Thermodynamic Measurements May Reveal a New Ion/Substrate Stoichiometry for the $\gamma$-Aminobutyric Acid Transporter 1

Samantha L. Willford
Faculty Mentor: Sepehr Eskandari

Biological Sciences Department, California State Polytechnic University, Pomona, CA 91768-4032, USA
Introduction

Neurons communicate with one another via chemical messenger molecules called neurotransmitters. Neurotransmitters are released from pre-synaptic neurons into the synaptic cleft that separates the pre-synaptic and post-synaptic neurons (Borden, 1996). After release into the synaptic cleft, neurotransmitter molecules diffuse to the post-synaptic cell where they bind to receptors, thereby leading to excitation (depolarizing post-synaptic potentials) or inhibition (hyperpolarizing post-synaptic potentials) of the post-synaptic neuron. While this mechanism for information transmission from the pre- to post-synaptic neuron is well-studied, the mechanism used to bring about the termination of the signal is not as clearly understood. In most synapses, signal termination is accomplished by neurotransmitter transporters, which remove the neurotransmitter molecules from the synapse and transport them back to the pre-synaptic cell or surrounding glial cells (Borden, 1996; Nelson, 1998).

γ-Aminobutyric acid (GABA) is the most abundant inhibitory neurotransmitter in the adult mammalian central nervous system. Proper regulation of the GABA concentration is critical for normal brain function, and is accomplished by Na⁺ and Cl⁻-coupled GABA transporters found in the plasma membrane of pre-synaptic neurons and glial cells (Borden, 1996, Nelson, 1998, Conti et al., 2004; Kristensen et al., 2011). The GABA transporters couple the energy stored in the electrochemical gradients of Na⁺ and Cl⁻ to drive GABA against its concentration gradient across neuronal and glial plasma membranes. By regulating the extracellular concentrations of GABA, the GABA transporters regulate the level of neuronal excitability and, thus, are implicated in the pathophysiology of seizures and stroke (Kristensen et al., 2011).

The ion/substrate stoichiometry of the GABA transporter determines the ability of the transporter to regulate extracellular GABA concentrations, however, there has not been a definitive determination transporter coupling stoichiometry (Pastuszko et al., 1982; Radian and
Kanner, 1983; Keynan and Kanner, 1988; Loo et al., 2000). The GABA transporter can work in forward and reverse modes and this property allows us to use a thermodynamic approach to determine the ion/substrate coupling stoichiometry of the transport cycle (Lu and Hilgemann, 1999).

**Materials and Methods**

Stage V–VI *Xenopus laevis* oocytes were injected with 50 ng of cRNA for human GAT1 (hGAT1; SLC6A1) or mouse GAT3 (mGAