]_i$  or  $E_{\text{gly/GABA}}$  has received little attention (see Gulacsi et al., 2003), perhaps due to the assumption that intracellular pH, which determines the driving force for  $\text{HCO}_3^-$ , is stable.

The cartwheel cell (CWC) is a glycinergic interneuron in the dorsal cochlear nucleus (DCN). CWCs form synapses among themselves and with the principal cells of DCN (Wouterlood and Mugnaini, 1984; Berrebi and Mugnaini, 1991), mediating strong feed-forward inhibition of principal cells upon somatosensory stimulation (Davis and Young, 1997). Their electrical signature is the complex spike, a burst of fast spikes atop a  $\text{Ca}^{2+}$ -dependent slow depolarization (Zhang and Oertel, 1993; Manis et al., 1994; Golding and Oertel, 1996; Kim and Trussell, 2007). Here, we investigated glycinergic responses in CWCs with the gramicidin perforated-patch method and found that complex-spike activity triggered a negative shift in  $E_{\text{gly}}$ . This shift occurred as a result of a  $\text{Ca}^{2+}$ -dependent acidification and a consequent decrease in  $[\text{Cl}^-]_i$  or  $[\text{HCO}_3^-]_i$ , most likely involving the activity of  $\text{Na}^+$ -driven  $\text{Cl}^-$ - $\text{HCO}_3^-$  exchanger. To our knowledge, this is the first demonstration that the anion exchanger, working against an activity-dependent intracellular acid load, can control the glycinergic/GABAergic reversal potential.

## METHODS

### Slice preparation and recording

Brainstem slices containing the DCN were prepared from ICR mice aged 17–27 days (Harlan, Indianapolis, IN). Mice were anesthetized with isoflurane and then decapitated in accord with the regulations of the Institutional Animal Care and Use Committee of Oregon Health and Science University. Subsequently, a block of brainstem was isolated and horizontal slices of 200- $\mu\text{m}$  thickness were cut with a vibrating slicer (VT1200S, Leica, Deerfield, IL). Dissection and slicing were done in a warm ( $\sim 30^\circ\text{C}$ ) solution saturated with 95%  $\text{O}_2$ /5%  $\text{CO}_2$  and composed of (in mM): 1) 129 NaCl, 3 KCl, 1.2  $\text{KH}_2\text{PO}_4$ , 2.4  $\text{CaCl}_2$ , 1.3  $\text{MgSO}_4$ , 20  $\text{NaHCO}_3$ , 3 HEPES, 10 glucose (“ACSF”) or 2) 73 sucrose, 81 NaCl, 3 KCl, 1.2  $\text{NaH}_2\text{PO}_4$ , 1  $\text{CaCl}_2$ , 0.7  $\text{MgCl}_2$ , 1.3  $\text{MgSO}_4$ , 0.5 ascorbic acid, 25  $\text{NaHCO}_3$ , 3 HEPES, 10 glucose. The latter solution was used in about half of dissections. The slices were kept in ACSF at  $34.5^\circ\text{C}$  for the first hour and then left at room temperature.

ACSF was the standard bathing medium for recordings, except for the following. For inducing intracellular acidification, modified ACSFs were used, in which NaCl was reduced by 20 mM and equiosmolar amount ( $\sim 22$  mM, “20 mM” in Results) of either Na-methanesulfonate ( $\text{NaCH}_3\text{O}_3\text{S}$ ) or Na-propionate was introduced. For inducing intracellular alkalinization, 10

mM trimethylamine hydrochloride (TMA·HCl) replaced equimolar NaCl in the ACSF. A 3M KCl/3% agarose salt bridge reference electrode was used in experiments utilizing these three modified ACSFs. For bicarbonate- and CO<sub>2</sub>-free condition, the ACSF was buffered only with HEPES and equilibrated with 100 % O<sub>2</sub> (“HEPES/O<sub>2</sub>”): 20 mM NaHCO<sub>3</sub> was replaced with 20 mM HEPES (in addition to the original 3 mM), and glucose was increased to 20 mM to achieve a similar osmolality to that of ACSF (~303 mosmol/L). The pH of “HEPES/O<sub>2</sub>” was adjusted with NaOH at 34 °C to 7.32–7.34, which is the pH range of ACSF equilibrated with 5% CO<sub>2</sub>/95% O<sub>2</sub>, measured at 34 °C.

DCN cells in the slice were visualized by infrared differential interference contrast videomicroscopy on an Olympus BX51WI microscope with a 60X water-immersion objective (0.9 N.A., LUMPlanFL, Olympus, Japan). The ACSF or other saline solution was perfused at 2–3 ml/min through the recording chamber by a peristaltic pump (Minipulse 3, Gilson, Middleton, WI), and the temperature of the solution at the recording chamber was maintained at 34±0.5°C by an in-line heater (SH27B, Warner Instruments, Hamden, CT). Medium-sized cells in the molecular and fusiform cell layers of DCN were identified as CWCs if they showed complex spikes spontaneously or upon injection of depolarizing current. The data presented in this study were obtained with gramicidin perforated-patch recording (Rhee et al., 1994). The standard pipette solution for perforated patch recording contained (in mM) 140 KCl, 10 NaCl and 10 HEPES (pH adjusted to 7.25 with KOH, 290 mOsm). Gramicidin was then added to this solution on the day of experiment at a final concentration of 40–100 µg/ml from a stock solution of 30–50 mg/ml DMSO. For simultaneous imaging, one or two acetoxymethyl (AM) ester fluorescent dyes (see below) were added along with gramicidin. The maximum v/v% of DMSO reached in the recording solution was 0.48 % when two AM dyes were included. The tip of the recording pipette was filled with the standard solution (without gramicidin or indicator dyes). The recording pipettes had a resistance of 3–6 MΩ when filled with the standard solution, and were prepared by pulling thick-walled filamented borosilicate glass capillaries (1B120F-4, World Precision Instruments, Sarasota, FL), and wrapped with Parafilm along one third the pipette's length from the tip to reduce capacitance. The detection of patch rupture was by a sudden offset in membrane potential. The liquid junction potential was not corrected, as discussed previously (Kim and Trussell, 2007).

Recordings were made with a BVC-700A (Dagan, Minneapolis MN) or MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA) in conjunction with Digidata 1322A digitizer (Molecular Devices) and ClampEx software (pClamp 9.2, Molecular Devices). After the electrode had formed a seal (> 1GΩ) on the cell membrane in voltage-clamp (v-clamp), the progression of perforation (reduction in series resistance, R<sub>s</sub>) was monitored in current-clamp (i-clamp) by periodic bridge balancing and by observing the growth in amplitudes of spontaneous fast spikes. The effect of glycine on spontaneous activity or on membrane potential (V<sub>m</sub>) was monitored early on during perforation because it could be distinguished even with a relatively high R<sub>s</sub>. Glycine or GABA was pressure-ejected (Picospritzer II, General Valve, Fairfield, NJ) from a patch pipette pointed toward the cell body from 25–30 µm away. The duration and pressure of a puff were adjusted for each cell to elicit a 500-msec response: the ranges were 5–20 msec and 0.5–2 psi with 2 mM glycine, 100–300 msec and 2–4 psi with 0.5 mM glycine or 0.5 mM GABA. Care was taken to minimize glycine/GABA applications, to avoid causing a significant change in intracellular [Cl<sup>-</sup>] or [HCO<sub>3</sub><sup>-</sup>] due to the flux through the glycine/GABA receptors. Glycine and GABA solutions were prepared in ACSF except during experiments involving HCO<sub>3</sub><sup>-</sup>/CO<sub>2</sub> removal, for which glycine was dissolved in HEPES-buffered saline. Glycinergic/GABAergic postsynaptic potential (PSP) was evoked by 200-µsec 30–60 V pulses given through a glass stimulating electrode filled with ACSF. The recorded cell was kept in i-clamp mode except when E<sub>gly</sub> was measured. After the glycine response of spontaneous activity/V<sub>m</sub> had been examined, the Na<sup>+</sup> channel blocker tetrodotoxin (TTX, 0.4 µM) and glutamate receptor blockers, DNQX (6,7-dinitroquinoxaline-2,3-dione, 10

$\mu\text{M}$ ) and APV (2-amino-5-phosphonovaleric acid,  $100 \mu\text{M}$ ) were added, and a bias current was given to keep  $V_m$  at  $-75 \sim -80 \text{ mV}$ . I-clamp recording was sampled at  $20 \text{ kHz}$  and low-pass filtered at  $10 \text{ kHz}$ . The pipette capacitance was compensated in both i-clamp and v-clamp mode.

### **$E_{\text{gly}}$ measurements**

Voltage ramps (v-ramp) in v-clamp were used for measurement of  $E_{\text{gly}}$ , and were sampled at  $40 \text{ kHz}$  and low-pass filtered at  $10 \text{ kHz}$ . The v-ramp protocol consisted of 4 consecutive runs of a 1-sec-long unit stimulus that included a short  $-5 \text{ mV}$  step followed by a 130-msec depolarizing ramp spanning  $18\text{--}20 \text{ mV}$  around the expected  $E_{\text{gly}}$  of the recorded cell ( $V_{\text{HOLD}} = -75 \text{ mV}$ ) (Fig. S1Bi). Glycine was puffed after the  $-5 \text{ mV}$  step during the 1st and 3rd run. The average ramp voltage where the glycine responses and control responses crossed was taken as the raw  $E_{\text{gly}}$ . The  $R_s$  during an  $E_{\text{gly}}$  measurement was estimated from dividing  $5 \text{ mV}$  by the average amplitude of the capacitive transient current (in nA) of the  $-5 \text{ mV}$  step. The raw  $E_{\text{gly}}$  was corrected for the voltage error from the  $R_s$  with the following formula:  $E_{\text{gly}} = \text{raw } E_{\text{gly}} - ((\text{average clamp current at raw } E_{\text{gly}}) \times R_s)$ . The inclusion criteria for  $E_{\text{gly}}$  data was  $R_s < 60 \text{ M}\Omega$ . The  $R_s$  dropped to  $20\text{--}40 \text{ M}\Omega$  within 40 minutes of forming a seal in most cells used in  $E_{\text{gly}}$  measurements.

The resting  $E_{\text{gly}}$  (measured  $>150 \text{ sec}$  after any depolarization-induced spiking) was measured after an initial  $\sim 10 \text{ sec}$  clamp to  $-75 \text{ mV}$ . As shown in Fig. S1Di, a slow, negative drift in the resting  $E_{\text{gly}}$  was found in most cells. As the perforated-patch recording pipette contained  $150 \text{ mM Cl}^-$ , the negative drift was opposite to that expected if the pipette  $\text{Cl}^-$  leaked into the cell. The negative drift in  $E_{\text{gly}}$  was also recognized in i-clamp mode from the change in  $V_m$  response to glycine over time. The negative drift occurred both with the dye-free and dye-containing recording solution and was still observed when different pipette salts ( $145 \text{ K-gluconate}$ ,  $4 \text{ NaCl}$ ,  $4 \text{ NaOH}$ ,  $10 \text{ HEPES}$  or  $140 \text{ KCl}$ ,  $6 \text{ MgCl}_2$ ,  $4 \text{ KOH}$ ,  $10 \text{ HEPES}$ ) were used. Occasionally, a positive drift in resting  $E_{\text{gly}}$  was seen under control conditions and was followed within a few minutes by the rupture of perforated patch; data from these recordings were not included.

We did not determine resting  $E_{\text{gly}}$  by plotting the amplitude of glycine-induced currents versus holding potential in v-clamp because the  $E_{\text{gly}}$  seemed to drift towards the holding potentials, particularly when the new potential was below  $-80 \text{ mV}$  or above  $-60 \text{ mV}$  suggestive of a passive  $\text{Cl}^-$  conductance. The V-ramp protocol was adopted to minimize the drift of  $E_{\text{gly}}$  during measurements and to be able to follow the activity-induced change in  $E_{\text{gly}}$  (see Results).

### **Fluorescent imaging**

Imaging experiments with the pH-sensitive dye SNARF-5F were generally performed with a monochromator-based imaging system (Polychrome V, Till Photonics, Munich, Germany) attached to the Olympus BX51WI microscope. Excitation wavelength was  $547 \text{ nm}$ , and emission was long-pass filtered above  $600 \text{ nm}$  (Chroma E590LPv2). Fluorescent images were acquired with IMAGO QE cooled CCD camera (Till Photonics) controlled by the TILLvisION 4.0 software. SNARF-5F was loaded into a cell by including the AM ester form ( $43 \mu\text{M}$ ) in the recording pipette from which it diffused through the perforated patch and was de-esterified inside the cell (Filosa et al., 2002). The fluorescent intensity of the recorded cell increased with time as the dye accumulated (Fig. S2A). To minimize phototoxicity and to keep the average raw intensity of cell body in different image series within a narrow range, the images were obtained (at  $1 \text{ Hz}$ ) with  $4 \times 4$  binning and the exposure time was adjusted as the baseline intensity increased (e.g.  $150 \text{ msec}$  for first imaging series collected  $\sim 30 \text{ min}$  after the seal, gradually shortened to  $50 \text{ msec}$  over the next hour). For simultaneous pH-Ca or pH-Cl imaging, 2-photon scanning microscopy was utilized. Images of  $256 \times 256$  pixels were acquired with the Ultima system by Prairie Technologies (Middleton, WI) using a Chameleon Ultra II Ti:sapphire pulsed laser (Coherent, Santa Clara, CA) (Roberts et al. 2008). For pH and  $\text{Ca}^{2+}$  imaging, the AM

forms of SNARF-5F and either Fura-2 (100  $\mu\text{M}$ ) or Fluo-4 (76  $\mu\text{M}$ ) were included in the recording pipette for loading.  $\text{Ca}^{2+}$  dyes did not load as well as SNARF-5F through the patch membrane, but Fluo-4 loading was improved by adding Pluronic F-127 (final concentration, 0.017%). The excitation wavelength was 800 nm for simultaneous pH-Ca imaging with Fluo-4, and 780 nm with Fura-2. For concurrent pH and  $\text{Cl}^-$  imaging,  $\text{Cl}^-$ -sensitive dye MQAE (N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide) was loaded into the DCN slice by incubating the slice in 3 mM dye in ACSF for 8 min at 34  $^{\circ}\text{C}$ , and SNARF-5F was loaded from the recording pipette. MQAE and SNARF-5F were both excited by 750 nm laser light. For pH and  $\text{Ca}^{2+}$  imaging, fluorescent emission was split into two photomultiplier tubes using a dichroic mirror and band-pass filters for red (SNARF-5F) and green (Fluo-4 and Fura-2) light. For pH and  $\text{Cl}^-$  imaging, the green band pass filter was removed to collect all emission below 560 nm for the weak MQAE signal. As shown in an example of simultaneously recorded MQAE and SNARF-5F images in Figure 6Bi, some of MQAE's fluorescence was caught in the red channel for SNARF-5F, but its impact on the SNARF signal was probably negligible, considering the weak MQAE signal. The baseline MQAE fluorescence did not noticeably decrease, as expected from dye leakage or bleaching, either during a 150-sec run of experiment or over the  $\sim 1$  hour of recording period. However, as experiments evoking a change in MQAE signal were repeated over time, the response magnitude became attenuated. With time, the typical inhomogeneous MQAE staining of the cell body (Marandi et al., 2002) also became more homogeneous. We suspect that the time-dependent loss of response is due to MQAE's conversion to a hydrolyzed (de-esterified) form having a reduced  $\text{Cl}^-$  sensitivity (Verkman, 1990; Koncz and Daugirdas, 1994). No glycine puff was given during 2-photon imaging experiments.

For both single-photon and 2-photon image series obtained, the "signal" was extracted from the average intensity (in arbitrary units) of a region of interest (ROI) drawn along the periphery of the cell body (Fig. S2A). The average intensity of a background region was subtracted from the SNARF signal but not from other dyes' signal because the signal-to-noise ratio was worsened by background subtraction. The time plot of fluorescence signal over a 150–200 sec period was corrected for the up-sloping baseline by fitting a straight line along the control period and subtracting the line from the signal. The initial intensity value before the line subtraction ( $F_0$ ) was then added back to all points and the plot of  $(F-F_0)/F_0$  (" $\Delta F/F$ ") was produced. For the 800-sec image series involving weak acid or weak base challenge, the fitted straight line was not subtracted but used as  $F_0$  in calculating  $(F-F_0)/F_0$  to prevent the overestimation of  $\Delta F$  in the later part of the 800-sec period. Although a linear increase in the baseline fluorescence was assumed for convenience, not infrequently the baseline-corrected SNARF-5F signals were found to deviate from 0 at the end of a 160-sec series when an intensity-attenuating response (acidification) was expected to have terminated. In such cases it was unclear whether it was a true signal or due to the failure of linear baseline correction. We mostly focused on the peak change in signal occurring within 30 sec from the control period, during which the linear extension of baseline is less likely to fail. Occasionally, a small, abrupt increase in SNARF signal occurred that was not associated with a stimulus or a change in  $V_m$  (e.g. Fig. 5A black, 6A, 8Aii gray); the origin of this change is unknown. The peak signal in a time plot was selected by eye for single-photon SNARF-5F data, and by curve fitting or from the intersection of two fitted straight lines for the 2-photon MQAE or SNARF-5F data, respectively.

### Drug application

All the pharmacological agents except glycine and GABA were applied by bath perfusion. DNQX (10  $\mu\text{M}$ ) and APV (100  $\mu\text{M}$ ) were co-applied with TTX.  $\text{H}_2\text{DIDS}$  (4,4'-diisothiocyano-1,2-diphenylethane-2,2'-disulphonic acid) was directly dissolved in ACSF on the day of each experiment, but other drugs were diluted from a stock solution in water (TTX,



APV, glycine, GABA) or DMSO (all others). Drugs were obtained from Sigma-Aldrich (St Louis, MO) with the exception of TTX (Alomone labs, Jerusalem, Israel), APV, DNQX (Ascent scientific, Weston-Super-Mare, UK) and H<sub>2</sub>DIDS (Invitrogen, Carlsbad, CA). Carboxyeosin diacetate used was from Sigma-Aldrich (n = 3) or Invitrogen (n = 2). All fluorescent dyes and Pluronic F-127 were from Invitrogen. When CdCl<sub>2</sub> or CoCl<sub>2</sub> was used, KH<sub>2</sub>PO<sub>4</sub> in the bathing solution was replaced with KCl to prevent precipitation.

### Data analysis

Data were analyzed with Clampfit (Molecular Devices), Microsoft Excel and KyPlot (KyensLab, Tokyo, Japan). Numerical values were given as mean ± S.D. where available. Two-tailed *t*-test (paired or unpaired) or Kolmogorov-Smirnov test was used to compare two groups of data, and one-way ANOVA and the multiple comparisons (Tukey-Kramer test) were used for three or more groups of data. Level of significance was at 0.05 for all statistical tests.

### Immunohistochemistry

Mice, ages P23–27, were anesthetized with isoflurane and then perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (in PBS). The brain was removed and post-fixed in 4% paraformaldehyde at 4 °C for 2.5 hr. 30 μM-thick coronal brainstem sections containing the DCN were cut using Leica VT1000S and then boiled in 10 mM sodium citrate, pH 6.0, for 20 min using a microwave oven. After cooling, sections were blocked for 1 hr in 2% normal goat serum/0.2% Triton X-100 (in PBS) and then incubated overnight at 4 °C with one of two clones of mouse monoclonal antibodies against the *SLC4A8* gene product, which is human NDCBE (clone 1G10 and 6E11; Abnova, Taiwan). Clone 1G10 was diluted at 1:100 and 6E11 was at 1:75 in the block solution. Clone 1G10 has been tested by the manufacturer to be cross-reactive to mouse protein in western blot analysis. The next day, sections were washed in PBS and incubated with goat anti-mouse IgG conjugated to Alexa Fluor 488 (Invitrogen; 1:500 in block solution). After 2 hr, sections were washed in PBS, mounted on gelatin-coated slides and dried. Section were then de-lipidized by going through ascending series of alcohols, xylene, descending series of alcohols and water, in sequence. Slides were coverslipped using Fluoromount G medium (Southern Biotech, Birmingham, AL). Confocal laser-scanned images of sections were obtained with Olympus FV1000 microscope with a 60X oil-immersion objective (N.A. 1.42) under the control of Olympus Fluoview-1000 software.

## RESULTS

### Glycine response of cartwheel cells

Depolarizing, excitatory glycine responses have been reported in CWCs using microelectrode or extracellular cell-attached recording (Golding and Oertel, 1996 (mice, P18–26); Tzounopoulos et al., 2004 (mice, P18–22)). We reevaluated the prevalence of depolarizing glycine responses using gramicidin perforated-patch recording, and found that cells varied in their response from depolarization to hyperpolarization, suggestive of variable glycine reversal potential ( $E_{\text{gly}}$ ). For spontaneously spiking cells, the effect of glycine was categorized as excitatory, inhibitory, or mixed. Excitation (Fig. 1A) was recognized by increase in spike frequency with an obvious depolarization. Mixed responses (Fig. 1B) consisted of a depolarized pause in spiking followed by higher frequency firing at the decay of the response. Reducing the puff pressure or duration for this group did not reveal an increase but rather a decrease or no change in spiking at the onset of response. This is expected if  $E_{\text{gly}}$  is a few mV below spike threshold, such that shunting depolarization provided a platform for higher frequency spiking after glycine receptors close (Gulledge and Stuart, 2003). An inhibitory glycine response (Fig. 1C), a decrease or pause in spiking, was observed with the  $V_m$  driven to levels from about 5 mV below the fast spike threshold (~ -65 mV) to -84 mV. The glycine

responses in CWCs that did not spike spontaneously ( $V_m -81.4 \pm 2.8$ ,  $n = 127$ ) consisted of depolarizing or hyperpolarizing deflections of  $V_m$ ; in some of these cases (11 of 127), spikes were evoked at the peak of a depolarizing response. The proportions of cells showing the three types of glycine response are shown in Table 1; for the standard pipette solution, the excitatory, mixed and inhibitory proportions were 31, 12, and 57 %, respectively ( $n=113$ ).

Several potential factors, either biological or experimental, could influence the distribution of response polarity. For example, higher proportion of inhibitory responses was observed in cells recorded with a pipette solution containing an AM-ester dye (SNARF-5F or Fluo-4, see data below on imaging). Another potential factor that could impact the distribution is postnatal age, as intracellular  $[Cl^-]$  is higher during the first 1-2 weeks after birth, resulting in transient excitatory response to GABA or glycine (Ben-Ari et al., 2007). However, no significant difference was found in the age distribution of all cells showing excitatory responses vs. inhibitory responses (Kolmogorov-Smirnov test,  $p = 0.99$ ;  $22.4 \pm 2.4$  days,  $n = 57$ , excitation vs.  $22.4 \pm 2.6$  days,  $n = 148$ , inhibition). No difference was present in the ages of mice recorded with or without AM dyes (Kolmogorov-Smirnov test,  $p = 0.65$ ;  $22.3 \pm 2.5$  days,  $n = 113$ , no dye vs.  $22.5 \pm 2.5$  days,  $n = 129$ , with dye). Thus, age is not a factor affecting glycine responses in our data set. More interestingly, we observed an association between the type of glycine response and whether the cell's spontaneous activity included complex spikes or was all simple spiking. In the current study, 19 % of spontaneously spiking cells (47 of 242) were complex-spiking, and among these the excitatory response was observed in only one, while the inhibitory response was seen in 85.1 % of them (Table 1). This suggests a possible relation between  $E_{gly}$  and complex spike activity, as described below.

### Activity-dependent shift in the glycine response

A series of experiments were designed to obtain a qualitative description of the effects of spike activity on glycine responses, and the results suggested the cellular mechanisms tested later in this study. Four cells showing depolarizing glycine responses, two simple-spiking cells and two silent cells, were induced to fire complex spikes for a long period by sustained depolarizing current injection (50–150 pA for 57–137 sec in different cells), and their glycine responses were monitored in 10–15 sec intervals. As shown in Figure 2A, the glycine responses shifted negative, i.e., becoming less excitatory, or more inhibitory, as complex spiking continued, and when the spiking was terminated, a hyperpolarizing glycine response was observed, which shifted back to depolarizing over the next 100–200 sec. These results suggest a negative shift in  $E_{gly}$ .

To contrast the relative effects of simple and complex spikes, the type of spikes in the train were controlled by the magnitude of a given duration of the stimulus. To facilitate the comparison of glycine response amplitudes, cells were silenced to  $-80$  mV, and 6 cell showing depolarizing glycine response at  $-80$  mV were chosen for further analysis. As shown in Figure 2B (top), although a reversal to a hyperpolarizing glycine response did not occur, complex spiking resulted in a reduction in the peaks of glycine responses (contrast responses 1 and 2; mean shift  $-1.7 \pm 0.7$  mV,  $n = 6$ ) 20–30 sec after the stimulus ended. By contrast, after simple spiking (including cases where the single onset complex spike was present; Fig. 2B bottom) such a reduction was barely noticeable ( $-0.1 \pm 0.2$  mV,  $n = 6$ ,  $p = 0.005$ , paired  $t$ -test). Interestingly, however, the peak of the first glycine response after simple spiking (o) was 0.1–3.3 mV more *positive* than that before the stimulus, despite riding on a prominent afterhyperpolarization (4 of 6 cells). A positive shift in  $E_{gly}$  is expected to develop during a long depolarization and to decay on repolarization in the presence of a passive  $Cl^-$  conductance, as  $[Cl^-]_i$  would change along with the  $V_m$ . A similar negative shift was seen in GABA<sub>A</sub> component of the responses to puffing 500  $\mu$ M GABA after complex spiking (8–14 sec duration,  $n = 7$  cells, not shown).

CWCs receive mixed glycinergic/GABAergic synapses from other CWCs (Roberts et al., 2008). We evoked glycinergic/GABAergic PSPs by stimulating (0.5–0.7 Hz) in the deep layer of DCN in the presence of glutamate receptor blockers (100  $\mu$ M APV, 10  $\mu$ M DNQX). Complex spiking induced a reversal in the polarity of evoked PSPs from depolarizing to hyperpolarizing, indicating a negative shift in  $E_{\text{gly}}$  ( $n=8$ , Fig. 3A,B). For 3 cells in which the evoked PSPs were hyperpolarizing even when  $V_m$  was brought near  $-80$  mV, 10-sec of complex spiking made the PSPs still more hyperpolarizing (Fig. 3B cell 3). The difference in the average amplitude of PSPs between the pre-activity period and the 15th–20th sec post-activity period was from  $-0.4$  to  $-2.2$  mV in 10 cells (average  $-1.3$  mV, complex spiking induced for 10–16 sec by 100–250 pA).

The CWC's complex spike requires  $\text{Ca}^{2+}$  channels (Kim and Trussell, 2007), and thus a complex spike generates a larger rise in intracellular  $\text{Ca}^{2+}$  than does a simple spike (Molitor and Manis, 2003; Roberts et al., 2008). To investigate whether the  $\text{Ca}^{2+}$  influx is sufficient for the negative shift in the glycine response to occur, high-threshold  $\text{Ca}^{2+}$  spikes were evoked in the presence of  $\text{Na}^+$  channel blocker TTX, 0.4  $\mu$ M.  $\text{Ca}^{2+}$  spikes were evoked in seven cells that had a depolarizing glycine response at  $V_m = -80$  mV, and glycine was puffed every 8 sec (Fig. 4A). In all 7 cells, the glycine response increased by the third puff during  $\text{Ca}^{2+}$  spiking. After  $\text{Ca}^{2+}$  spike trains, the  $V_m$  was depolarized compared to the pre- $\text{Ca}^{2+}$  spiking level, lacking the afterhyperpolarization seen after complex spiking. This depolarization actually helped reveal the negative shift in  $E_{\text{gly}}$  as the peaks of hyperpolarizing glycine responses after  $\text{Ca}^{2+}$  spiking (at  $-78$  to  $-93$  mV in 5 cells) were clearly more negative than the depolarizing peaks of glycine responses before  $\text{Ca}^{2+}$  spiking (at  $-79$  to  $-74$  mV).

### Measurement of $E_{\text{gly}}$

We used a voltage-ramp protocol to measure  $E_{\text{gly}}$  and document the time course of its change after spike-train stimuli (Fig. S1Bi, see Methods). To avoid the slow negative drift in  $E_{\text{gly}}$  (see Methods, Fig. S1Di), representative resting  $E_{\text{gly}}$  was assessed as early as possible during patch perforation, as long as  $R_s < 60$  M $\Omega$  (in TTX). With this criterion, the mean resting  $E_{\text{gly}}$  from 164 cells was  $-74.3 \pm 5.8$  mV. Table 2 lists  $E_{\text{gly}}$  values subgrouped with respect to spontaneous activity, response to glycine, and presence of AM dye in the recording pipette. The difference between spiking cells'  $E_{\text{gly}}$  and that of silent cells was insignificant ( $t$ -test,  $p = 0.60$ ).  $E_{\text{gly}}$  was most negative in cells with inhibitory responses and most positive for those with excitatory responses. The difference among the three response groups was significant (one-way ANOVA,  $p < 0.001$ ). While the  $E_{\text{gly}}$  difference between the excitatory group and mixed group was not significant in pairwise comparison (Tukey-Kramer test,  $p = 0.56$ ), the differences in the other pairwise group comparisons were significant ( $p < 0.001$ ). The mean  $E_{\text{gly}}$  of complex spiking cells, which showed a higher proportion of inhibitory glycine responses, was not significantly more negative than that of simple spiking cells ( $-76.2 \pm 5.6$ ,  $n = 17$ , vs.  $-73.7 \pm 5.8$ ,  $n = 84$ ;  $p = 0.12$ ,  $t$ -test). For comparison with CWCs, we examined glycine responses in fusiform cells which are the principal neuron of the DCN and are postsynaptic to CWCs. All showed hyperpolarizing, inhibitory responses to glycine ( $n = 28$ ) and the  $E_{\text{gly}}$  measured in 4 cells was  $-83.9 \pm 0.7$  mV (in TTX). That the fusiform cell showed more negative  $E_{\text{gly}}$  than the CWC and only inhibitory responses to glycine is in agreement with Golding and Oertel (1996).

A protocol using mixed voltage and current clamp recording modes was used to monitor the time course of  $E_{\text{gly}}$  shifts in relation to a period of activity.  $V_m$  and glycine responses were recorded in current clamp for 150–180 sec interrupted every 15 sec or less by the ramp protocol; an  $\sim 8$  sec burst of complex or  $\text{Ca}^{2+}$  spiking was induced at  $t = 35$  sec (Fig. S1A–C). Shown in Figure 4B is the distribution of peak negative  $E_{\text{gly}}$  shifts (difference between the most negative  $E_{\text{gly}}$  after  $\text{Ca}^{2+}$  spiking and the mean of pre- $\text{Ca}^{2+}$  spiking values) versus the number of  $\text{Ca}^{2+}$  spikes evoked during the 8-sec depolarization ( $n = 148$  measurements; Pearson's  $r =$



-0.66,  $p < 0.001$ ). Initially, we evoked enough  $\text{Ca}^{2+}$  spiking in each cell to recognize a clear negative shift in glycine responses during current-clamp after the stimulus (e.g., Fig. S1A–C, Fig. 5A) and confirmed that the  $E_{\text{gly}}$  shifted negative. However, for cells with the largest shifts ( $> 5$  mV),  $E_{\text{gly}}$  would often fail to recover fully within 120 sec after the  $\text{Ca}^{2+}$  spiking or would settle to a more negative level than the control level. Additionally in some cases  $\text{Ca}^{2+}$  spiking would continue beyond the 8-sec current injection. Therefore, we adjusted the current injection in each cell to prevent runaway spiking and to achieve  $\leq 5$  mV of negative  $E_{\text{gly}}$  shift. As shown in Figure 5A, the most negative shift in  $E_{\text{gly}}$  occurred at one of the first three measurements after  $\text{Ca}^{2+}$  spiking (at 2, 8.5, or 19.5 sec, mean  $9.3 \pm 5.9$  sec,  $n = 61$  series with  $\geq 1.5$  mV peak negative shifts from 61 cells). Restoration of  $E_{\text{gly}}$  occurred over 100–130 sec, and single exponential fits for recovery gave a mean time constant of  $35 \pm 11$  sec ( $n = 34$  cells).

The 8-s challenge protocol was also run in cells in the absence of TTX to examine the  $E_{\text{gly}}$  shift with complex or simple spiking.  $E_{\text{gly}}$  shifted negative by up to 4 mV after an 8-sec stimulus evoked with a maximum of 250 pA injection (Fig. 4Cii). In 32  $E_{\text{gly}}$  series (from 32 cells, 29 with AM dye and 3 without dye) where  $> 1$  mV peak negative shift occurred with complex spiking, the time of peak was most often (18 of 32 cells) at the 19.5 sec point, and two had the peak at 34 sec. Thus, the average peak time ( $16.3 \pm 7.8$  sec,  $n = 32$ ) was later than with  $\text{Ca}^{2+}$  spiking. The first data point (2 sec) after complex spiking was not as negatively shifted as that after  $\text{Ca}^{2+}$  spiking, and showed a small positive shift in some cases. Figure 4C shows the maximal negative shift in  $E_{\text{gly}}$  plotted against the shift at the 2-sec time point for individual experiments obtained with  $\text{Ca}^{2+}$  spiking (limited to 1–4 mV shifts for comparison with complex spiking-induced shifts), complex spiking and simple spiking. These data show that with only  $\text{Ca}^{2+}$  spiking, the magnitude of peak negative shifts was greater, and an initial positive shift was rarely observed. Such initial positive shifts were more common with complex spiking, and especially with simple spiking; as discussed below, these positive shifts appear to reflect passive elevation in intracellular  $\text{Cl}^-$  that are opposed by a  $\text{Ca}^{2+}$  dependent mechanism.

### **$\text{Ca}^{2+}$ -dependent and passive $E_{\text{gly}}$ shifts**

To test whether  $\text{Ca}^{2+}$  influx triggers the negative shift of  $E_{\text{gly}}$ ,  $\text{Ca}^{2+}$  was removed from the ACSF (“zero- $\text{Ca}^{2+}$ ”,  $\text{MgCl}_2$  substitution of  $\text{CaCl}_2$ , in TTX,  $n = 8$ ). Removal of  $\text{Ca}^{2+}$  depolarized  $V_m$  (restored to between -75 and -80 mV with bias current), and shifted resting  $E_{\text{gly}}$  between +2 to -4 mV in different cells. As illustrated in Figure 5B, 8-sec current injection in TTX and zero- $\text{Ca}^{2+}$  depolarized the cells strongly but without spiking activity. Following the stimulus, there was no negative shift in  $E_{\text{gly}}$ , even with currents as large as 500 pA ( $n = 8$  cells). Rather, the  $E_{\text{gly}}$  immediately shifted positive after the strongest depolarization, and this positive shift decayed within 60 sec (Fig. 5Bii). While the negative shift in  $E_{\text{gly}}$  when spikes were present peaked at 8.5 sec or 19.5 sec after stimulus in 6 of the 8 cells, the positive shift in zero- $\text{Ca}^{2+}$  peaked at 2 sec (the first measured time point) in 7 of 8 cells. The simplest explanation for the transient positive shift in  $E_{\text{gly}}$  is a rise in intracellular  $\text{Cl}^-$  through a passive conductance during the depolarization. Such a  $\text{Cl}^-$  conductance is expected also to mediate the influx of  $\text{Cl}^-$  during the control condition with  $\text{Ca}^{2+}$  spiking. The fact that the  $E_{\text{gly}}$  was often more negative than the control level soon after (at 2 sec)  $\text{Ca}^{2+}$  spiking and that it could drop further during the next 10 or 20 sec indicates that  $\text{Cl}^-$  was actively removed or that a constitutive  $\text{Cl}^-$  accumulation was inhibited by a  $\text{Ca}^{2+}$ -triggered mechanism. Figure 5C illustrates in 6 cells the contrasting direction of  $E_{\text{gly}}$  shifts induced by the same amount of current injection in control and zero- $\text{Ca}^{2+}$ , as well as the tendency for both directions of shifts to increase with the amount of current injected. The average of the largest negative shift from each cell was  $-2.4 \pm 2.2$  mV and that for the positive shift was  $2.2 \pm 1.2$  mV. Thus, the  $\text{Ca}^{2+}$ -dependent process opposed a passive process, shifting  $E_{\text{gly}}$  negative by nearly 5 mV in this protocol.

### A hypothesis for the mechanism of $\text{Ca}^{2+}$ -dependent negative $E_{\text{gly}}$ shift

A negative shift in  $E_{\text{gly}}$  is expected to result from a reduction in intracellular  $\text{Cl}^-$  and/or  $\text{HCO}_3^-$ .  $\text{HCO}_3^-$  is less permeable than  $\text{Cl}^-$  through glycine/GABA receptors (Bormann et al., 1987), and at constant  $P_{\text{CO}_2}$ , the intracellular concentration,  $[\text{HCO}_3^-]_i$ , is expected to be set by the intracellular pH ( $\text{pH}_i$ ) (Roos and Boron, 1981). On the other hand,  $[\text{Cl}^-]_i$  in neurons may be regulated by KCC2, NKCC1, and the anion ( $\text{Cl}^-$ - $\text{HCO}_3^-$ ) exchangers, either  $\text{Na}^+$ -independent (AE) or  $\text{Na}^+$ -driven (NDCBE) forms (Farrant and Kaila, 2007) (Fig. 9Di). To date, the specific expression of each transporter species in CWCs is not known, but we assumed that all four kinds are functioning in order to develop a hypothesis for the mechanism of  $\text{Ca}^{2+}$ -dependent negative  $E_{\text{gly}}$  shift. For KCCs or NKCCs to cause a decrease in  $[\text{Cl}^-]_i$  with  $\text{Ca}^{2+}$  spiking, their activity needs to be increased or decreased, respectively, with a rise in intracellular  $\text{Ca}^{2+}$ . However, given the depolarized  $V_m$  during  $\text{Ca}^{2+}$  spiking, extracellular  $\text{K}^+$  could elevate, and the electrochemical driving force thus may not be in favor of KCC transporting  $\text{Cl}^-$  out of the cell. Moreover, blockade of NKCC with bumetanide did not prevent the effect of  $\text{Ca}^{2+}$  spiking on  $E_{\text{gly}}$  (Supplemental Results). The  $\text{Cl}^-$ - $\text{HCO}_3^-$  exchangers, AE and NDCBE, have been studied mostly in the context of  $\text{pH}_i$  regulation: AE mediates influx of  $\text{Cl}^-$  while exporting  $\text{HCO}_3^-$  activated by intracellular alkalinization, and NDCBE, known as an acid extruder, moves  $\text{Cl}^-$  out in exchange for  $\text{HCO}_3^-$  driven by the  $\text{Na}^+$  gradient (Chesler, 2003; Romero et al., 2004). Intracellular acidification has been shown to occur with spiking activity or depolarization in various types of neurons often in a  $\text{Ca}^{2+}$ -dependent way (e.g. Trapp et al., 1996a), and the proposed mechanisms are replacement of  $\text{H}^+$  by  $\text{Ca}^{2+}$  in intracellular binding sites, mitochondrial  $\text{Ca}^{2+}$  uptake leading to  $\text{H}^+$  release and cytosolic  $\text{Ca}^{2+}$  removal by  $\text{Ca}^{2+}$ - $\text{H}^+$  ATPases of plasma membrane (PMCA) or endoplasmic reticulum (SERCA) (reviewed in Ballanyi and Kaila, 1998; Chesler, 2003).

Therefore, we propose that complex or  $\text{Ca}^{2+}$  spiking leads to a  $\text{Ca}^{2+}$ -dependent intracellular acidification, which leads NDCBE to extrude  $\text{Cl}^-$  and cause a negative shift in  $E_{\text{gly}}$  (Fig. 9Dii). Also contributing to the negative shift in  $E_{\text{gly}}$  could be the lowering of  $[\text{HCO}_3^-]_i$  during the intracellular acidification (Kaila et al., 1993), provided that  $P_{\text{CO}_2}$  is constant and carbonic anhydrase is present, thus promoting fast equilibration of the reaction,  $\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{H}^+ + \text{HCO}_3^-$  (Roos and Boron, 1981). We tested key elements of this hypothesis, examining whether i) the  $\text{pH}_i$  decreased with  $\text{Ca}^{2+}$ /complex spiking and the decrease was  $\text{Ca}^{2+}$ -dependent, ii)  $E_{\text{gly}}$  could shift negative with an intracellular acidification not associated with electrical activity, and iii) blocking NDCBE could eliminate the negative  $E_{\text{gly}}$  shift.

### Activity-dependent and $\text{Ca}^{2+}$ -dependent intracellular acidification

Changes in  $\text{pH}_i$  were monitored with the fluorescent indicator SNARF-5F ("SNARF"). To maintain the perforated patch condition, the dye was introduced into the cell by including the AM ester form in the recording pipette, which we found would diffuse through the perforated patch and become de-esterified. This loading method led to a steady increase in the baseline intracellular fluorescence over the course of the recording due to accumulation of the de-esterified indicator (Fig. S2A). A change in  $\text{pH}_i$  was detected by measuring SNARF emission at wavelengths  $> 600$  nm, where the fluorescence intensity decreased with a decrease in pH. SNARF images were taken at 1 Hz during a run of the 8s challenge protocol to follow simultaneously the change in  $\text{pH}_i$  and  $E_{\text{gly}}$  after complex/ $\text{Ca}^{2+}$  spiking. Shown in Figure 6A are the concurrent changes in SNARF signal and  $E_{\text{gly}}$  induced by simple (red) and complex (black) spiking in one cell. Similar profiles were observed in 9 other cells. In all cells, the  $\text{pH}_i$  fell during both complex and simple spiking, and the recovery to near control level occurred within the next 120 sec beginning immediately after the spikes terminated. The degree of acidification, however, was greater with complex spiking than with simple spiking in each cell, with the peak SNARF signal 119–240 % (mean 171 %) larger with complex spiking ( $p < 0.001$ , paired  $t$ -test,  $n = 10$ ). Complex spiking was evoked with 150–250 pA in different cells, and the

resulting acidification and peak negative  $E_{\text{gly}}$  shift were  $-9.5 \pm 1.6$  in  $\% \Delta F/F$  ( $n = 10$ ) and  $-1.9 \pm 0.2$  mV ( $n = 6$ ). The corresponding values for simple spiking, evoked with 70–200 pA in the same group of cells, were  $-5.7 \pm 1.1$  in  $\% \Delta F/F$  ( $n = 10$ ) and  $-0.8 \pm 0.2$  mV ( $n = 6$ ). The fact that complex spiking led to a larger  $\text{pH}_i$  decrease than simple spiking suggests that the intracellular acidification may be proportional to the increase in  $[\text{Ca}^{2+}]_i$ . However, that an obvious negative  $E_{\text{gly}}$  shift was only observed with complex spiking suggests that the intracellular acidification may need to be larger than a threshold level to be associated with a negative  $E_{\text{gly}}$  shift.

$\text{Ca}^{2+}$  spiking (in TTX) was found to cause the same response pattern, a drop in  $\text{pH}_i$  during the activity, with recovery beginning upon termination of the activity (Fig. 6Bi-ii, controls). As illustrated in Figure 6Bi-ii, the acidification elicited by  $\text{Ca}^{2+}$  spiking under control conditions became unnoticeable (Fig. 6Bi,  $n = 5$  cells) or largely reduced (Fig. 6Bii,  $n = 3$  cells) when the same amount of current injection was given in zero- $\text{Ca}^{2+}$  (replaced with  $\text{Co}^{2+}$  or  $\text{Mg}^{2+}$ ) or in 300  $\mu\text{M}$   $\text{Cd}^{2+}$ . Switching to solutions containing 2.4 mM  $\text{Co}^{2+}$  (zero- $\text{Ca}^{2+}$ ) or 300  $\mu\text{M}$   $\text{Cd}^{2+}$  caused the resting  $E_{\text{gly}}$  to shift positive by 4–7 mV with an apparent shrinkage of the cell body. Nevertheless, there was an additional positive shift in  $E_{\text{gly}}$  immediately after depolarization ( $n = 8$  cells), just as in the previous experiments without SNARF. The  $\% \Delta F/F$  values at the end of the 8-sec current injection are plotted against the peak negative  $E_{\text{gly}}$  shift (in control condition) and the peak positive  $E_{\text{gly}}$  shift (in  $\text{Ca}^{2+}$  block) for the 8 cells in Figure 6Biii. The average reduction in acidification by  $\text{Ca}^{2+}$  block was  $6.6 \pm 0.9$  in  $\% \Delta F/F$  for the 8 data series in the plot. In  $\text{Ca}^{2+}$  block conditions, another current injection 100–250 pA larger than that used for evoking  $\text{Ca}^{2+}$  spikes was given in 7 of the 8 cells. A larger current injection, i.e. larger depolarization, caused a more positive  $E_{\text{gly}}$  shift in all cells, but no change in the  $\text{pH}_i$  response (5 of the 7 cells; Fig. 6Bi-ii).

Change in  $[\text{Ca}^{2+}]_i$  and  $\text{pH}_i$  were monitored simultaneously with respect to an 8-sec complex spiking taking advantage of the overlapping 2-photon excitation spectra of the fluorescent indicators. The  $\text{Ca}^{2+}$  indicator Fluo-4 ( $n = 3$ ) or Fura-2 ( $n = 5$ ) was loaded into the cell along with SNARF by including their AM forms in the recording pipette. As shown in Figure 6C, the rise and fall in  $[\text{Ca}^{2+}]_i$  was rapid, and thus restricted to the period of spiking, in sharp contrast to the prolonged decrease in  $\text{pH}_i$ . The  $[\text{Ca}^{2+}]_i$  peaked at the end of the depolarization and then within 5 sec fell to about 10–15 % (3 cells with Fluo-4) or 20–25 % (5 cells with Fura-2) of the peak. The relatively fast clearance of the  $\text{Ca}^{2+}$  rise compared to that of  $\text{H}^+$  ( $\text{pH}_i$  recovery) after complex spiking suggests that the slow recovery of  $\text{pH}_i$  is not secondary to a gradual recovery of  $\text{Ca}^{2+}$  but rather attributable to the rate of  $\text{H}^+$  removal and the intracellular  $\text{H}^+$  buffering capacity. These data confirm that both intracellular acidification and negative  $E_{\text{gly}}$  shift are  $\text{Ca}^{2+}$  dependent.

### Activity-independent changes in $\text{pH}_i$ and $E_{\text{gly}}$

We next explored whether the  $\text{pH}_i$  decrease is a necessary intermediate in the process of  $\text{Ca}^{2+}$ -induced negative  $E_{\text{gly}}$  shift. Preventing or reducing the  $\text{pH}_i$  decrease during  $\text{Ca}^{2+}$  spiking by blocking the PMCA and SERCA was attempted using carboxyeosin, an inhibitor of  $\text{Ca}^{2+}$ - $\text{H}^+$  ATPases (Gatto et al., 1995). Unfortunately, this compound (diacetate form bath-applied at 40 or 80  $\mu\text{M}$ ) was not useful, due both to its intrinsic fluorescence, which precluded monitoring of  $\text{pH}_i$  with SNARF, and to its suppression of  $\text{Ca}^{2+}$  spikes, with only 1–3 spikes evoked at the onset of an 8-sec depolarization (see Choi and Eisner, 1999).

We asked instead whether  $E_{\text{gly}}$  shifts negative with an activity-independent intracellular acidification induced by a weak acid (Roos and Boron, 1981) in CWCs. Kaila et al. (1993) had shown that the IPSP reversal potential shifted negative or positive during application of weak acid or weak base, respectively, in neocortical pyramidal cells. Sodium propionate, 20 mM, was perfused for 100–120 sec while cells were held at  $-75$  mV in voltage clamp (in TTX),

with periodic  $E_{\text{gly}}$  measurements. In order to maintain the same  $[\text{Cl}^-]_o$  throughout the period of  $E_{\text{gly}}$  measurements, cells were bathed in a solution containing 20 mM Na-methanesulfonate instead of Na-propionate before and after the propionate challenge (Kaila et al., 1993). Simultaneous records of  $\text{pH}_i$  change and  $E_{\text{gly}}$  during propionate wash-in and wash-out were obtained from 5 cells as shown in Figure 7Ai. In all cases,  $\text{pH}_i$  fell during propionate perfusion (peak acidification of  $-8.4 \pm 3.1$  in  $\% \Delta F/F$ ) and this change was correlated with a negative shift in  $E_{\text{gly}}$  (peak shift of  $-4.9 \pm 1.6$  mV compared to the mean  $E_{\text{gly}}$  during 100 sec before propionate). Upon propionate wash-out, a small overshoot in pH above baseline level was observed ( $3.7 \pm 2.6$  in  $\% \Delta F/F$ ), accompanied in 4 of 5 cases by a concurrent overshoot in  $E_{\text{gly}}$ ,  $1.6 \pm 0.6$  mV.

We then applied the weak base trimethylamine (“TMA”, 10 mM) to see if an alkalization would shift  $E_{\text{gly}}$  in the depolarizing direction in CWCs. A parallel positive shift in  $E_{\text{gly}}$  was observed along with the TMA-induced intracellular alkalization in all 4 cells examined (Fig 7Aii; peak shifts were  $1.9 \pm 0.3$  mV in  $E_{\text{gly}}$  and  $4.0 \pm 0.9$  in  $\% \Delta F/F$ ). Moreover, an undershoot occurred upon TMA washout in  $\text{pH}_i$  and  $E_{\text{gly}}$ , of  $-5.1 \pm 0.9$  in  $\% \Delta F/F$  and  $-2.6 \pm 0.9$  mV, respectively. The overshoot and undershoot in  $\text{pH}_i$  during wash-out of weak acid and weak base may reflect the activation of  $\text{pH}_i$  regulation mechanisms during intracellular acidification and alkalization, respectively (Roos and Boron, 1981). With both weak acid and weak base challenges, the  $E_{\text{gly}}$  was found to shift negative with a  $\text{pH}_i$  decrease and to shift positive with a  $\text{pH}_i$  increase. The Spearman's correlation coefficient ( $\rho$ ) between  $E_{\text{gly}}$  and decimated SNARF signal (in  $\% \Delta F/F$ ) was calculated for each run of propionate or TMA challenge. With propionate, the average correlation coefficient for the full duration (800 sec) of the trial was  $0.61 \pm 0.09$  ( $p < 0.05$  for all 5 cases) and that for the 135-sec period of wash-in to wash-out was  $0.88 \pm 0.10$  ( $p < 0.05$  for all 5 cases). For TMA runs, the coefficient for whole duration was  $0.73 \pm 0.11$  ( $p < 0.05$  for all 4 cases) and that for the 160-sec period of wash-in and out was  $0.78 \pm 0.23$  ( $p < 0.05$  in 3 cases,  $p > 0.05$  in one). Thus, the activity-independent  $\text{pH}_i$  decrease or increase caused negative or positive shifts in  $E_{\text{gly}}$  in CWCs (see Supplemental Results for the issue of possible changes in  $[\text{Ca}^{2+}]_i$  by weak acid/base).

### Simultaneous monitoring of $\text{Cl}^-_i$ and $\text{pH}_i$

$\text{pH}_i$  may affect  $E_{\text{gly}}$  through the NDCBE/AE-mediated change in  $[\text{Cl}^-]_i$ , and/or through the passive change in  $[\text{HCO}_3^-]_i$ . To examine whether  $[\text{Cl}^-]_i$  actually falls during the activity-dependent negative  $E_{\text{gly}}$  shift, we used MQAE, a fluorescent dye quenched by  $\text{Cl}^-$  and relatively insensitive to pH changes (Verkman, 1990; Marandi et al., 2002). MQAE loading of CWCs was done by incubating slices in the indicator. MQAE (3 mM) was loaded for 8 min at 34 °C; longer incubations or higher concentrations led to excessive depolarization and inability to maintain firing. With SNARF loaded from the recording pipette, simultaneous monitoring of  $\text{pH}_i$  and  $\text{Cl}^-_i$  by 2-photon imaging (Fig 7Bi) was done in CWCs held silent in current clamp. The MQAE fluorescence was found to increase (a decrease in  $\text{Cl}^-_i$ ) after sufficient complex spiking in each cell examined, and the return to baseline took place over similar time scale as that of  $\text{pH}_i$  (Fig. 7Bii). Eight sec of depolarization-induced simple spiking examined in 4 cells did not induce a change in MQAE fluorescence while complex spiking did so in the same cells (not shown). The peak increase in MQAE signal was 3.3–7.3 in  $\% \Delta F/F$  (average 4.7,  $n = 15$ ) after an 8-sec complex spiking evoked by 100–250 pA in different cells, and the corresponding peak decrease in SNARF signal ranged  $-6.4$  to  $-13.5$  in  $\% \Delta F/F$  (average  $-8.8$ ). Interestingly, the peak in MQAE fluorescence occurred at  $15.4 \pm 5.4$  sec from the end of complex spiking while the peak acidification was at  $1.9 \pm 1.5$  sec (average difference,  $13.5 \pm 5.3$  sec, paired  $t$ -test,  $p < 0.001$ ,  $n = 15$ ). The large difference in time course for MQAE and SNARF signals argues against the possibility that MQAE fluorescence might have originated from a decrease in non- $\text{Cl}^-$  quencher anion, such as  $\text{HPO}_4^{2-}$  and  $\text{HCO}_3^-$ , whose concentration is dependent on  $\text{pH}_i$  (Koncz and Daugirdas, 1994). The time-to-peak of MQAE fluorescence

(15 sec) was similar to that of the  $E_{\text{gly}}$  shift (16.3 sec), indicating that a change in  $E_{\text{gly}}$  reflects a change in  $[\text{Cl}^-]_i$ .

### Block of the negative $E_{\text{gly}}$ shift by $\text{H}_2\text{DIDS}$

Anion exchangers, NDCBE and AE, are known to be sensitive to block by disulfonic stilbene derivatives such as DIDS and SITS (Romero et al., 2004). Using  $\text{H}_2\text{DIDS}$ , an analog of DIDS, to block NDCBE, we asked whether the change in  $E_{\text{gly}}$  can be blocked despite activity-dependent intracellular acidification.  $\text{H}_2\text{DIDS}$  (100  $\mu\text{M}$ ) attenuated the glycine responses but did not prohibit determination of  $E_{\text{gly}}$ . Several effects of  $\text{H}_2\text{DIDS}$  were observed prior to testing its effects on the activity-dependent shift. In  $\text{H}_2\text{DIDS}$ , the *resting*  $E_{\text{gly}}$  (in TTX) changed by  $-3.6 \pm 2.0$  mV (from control resting  $E_{\text{gly}}$  ranging from  $-72$  to  $-85$  mV,  $p = 0.007$ , paired  $t$ -test,  $n = 6$ ) and  $V_m$  hyperpolarized (not measured but evident from the positive shift in holding current at  $-75$  mV).  $\text{H}_2\text{DIDS}$  increased the  $\text{Ca}^{2+}$  spikes number in response to current injection, and often the spiking did not cease immediately after termination of the stimulus. Therefore, cells were kept in voltage clamp at  $-75$  mV throughout a run of simultaneous  $\text{pH}_i$  and  $E_{\text{gly}}$  monitoring except for 10 sec when a glycine puff and an 8-sec depolarization were given in current clamp. The greater number of  $\text{Ca}^{2+}$  spikes evoked in  $\text{H}_2\text{DIDS}$  led to a larger  $\text{pH}_i$  decrease than under control condition in each cell examined ( $219 \pm 58$  % increase in number of spikes evoked with the same current, 70–300 pA in different cells, and  $168 \pm 34$  % larger acidification peak in SNARF signal,  $n = 6$ ) (Fig. 8A, B). Importantly, however, the negative  $E_{\text{gly}}$  shift was eliminated by  $\text{H}_2\text{DIDS}$  after the  $\text{Ca}^{2+}$  spiking ended ( $+0.4 \sim +1.5$  mV (mean  $+0.9$ ) shift compared to  $-1.4 \sim -4.1$  mV (mean  $-2.6$ ) in control conditions,  $n = 5$ ,  $p = 0.001$ , paired  $t$ -test; Fig. 8C). The small positive  $E_{\text{gly}}$  shift peaked at 2-sec point after  $\text{Ca}^{2+}$  spiking in  $\text{H}_2\text{DIDS}$  and is probably due to accumulation of  $\text{Cl}^-$  through a passive  $\text{H}_2\text{DIDS}$ -insensitive mechanism. The elimination of negative  $E_{\text{gly}}$  shift in  $\text{H}_2\text{DIDS}$  excludes the possibility that the negative  $E_{\text{gly}}$  shift in control conditions was primarily caused by the reduction in  $[\text{HCO}_3^-]_i$  during the  $\text{pH}_i$  decrease rather than by the reduction in  $[\text{Cl}^-]_i$ , consistent with our conclusions with MQAE.

The recovery time course of  $\text{pH}_i$  appeared slower in  $\text{H}_2\text{DIDS}$  in all cases examined, and this was evident in the cell of Figure 8A, in which an acidification of similar magnitude to that in control experiment was obtained with a smaller current injection in  $\text{H}_2\text{DIDS}$ . In addition, the peak acidification in  $\text{H}_2\text{DIDS}$  did not occur at or immediately after the end of 8-sec depolarization (at  $1.3 \pm 0.6$  sec in controls,  $n = 6$ ) but was delayed to a later time than in control conditions (difference in time to peak  $15.2 \pm 8.2$  sec,  $n = 6$ ) (Fig. 8A, B). This suggests that the production of acid continues beyond the duration of depolarizing current injection, and the mechanism inhibited by  $\text{H}_2\text{DIDS}$  normally works to remove the acid fast enough for the  $\text{pH}_i$  to begin rising immediately after the depolarization. Though the  $\text{H}_2\text{DIDS}$ -sensitive acid removal mechanism may be the  $\text{Na}^+\text{-HCO}_3^-$  cotransporter (Romero et al., 2004) as well as the NDCBE, that the slowed  $\text{pH}_i$  recovery occurred together with the block of negative  $E_{\text{gly}}$  shift in  $\text{H}_2\text{DIDS}$  supports the involvement of NDCBE.

### Block of the negative $E_{\text{gly}}$ shift by $\text{HCO}_3^-/\text{CO}_2$ removal

Although we interpreted the effects of  $\text{H}_2\text{DIDS}$  in terms of the block of NDCBE, the drug may also have inhibited KCC. For example,  $\text{H}_2\text{DIDS}$  and DIDS block KCC in red blood cells (Delpire and Lauf, 1992; Culliford et al., 2003). To confirm that the  $\text{Cl}^-/\text{HCO}_3^-$  exchangers, rather than the KCC, is involved in the negative  $E_{\text{gly}}$  shift after  $\text{Ca}^{2+}$  spiking, we investigated whether removal of  $\text{HCO}_3^-$  and  $\text{CO}_2$  could block the negative  $E_{\text{gly}}$  shift. In addition to disabling NDCBE,  $\text{HCO}_3^-$  removal is expected to eliminate the contribution of lowered  $[\text{HCO}_3^-]_i$  to the negative  $E_{\text{gly}}$  shift during a  $\text{pH}_i$  decrease. For  $\text{HCO}_3^-/\text{CO}_2$ -free condition,  $\text{NaHCO}_3$  in ACSF was replaced with equimolar HEPES, and the solution was gassed with 100 %  $\text{O}_2$  (“HEPES/ $\text{O}_2$ ”). After  $>10$  min of perfusion with HEPES/ $\text{O}_2$ , the resting  $E_{\text{gly}}$  of CWCs shifted by  $+0.4$



$\pm 1.8$  mV (range  $-3.4 \sim +2.7$  mV,  $n = 12$ ,  $p = 0.50$  with paired  $t$ -test), and the excitability slightly increased in 9 of 12 cells. Although reduced, the negative  $E_{\text{gly}}$  shift with 8-sec  $\text{Ca}^{2+}$  spiking persisted in HEPES/ $\text{O}_2$  in all cells examined (Fig. 9Ai), contrary to our expectation (see Supplemental Results). However, it is possible that  $\text{CO}_2$  may still be produced by oxidative metabolism in cells despite perfusion of a nominally  $\text{HCO}_3^-/\text{CO}_2$ -free solution (Voipio and Ballanyi, 1997). Hydration of  $\text{CO}_2$  could then generate sufficient  $\text{HCO}_3^-$  in the slice to drive NDCBE. Therefore, we minimized endogenous  $\text{HCO}_3^-$  production by blocking carbonic anhydrase which catalyzes the hydration of  $\text{CO}_2$ . Acetazolamide (“AZA”), a membrane-permeable inhibitor of carbonic anhydrase, added in HEPES/ $\text{O}_2$  at  $50 \mu\text{M}$  caused the resting  $E_{\text{gly}}$  of CWCs to shift negative by  $4.6 \pm 2.6$  mV (from  $-64 \sim -81$  mV,  $n = 10$ ,  $p < 0.001$ , paired  $t$ -test). The excitability increased in AZA/HEPES/ $\text{O}_2$  to such a degree that cells would not stop  $\text{Ca}^{2+}$  spiking after an 8-sec depolarization; thus,  $E_{\text{gly}}/\text{pH}_i$  series were obtained in voltage clamp as described above for  $\text{H}_2\text{DIDS}$  experiments. In AZA/HEPES/ $\text{O}_2$ , the negative  $E_{\text{gly}}$  shift was absent after an 8-sec  $\text{Ca}^{2+}$  spiking in all 10 cells examined (Fig. 9Aii). Instead,  $E_{\text{gly}}$  was positively shifted, which peaked at 8–55 sec after spiking, rather than immediately ( $\sim 2$  sec) (9 of 10 cells; Fig. 9Aii, B). The maximal positive  $E_{\text{gly}}$  shift over several runs of  $E_{\text{gly}}/\text{pH}_i$  series with different current injections in single cells ranged  $1.6\text{--}3.1$  mV (average  $2.3$  mV, after 22–96  $\text{Ca}^{2+}$  spikes evoked by 27–190 pA in different cells,  $n = 10$ ).

AZA/HEPES/ $\text{O}_2$  also had effects indicative of reduced control of  $\text{pH}_i$ . During experiments with AZA/HEPES/ $\text{O}_2$ , attempts were made to evoke similar numbers of  $\text{Ca}^{2+}$  spikes in AZA to that before AZA addition by reducing the amount of injected current during  $E_{\text{gly}}/\text{pH}_i$  series. Figure 9C shows the peak acidification (i) and half-time of  $\text{pH}_i$  recovery (ii) plotted against the peak negative and positive  $E_{\text{gly}}$  shift for HEPES/ $\text{O}_2$  and AZA/HEPES/ $\text{O}_2$ , respectively, for 6 cells in which the numbers of evoked  $\text{Ca}^{2+}$  spikes were similar between the two conditions ( $\leq 3$  spike difference). The difference between the HEPES/ $\text{O}_2$  and AZA/HEPES/ $\text{O}_2$  conditions in peak acidification was not significant ( $p = 0.24$ , paired  $t$ -test), but the half-recovery time was longer by  $166 \pm 21\%$  in AZA ( $p < 0.001$ , paired  $t$ -test) as well as the time of peak acidification (delayed by  $3.2 \pm 1.7$  sec in AZA,  $p = 0.006$ , paired  $t$ -test). Thus, like  $\text{H}_2\text{DIDS}$  in  $\text{HCO}_3^-/\text{CO}_2$ -buffered conditions, the addition of AZA in HEPES/ $\text{O}_2$  slowed the  $\text{pH}_i$  recovery. We also tested AZA in  $\text{HCO}_3^-/\text{CO}_2$ -buffered condition. Resting  $E_{\text{gly}}$  again shifted negative by  $1.8 \pm 1.0$  mV in AZA ( $p = 0.002$ , paired  $t$ -test,  $n = 8$ ), and the excitability increased. After  $\text{Ca}^{2+}$  spiking under AZA, the  $E_{\text{gly}}$  shifted negative, and the recovery from acidification began without delay in all cells examined. Comparing control-AZA pairs of  $E_{\text{gly}}/\text{pH}_i$  series with similar number of evoked  $\text{Ca}^{2+}$  spikes (1 to 7 more spikes in AZA,  $n = 5$  cells), however, the negative  $E_{\text{gly}}$  shift was smaller by  $1.2 \pm 0.5$  mV in AZA ( $p = 0.005$ , paired  $t$ -test) while the peak acidification was  $139 \pm 20\%$  larger in AZA ( $p = 0.002$ , paired  $t$ -test). This effect, reduced coupling between  $\text{pH}_i$  decrease and  $E_{\text{gly}}$  shift, was similar to that observed in HEPES/ $\text{O}_2$  (Supplemental Results). Taken together, these results suggest that the mechanism responsible for the negative  $E_{\text{gly}}$  shift is  $\text{H}_2\text{DIDS}$ -sensitive and  $\text{HCO}_3^-$ -dependent, but may be able to function at low bicarbonate level (see Discussion).

### Immunohistochemical detection of NDCBE in the DCN

To confirm that NDCBE protein is indeed expressed in CWCs, we probed the DCN with two different clones of monoclonal antibodies against human NDCBE followed by fluorescent labeling with a secondary antibody. As expected from NDCBE's homeostatic function and as reported by Chen et al. (2008), wide-spread staining was found in the DCN, the adjoining brainstem and in the cerebellum. The two clones of NDCBE antibody resulted in identical staining pattern. In the DCN, the molecular layer was diffusely stained, and many cells, from small packed cells to large cells, were labeled over the entire DCN (Fig. 10A). No labeling was observed when the primary antibody was omitted (Fig. 10B). The labeling in DCN included numerous round, medium-sized cell bodies in the molecular and outer fusiform cell

layer, which corresponds to the size and distribution of CWCs (Wouterlood and Mugnaini, 1984; Berrebi and Mugnaini, 1991). Unexpectedly, the strongest labeling was found in the unipolar brush cell (UBC), identified by its single tufted dendrite (Dino and Mugnaini, 2008), both in the DCN and in the cerebellum (Fig. 10A, C). In the cerebellum, Purkinje cells were stained brightly in the soma and the primary dendrite as in previous reports (Damkier et al., 2007; Chen et al., 2008), and granule cells were also stained, as seen by a thin ring of labeled cytoplasm around the nuclei (Fig. 10C; Chen et al. 2008).

## DISCUSSION

We have identified a novel postsynaptic mechanism for enhancement of the effectiveness of inhibition during prolonged spiking activity or transiently following a period of increased activity. A shift in  $E_{GABA/IPSP}$  dependent on postsynaptic activity has been reported in hippocampal neurons (Fiumelli et al., 2005; Brumback and Staley, 2008) and subthalamic neurons (Wang et al., 2006). However, while the  $E_{GABA/IPSP}$  shift in both of these regions is  $Ca^{2+}$ -dependent, it differs from the situation in CWC in several major respects. These reported shifts were long-lasting (> 30 min) and often of *opposite* direction to that seen in CWC.  $E_{GABA}$  shifts in hippocampal cells were depolarizing and were induced with several minutes of 10–25 Hz firing. In subthalamic neurons, negative or positive shift in  $E_{IPSP}$  appeared after evoking rebound bursts at 0.1 Hz for 100 sec depending on intra-burst frequency. Unlike the positive  $E_{gly}$  shift in CWCs, the positive shift in this study was eliminated by application of  $Ca^{2+}$  channel blockers. The proposed mechanism for the  $E_{GABA}$  shift in hippocampal neurons was  $Ca^{2+}$ -mediated down-regulation of KCC2 (Fiumelli et al., 2005) or change in the set-point of NKCC following lowering of  $[Na^+]_i$  (Brumback and Staley, 2008). It may be that the mechanism we have identified is of general significance, but precedes the longer lasting  $Cl^-$  shifts described above. Given its dependence on dynamic control of intracellular  $Cl^-$  and pH, the drop in  $E_{gly}$  we describe may have been missed in studies that do not employ perforated patch recording.

### Negative $E_{gly}$ shift following intracellular acidification

Glycine responses ranged from depolarizing excitation to hyperpolarizing inhibition, reflecting the range of resting  $E_{gly}$  (–58 to –87 mV). These differences may be due to variation in the resting pH<sub>i</sub> and  $[Cl^-]_i$  among CWCs (see Supplemental Discussion). The negative  $E_{gly}$  shift following complex spiking likely arises from NDCBE-mediated decrease in  $[Cl^-]_i$ , given that the negative  $E_{gly}$  shift was sensitive to H<sub>2</sub>DIDS and removal of  $HCO_3^-$ . The contribution of lowered  $[HCO_3^-]_i$  to the activity-dependent negative  $E_{gly}$  shift does not appear as important as the decrease in  $[Cl^-]_i$  based on the following observations: 1) when  $Cl^-$ - $HCO_3^-$  exchangers were blocked by H<sub>2</sub>DIDS, the decrease in pH<sub>i</sub> was greater than control, but  $E_{gly}$  did not shift negative. 2) in nominally  $HCO_3^-$ /CO<sub>2</sub>-free Ringer (HEPES/O<sub>2</sub>), where the impact of change in  $[HCO_3^-]_i$  on  $E_{gly}$  is expected to be greatly reduced even if a few millimolar level of intra- and extracellular  $HCO_3^-$  is considered (Fig. S3C), the  $E_{gly}$  could shift negative similar to control conditions. It appears that the  $Na^+$ -driven  $Cl^-$ - $HCO_3^-$  exchange could run at a reduced rate with a few millimolar extracellular  $HCO_3^-$  in HEPES/O<sub>2</sub>, such that the  $E_{gly}$  shifted negative by the fall in  $[Cl^-]_i$ . This is consistent with the reported  $K_m$  or  $K_i$  for  $[HCO_3^-]_o$  of  $Na^+$ -driven or  $Na^+$ -independent  $Cl^-$ - $HCO_3^-$  exchange of 1 to 10 mM (Boron et al., 1981; Boron and Russell, 1983; Olsnes et al., 1986; Vaughan-Jones, 1986; Cassel et al., 1988). However, the amount of decrease in  $[Cl^-]_i$  associated with the activity-dependent negative  $E_{gly}$  that we observed, in normal  $HCO_3^-$ /CO<sub>2</sub>-buffered condition, may be small (<2 mM). The relation between  $E_{gly}$ ,  $[Cl^-]_i$ , and  $[HCO_3^-]_i$  according to the Goldman-Hodgkin-Katz (GHK) equation predicts that a drop in  $E_{gly}$  occurring with a pH<sub>i</sub> decrease involves less decrease in  $[Cl^-]_i$  than that occurring without a pH<sub>i</sub> change, and even an increase in  $[Cl^-]_i$  can be associated

with the negative  $E_{\text{gly}}$  shift depending on the magnitude of  $\text{pH}_i$  decrease (See Supplemental Discussion and Fig. S3).

The different contributors to  $\text{Cl}^-$  flux and their pH and voltage dependence likely underlies the timecourse of change in  $E_{\text{gly}}$ . During spiking, passive influx of  $\text{Cl}^-$  is expected to oppose efflux mediated by NDCBE. In the case of prolonged complex/ $\text{Ca}^{2+}$  spiking and large intracellular acidification, NDCBE-mediated  $\text{Cl}^-$  efflux may dominate the passive influx leading to fall in  $[\text{Cl}^-]_i$ . If the spiking-induced acidification did not drive NDCBE sufficiently,  $[\text{Cl}^-]_i$  may rise above baseline, and the outcome may be the small depolarizing shift in  $E_{\text{gly}}$  observed immediately after simple spiking and in some cases of complex spiking. After the voltage is restored and  $\text{pH}_i$  begins to normalize, remaining activity of NDCBE may become more prominent, which could account for the decrease in  $[\text{Cl}^-]_i$  during the initial 10 sec or so, i.e., the delayed peak in negative  $E_{\text{gly}}$  shift. It is possible that AE, KCC, and NKCC, which are inhibited at acidic  $\text{pH}_i$  (Olsnes et al., 1986; Boyarsky et al., 1988; Leem et al., 1999; Russell, 2000; Bergeron et al., 2003), may also be involved and contribute to the timecourse of  $E_{\text{gly}}$  change.

### Activity-induced intracellular acidification

Decrease in intracellular pH occurring with depolarization, spike firing, or  $\text{Ca}^{2+}$  rise, has been observed in a wide variety of neurons (reviewed in Ballanyi and Kaila, 1998; Chesler, 2003). The  $\text{pH}_i$  decrease during evoked spiking in CWCs required  $\text{Ca}^{2+}$  entry, as depolarization given in zero  $\text{Ca}^{2+}$  or after  $\text{Ca}^{2+}$  channel blockade induced little or no acidification. We did not investigate the mechanism of  $\text{Ca}^{2+}$ -dependent  $\text{pH}_i$  decrease in CWCs, but it could occur via multiple pathways, consistent with previous studies: displacement of  $\text{H}^+$  by  $\text{Ca}^{2+}$  in intracellular binding sites, mitochondrial  $\text{Ca}^{2+}/\text{H}^+$  exchange, PMCA or SERCA-mediated  $\text{Ca}^{2+}$  extrusion, and stimulation of metabolic acid production (Ballanyi and Kaila, 1998; Chesler, 2003). Considering the magnitude of change in  $\text{Cl}^-$ , and the proposed coupling between  $\text{Ca}^{2+}$ ,  $\text{H}^+$ , bicarbonate and  $\text{Cl}^-$ , leads to the conclusion that total  $\text{Ca}^{2+}$  flux during spike trains must have been quite large. Direct measurements of ion concentration and buffer capacity will be needed to confirm these relations. A novel aspect of present study was that the spiking-induced  $\text{pH}_i$  decrease was demonstrated with the gramicidin perforated-patch recording in mammalian neurons, while many previous studies utilized whole-cell recording (Trapp et al., 1996a; Trapp et al., 1996b; Meyer et al., 2000; Willoughby and Schwiening, 2002; Ritucci et al., 2005). We found that complex spiking-induced acidification was smaller and decayed faster in cells recorded whole-cell than in those recorded in perforated-patch condition (Fig. S2B). Willoughby and Schwiening (2002) showed in whole-cell recorded cerebellar Purkinje cells that acidic  $\text{pH}_i$  transients induced by depolarization or spiking were greater in dendrites than in soma. Given the large dendritic  $\text{Ca}^{2+}$  signals characteristic of CWCs (Roberts et al. 2008), complex/ $\text{Ca}^{2+}$  spiking could induce a larger negative  $E_{\text{gly}}$  shift in dendrites than we recorded at the soma, provided that NDCBE is expressed in dendrites.

### Functional relevance of shifts in $E_{\text{gly}}$

Golding and Oertel (1996) proposed that weakly excitatory glycinergic and GABAergic input in CWCs at rest could become inhibitory when the cell fires at high rates (i.e. during strong depolarization); this model assumed that during intense activity the voltage would be positive to a constant value of  $E_{\text{gly}}$  of about  $-55$  mV. A similar view in relation to change in  $E_{\text{GABA}}$ , was offered by Chavas and Marty (2003) regarding the polarity of GABAergic input to cerebellar molecular layer interneurons, which also was both excitatory and inhibitory. Unlike our study, Golding and Oertel (1996) did not observe cells with hyperpolarizing  $E_{\text{gly}}$  at rest. However, hyperpolarizing PSPs were seen at resting  $V_m$  of CWCs by Manis et al. (1994), and CWC spiking increased after strychnine or bicuculline *in vivo* (Davis and Young, 2000). Using perforated patch, Mancilla and Manis (2009) also observed hyperpolarizing IPSPs in CWCs

of young (P10–12) rats; however the resting potential in the cells they reported were relatively depolarized (mean  $-53$  mV). By contrast their mean reversal potential was  $-67$  mV, within the range of those we report here. These differences among studies may also be related to variation in the resting pattern of spontaneous activity of cells. We found that few spontaneously complex spiking cells showed excitatory responses, and many of these showed inhibitory responses to glycine. Indeed in Davis and Young (2000) only complex spiking units were selected for recording, and most CWCs in Manis et al. (1994) were predominantly complex spiking. It could be that the presumably more negative resting  $E_{\text{gly}}$  in these cells is associated with a lower resting  $\text{pH}_i$  or the dominance in activity of KCC over NKCC.

While the prolonged high-frequency complex spiking used in the present study may only rarely occur in physiological settings, it is likely that  $\text{pH}_i$  will fall during briefer periods of activity. Because such periods of activity will be associated with passive influx of  $\text{Cl}^-$ , we suggest that the  $\text{Ca}^{2+}$ -dependent mechanism will serve to minimize passive changes in  $E_{\text{gly}}$ . However, when such protracted spike activity does occur, the increase in glycinergic/GABAergic inhibition through the negative  $E_{\text{gly}}$  shift is expected to reduce the frequency of complex spikes in a CWC. For cells postsynaptic to a CWC, the DCN principal cells or other CWCs, this mechanism would limit the frequency of the large PSP bursts originating from presynaptic complex spikes (Tzounopoulos et al., 2004; Roberts et al., 2008), and thus limit the maximal glycinergic inhibition or excitation coming from a CWC. From the perspective of an inhibitory interneuron network, the use-dependent variation in resting  $E_{\text{gly}}$  which enables both excitatory and inhibitory connections may be more effective in stabilizing inhibition to principal neurons (Chavas and Marty, 2003).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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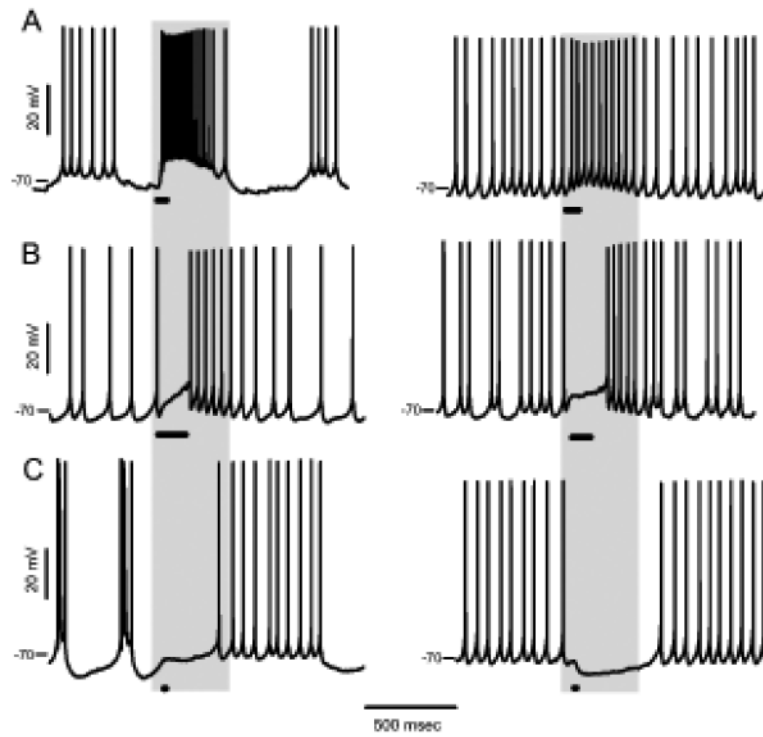
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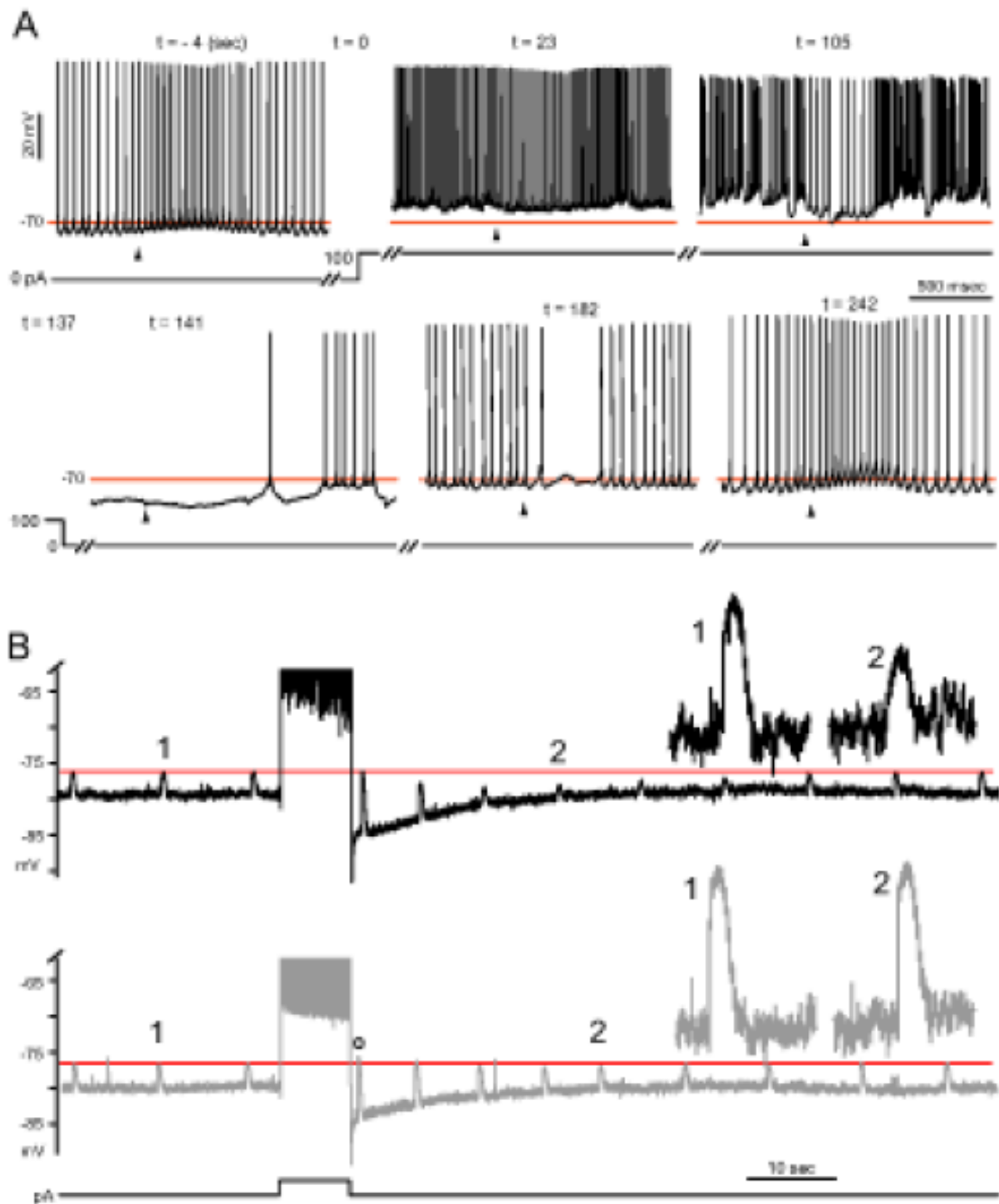
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**Figure 1. Three types of glycine response in spontaneously spiking CWCs**

Examples of excitatory, (A), mixed, (B), and inhibitory, (C), responses to a glycine puff during spontaneous activity. The duration of the glycine puff is indicated with a bar, and the glycine concentration was 0.5, 0.5, 0.5, 0.5, 2, 2 mM for the six cells in left-to-right, top-to-bottom order.

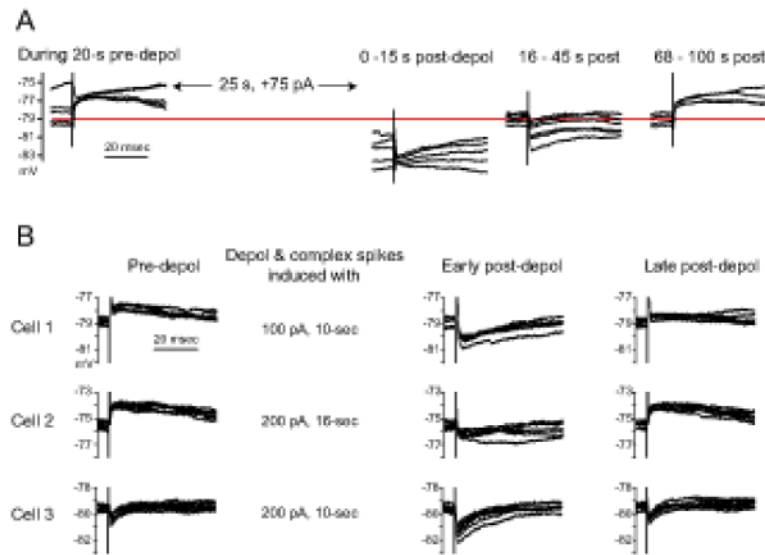


**Figure 2. Activity-dependent shifts in the glycine response**

(A) An example of negative glycine response shift occurring with prolonged complex spiking. An all-simple-spiking cell with an excitatory glycine response was injected 100 pA at  $t = 0$ . Complex spikes began appearing at  $t = 27$  s. The weakly inhibitory effect of glycine at  $t = 23$  s became more inhibitory ( $t = 105$  s) as complex spiking continued. After termination of depolarization, the glycine responses were hyperpolarizing until the 4th response ( $t = 182$ ), and the return of the excitatory response took  $\sim 100$  seconds ( $t = 242$  s). Arrowheads indicate time of a 500  $\mu\text{M}$  glycine puff, and the horizontal line is drawn at  $-70$  mV for reference. (B) Comparison of the change in glycine responses following simple and complex spiking. An all-simple-spiking cell with an inhibitory glycine (2 mM) response was silenced with  $-110$  pA

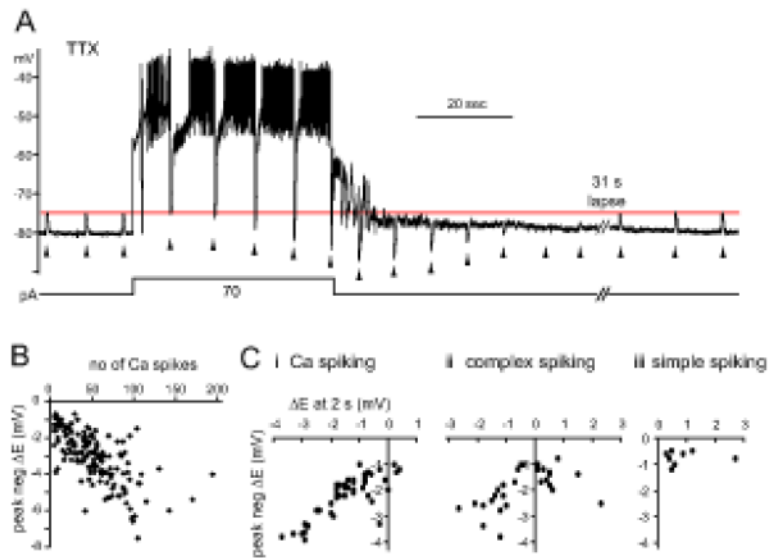
bias current and induced to fire late complex spikes (top) or trains of simple spikes (bottom), with an 8-sec step of 250 pA and 170 pA, respectively. The horizontal line is aligned to the peaks of glycine responses before the evoked activity. Insets show magnified responses at time points marked '1' and '2'. A positive shift in the glycine response occurred (o) immediately after simple spiking.





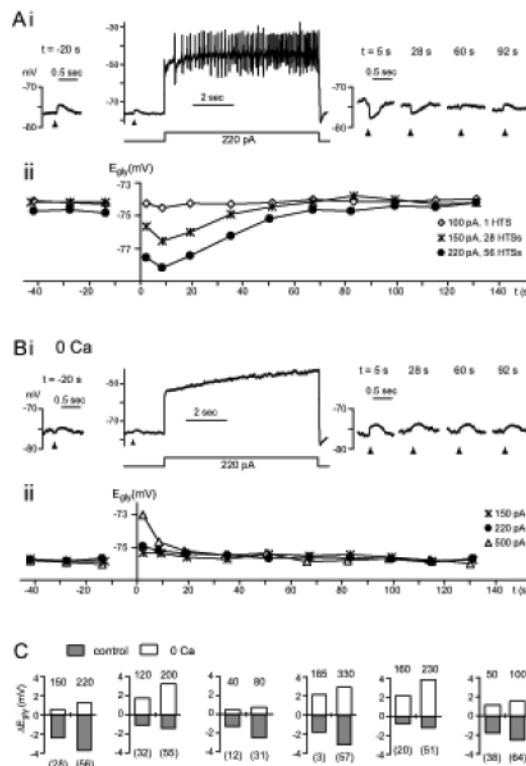
**Figure 3. Change in the reversal potential of glycinergic PSPs**

(A) Glycinergic/GABAergic PSPs were evoked at 0.5 Hz in a cell exhibiting spontaneous bursts of simple spikes. Four segments from a 150-sec-long  $V_m$  recording are shown in sequence. Complex spiking was induced after 20 sec of control period by 75 pA injection (depol) for 30 sec. The PSPs were depolarizing at  $V_m$   $-77$  mV during the pre-depolarization period. Just after the depolarization and complex spiking, PSPs were hyperpolarizing even at  $-81$  mV but became depolarizing at  $-77$  mV again over the next 100 sec. Recorded in the presence of 100  $\mu$ M APV and 10  $\mu$ M DNQX. (B) Evoked glycinergic/GABAergic PSPs shifting negatively after complex spiking. The bias current during the pre-depolarization period for cell 1, 2 and 3 was  $-45$ ,  $-60$  and  $0$  pA, respectively, and the current was adjusted post-depolarization to hold the  $V_m$  close to the pre-depolarization level.



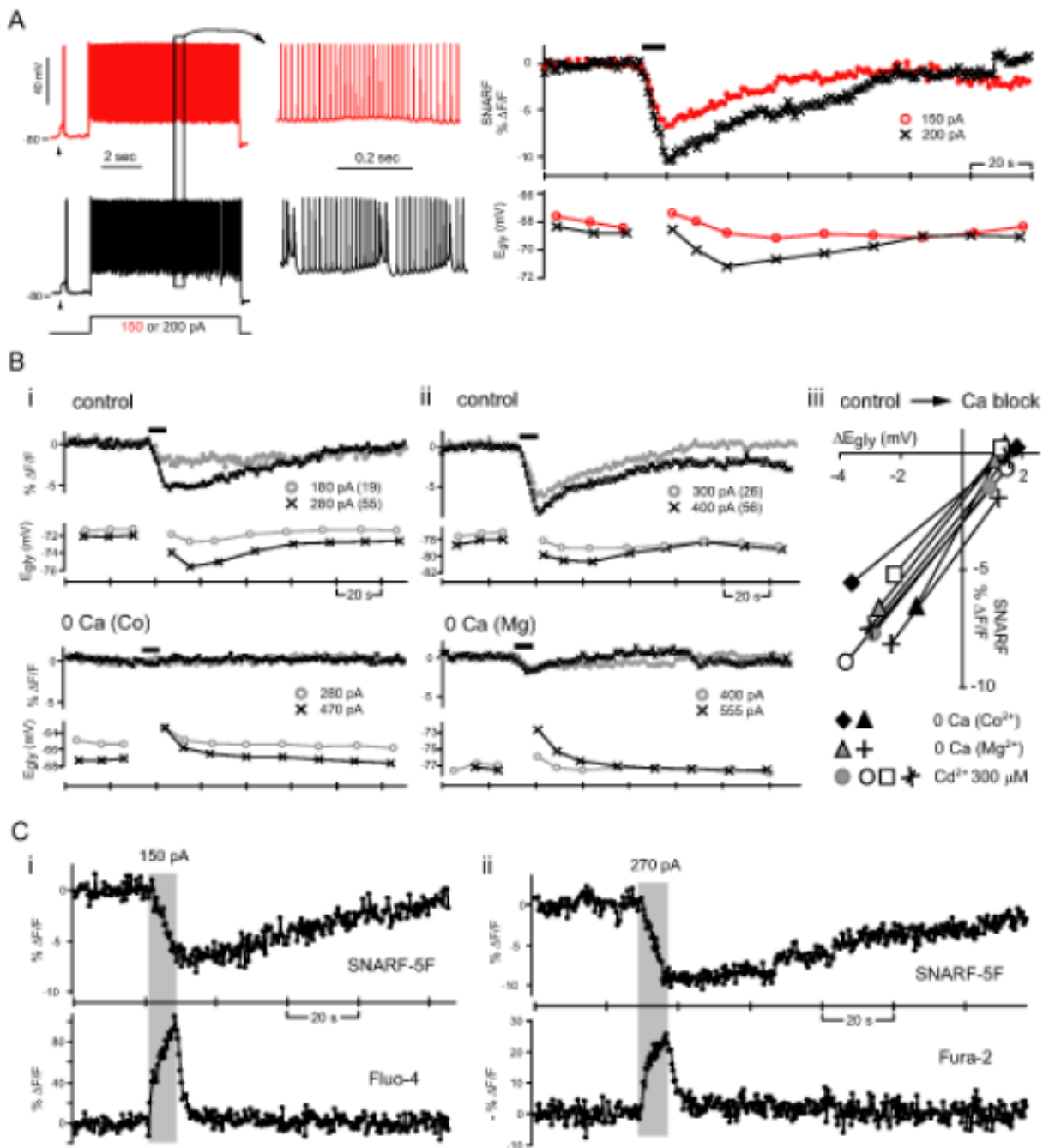
#### Figure 4. $\text{Ca}^{2+}$ spiking leads to $E_{\text{gly}}$ shifts

(A) Glycine responses (2mM, marked by arrowheads) shifted negative during long prolonged period of  $\text{Ca}^{2+}$  spiking. Spikes were evoked for 42 sec by +70 pA from -30 pA bias. A negative shift and recovery in  $E_{\text{gly}}$  was evident as the polarity of glycine responses changed from depolarizing to hyperpolarizing after  $\text{Ca}^{2+}$  spiking and back to depolarizing. The hyperpolarization to -80 mV at 2 sec into the stimulus was an afterhyperpolarization of the first  $\text{Ca}^{2+}$  spike, and the fluctuation of  $V_m$  during 10 sec after  $\text{Ca}^{2+}$  spiking is thought to be a manifestation of the intrinsic bistability of CWCs. (B) Plot of the peak negative shift in  $E_{\text{gly}}$  versus the number of high-threshold  $\text{Ca}^{2+}$  spikes evoked during an 8-sec challenge protocol. Dots represent 148  $E_{\text{gly}}$  series from 83 CWCs. 104 series are from 55 cells recorded with AM dye solution and 44 series from 28 cells without dye. (C) The shifts in  $E_{\text{gly}}$  at 2 sec after an 8-sec  $\text{Ca}^{2+}$  spiking (i), complex spiking (ii), or simple spiking (iii) are plotted against the peak negative shift of the  $E_{\text{gly}}$  series. Dots in each plot represent single  $E_{\text{gly}}$  series from different cells, with  $n=44$ , 29, and 8 for (i), (ii), and (iii), respectively. All data were obtained in TTX except (Cii-iii).



### Figure 5. $Ca^{2+}$ -dependent and independent change in $E_{gly}$

(A,B) Data from one cell comparing glycine responses and  $E_{gly}$  measurements in control condition (A) with those in zero- $Ca^{2+}$  (B). Time 0 is the moment the 8-sec depolarizing current injection terminated. (Ai) After 56 high-threshold  $Ca^{2+}$  spikes induced with a 220 pA injection, the glycine response shifted negative. (Aii) The  $E_{gly}$  series measured along with the  $V_m$  recording in (Ai) is shown (?) with two other series obtained with different amount of current injections. The negative  $E_{gly}$  shifts peaked at 8.5 sec. HTSs: high threshold spikes. (Bi,ii) The  $V_m$  and  $E_{gly}$  series in zero- $Ca^{2+}$  (replaced with  $Mg^{2+}$ ) are displayed in the same way as in (A). After a depolarization in zero- $Ca^{2+}$ , no negative shift but a positive shift in  $E_{gly}$  occurred. Bias current was  $-40$  pA in (Ai) and  $-55$  pA in (Bi). (C) Bar graphs showing the peak change in  $E_{gly}$  after an 8-sec current injection (amount in pA indicated above each bar) in control condition and in zero- $Ca^{2+}$ , from 6 cells. Number of  $Ca^{2+}$  spikes evoked is shown in parentheses under each bar belonging to control conditions. All data were obtained in TTX.

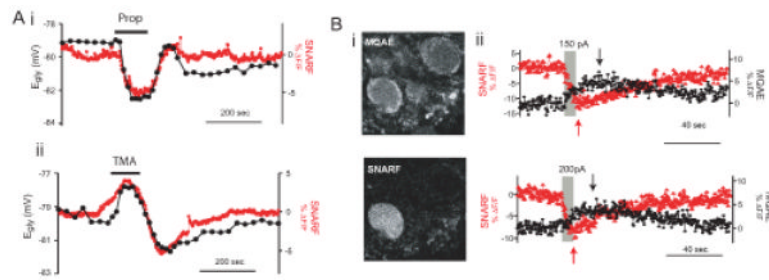


### Figure 6. $Ca^{2+}$ -dependent intracellular acidification

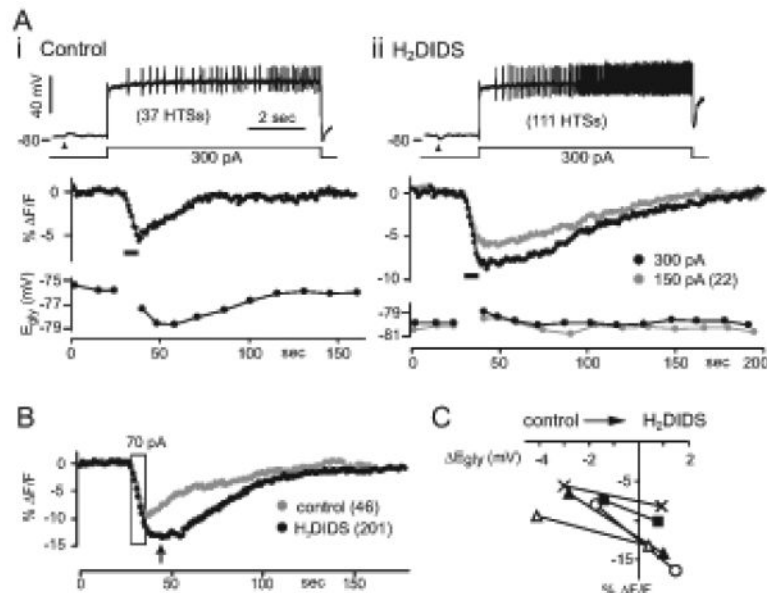
(A) Simultaneous monitoring of  $pH_i$  and  $E_{gly}$  with respect to 8-sec of simple spiking (red) and complex spiking (black) in one cell. Arrowheads indicate glycine (2 mM) responses. The pH-sensitive dye SNARF-5F's signal is the average fluorescence intensity of a region of interest drawn inside the cell body. An intracellular acidification, manifest as a decrease in SNARF's signal, occurred during both simple spiking and complex spiking. Black bar above SNARF traces indicate the duration of 8-sec depolarizing current injection. (B) The intracellular acidification was also seen with  $Ca^{2+}$  spiking (in TTX) and inhibited by zero- $Ca^{2+}$  (replaced with  $Co^{2+}$  or  $Mg^{2+}$ ) or 300  $\mu M$   $Cd^{2+}$ . (Bi-ii) Simultaneously recorded SNARF signal and  $E_{gly}$  from two cells. The 8-sec depolarizing current injections evoking  $Ca^{2+}$  spikes or just

depolarization after  $\text{Ca}^{2+}$  channel block are marked with thick bars above SNARF traces. The amount of injected current is shown beside each symbol along with the number of evoked  $\text{Ca}^{2+}$  spikes in parenthesis. An example of complete block (Bi) and incomplete block (Bii) of the depolarization-induced acidification by zero- $\text{Ca}^{2+}$  is shown. (Biii) Relation between the change in SNARF signal to the peak negative  $E_{\text{gly}}$  shift in control condition and the peak positive  $E_{\text{gly}}$  shift in  $\text{Ca}^{2+}$  block condition for 8 cells. (C) The change in  $\text{pH}_i$  and  $[\text{Ca}^{2+}]_i$  induced by 8-sec complex spiking were detected by simultaneous 2-photon imaging of SNARF-5F and Fluo-4 or Fura-2. Examples from 2 different cells are shown. The duration of 8-sec depolarizing current injection evoking complex spikes is indicated by the shaded rectangle. The excitation wavelengths were 800 nm (Ci) and 780 nm (Cii).





**Figure 7. Activity-independent  $pH_i$  and  $E_{gly}$  change and simultaneous  $Cl$  and  $pH$  imaging**  
 (A) Examples showing the concurrent changes in  $E_{gly}$  (left ordinate) and  $pH_i$  (right ordinate) induced during and after 20 mM sodium propionate (i) and 10 mM TMA (ii) perfusion for inducing intracellular acidification and alkalization, respectively. SNARF images were obtained at 0.5 Hz while cells were held at  $-75$  mV in  $v$ -clamp in TTX.  
 (B) Simultaneous 2-photon imaging of intracellular  $pH$  and  $Cl^-$  with SNARF and MQAE. Excitation wavelength at 750 nm. (Bi) Two images of one area taken at the same time through the red emission channel (SNARF) and green channel (MQAE). MQAE was bulk-loaded into DCN slice, and SNARF was loaded from the recording pipet on the left of the cell in lower left. Inhomogeneous staining with MQAE is typical. (Bii) Simultaneous records of  $pH_i$  (SNARF) and  $Cl^-_i$  (MQAE) obtained from two different cells. An 8-sec complex spiking was evoked during the period of gray rectangle with the indicated amount of current. The peak of each signal is indicated with an arrow.

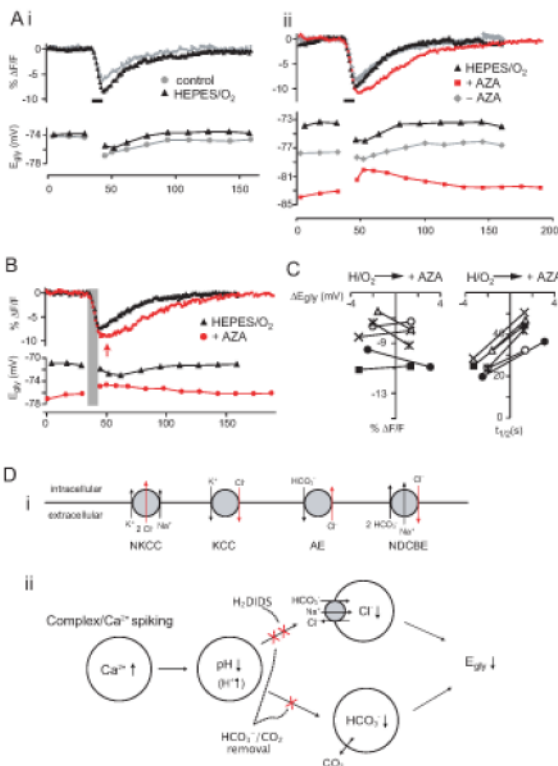


### Figure 8. Block of the negative $E_{gly}$ shift by H<sub>2</sub>DIDS

(A) Simultaneously obtained SNARF signal and  $E_{gly}$  from one cell are shown for control condition (i) and in 100  $\mu$ M H<sub>2</sub>DIDS (ii) along with the  $V_m$  trace of Ca<sup>2+</sup> spiking. Thick bars indicate periods of 8-sec Ca<sup>2+</sup> spiking. Arrowheads indicate time of 2 mM glycine puff. Number of evoked Ca<sup>2+</sup> spikes is shown in parenthesis. The bias currents during 8-sec current injection were  $-80$  pA and  $-55$  pA for (i) and (ii), respectively.

(B) Another example showing the difference in magnitude and time course of  $pH_i$  change between the control condition and in H<sub>2</sub>DIDS. The period of 8-sec Ca<sup>2+</sup> spiking evoked with 70 pA is bound by a rectangle. Numbers of evoked Ca<sup>2+</sup> spikes are shown in parenthesis. Arrow indicates the time of peak acidification in H<sub>2</sub>DIDS.

(C) Plot of peak acidification (in SNARF's  $\Delta F/F$ ) against the peak negative  $E_{gly}$  shift in control condition and the peak positive  $E_{gly}$  shift in H<sub>2</sub>DIDS. 5 cases (pairs) from different cells are shown. The same depolarizing current injection was used for control and H<sub>2</sub>DIDS conditions in each of the 5 cases, but the amount ranged from 70 to 300 pA in different cells. The average of peak negative  $E_{gly}$  shifts in controls is  $-2.6 \pm 1.1$  mV and that of peak positive shifts in H<sub>2</sub>DIDS is  $0.9 \pm 0.4$  mV for the 5 cases in the plot.



### Figure 9. Block of the negative E<sub>gly</sub> shift by HCO<sub>3</sub><sup>-</sup>/CO<sub>2</sub> removal

(A) The effect on E<sub>gly</sub> and pH<sub>i</sub> of removing HCO<sub>3</sub><sup>-</sup>/CO<sub>2</sub> from perfusion (HEPES/O<sub>2</sub>) (i), and then adding acetazolamide (AZA, 50 μM) (ii) in one cell. Superimposed control-treatment pairs of E<sub>gly</sub>/pH<sub>i</sub> series were chosen on the basis of similar number of evoked Ca<sup>2+</sup> spikes during the 8-sec depolarization. The periods of 8-sec Ca<sup>2+</sup> spiking are marked with thick bars.

(Ai) The injected current and number of evoked Ca<sup>2+</sup> spikes were 180 pA/16 and 120 pA/18 Ca<sup>2+</sup> spikes for the control and HEPES/O<sub>2</sub>, respectively. (Aii) The injected current and number of evoked Ca<sup>2+</sup> spikes were 130 pA/30, 110 pA/31, and 110 pA/15 Ca<sup>2+</sup> spikes for HEPES/O<sub>2</sub>, after AZA addition (+AZA), and 17 min after removing AZA (-AZA), respectively. The effects of AZA were fully reversible if it had been applied for less than about 15 min.

(B) Another example showing the slowed pH<sub>i</sub> recovery and positive E<sub>gly</sub> shift after addition of AZA in HEPES/O<sub>2</sub> after Ca<sup>2+</sup> spiking. Time of peak acidification in AZA is indicated with an arrow and the period of Ca<sup>2+</sup> spiking is shaded. The injected current and number of evoked Ca<sup>2+</sup> spikes were 170 pA/23 and 160 pA/22 Ca<sup>2+</sup> spikes for the HEPES/O<sub>2</sub> and +AZA, respectively.

(C) Plot of peak acidification (i) and half-recovery time (ii) against the peak negative E<sub>gly</sub> shift and the peak positive E<sub>gly</sub> shift for HEPES/O<sub>2</sub> and +AZA for 6 cases (from different cells) in which the number of evoked Ca<sup>2+</sup> spikes were similar between the two conditions. The number of evoked Ca<sup>2+</sup> spikes in +AZA condition ranged 20–47 in the 6 cases. The difference in peak acidification was not significant, but the half-recovery time was significantly lengthened in AZA added to HEPES/O<sub>2</sub>.

(D) Chloride transporters potentially affecting neuronal [Cl<sup>-</sup>]<sub>i</sub> (i), and the proposed mechanism of activity-dependent negative shift in E<sub>gly</sub> in CWCs (ii). KCC, the K<sup>+</sup>-Cl<sup>-</sup> cotransporters. NKCC, the Na<sup>+</sup>-K<sup>+</sup>-Cl<sup>-</sup> cotransporters. AE, the Na<sup>+</sup>-independent anion (Cl<sup>-</sup>-HCO<sub>3</sub><sup>-</sup>) exchanger. NDCBE, the Na<sup>+</sup>-driven Cl<sup>-</sup>-HCO<sub>3</sub><sup>-</sup> exchanger, also known as NDAE.



**Figure 10. Detection of NDCBE in the DCN by indirect immunofluorescence**

(A) A confocal laser scanned image showing labeling of NDCBE in the DCN. Putative CWCs are concentrated in the middle row of the image, and two of them are labeled with asterisks. Arrows, UBCs.

(B) Another DCN section processed at the same time with the one in (A), but without the primary antibody. Orientation of the DCN section in both (A) and (B) is the ependymal surface toward the bottom.

(C) A piece of cerebellum attached to the brainstem section containing the DCN of (A) shows labeling. Purkinje cells are marked with asterisks and one UBC with an arrow. Images of cerebellar sections not treated with the primary antibody were as dark as the one in (B) (not shown).

Scale bar, 20  $\mu\text{m}$ . NDCBE antibody was the 1G10 clone (1:100). Laser and confocal settings identical in all 3 micrographs.

**Table 1**  
Numbers and proportions of glycine response types in spontaneously spiking CWCs

|                 | n   | [%]    | exc. | (%)    | mix. | (%)    | inh. | (%)    |
|-----------------|-----|--------|------|--------|------|--------|------|--------|
| no dye          | 113 |        | 35   | (31.0) | 14   | (12.4) | 64   | (56.6) |
| complex-spiking | 30  | [26.5] | 1    | (3.3)  | 3    | (10.0) | 26   | (86.7) |
| simple-spiking  | 83  | [73.5] | 34   | (41.0) | 11   | (13.3) | 38   | (45.8) |
| AM dye          | 129 |        | 22   | (17.1) | 23   | (17.8) | 84   | (65.1) |
| complex-spiking | 17  | [13.2] | 0    | (0)    | 3    | (17.6) | 14   | (82.4) |
| simple-spiking  | 112 | [86.8] | 22   | (19.6) | 20   | (17.9) | 70   | (62.5) |
| total           | 242 |        | 57   | (22.3) | 37   | (15.3) | 148  | (61.2) |
| complex-spiking | 47  | [19.4] | 1    | (2.1)  | 6    | (12.8) | 40   | (85.1) |
| simple-spiking  | 195 | [80.6] | 56   | (28.7) | 31   | (15.9) | 108  | (55.4) |

% values in [] are the proportion of complex-spiking or simple-spiking cells.

Table 2

Resting  $E_{gly}$  of cartwheel cells

|                                | n          | $E_{gly}$ (mV) | SD         | range               |
|--------------------------------|------------|----------------|------------|---------------------|
| <b>Total</b>                   | <b>164</b> | <b>-74.3</b>   | <b>5.8</b> | <b>-87.4, -58.0</b> |
| spiking                        | 101        | -74.0          | 5.9        | -87.0, -58.0        |
| exc.                           | 24         | -68.5          | 4.6        | -75.9, -58.0        |
| mix.                           | 16         | -69.9          | 3.0        | -77.1, -65.5        |
| inh.                           | 61         | -77.3          | 4.5        | -87.0, -68.3        |
| silent ( $V_m$ -81.4 ± 2.8 mV) | 63         | -74.5          | 5.8        | -87.4, -60.8        |
| no dye                         | 92         | -73.1          | 5.3        | -85.0, -58.0        |
| SNARE-AM                       | 72         | -75.6          | 6.3        | -87.4, -63.2        |

See Supplemental Discussion for the possible cause of more negative  $E_{gly}$  in AM-dye loaded cells.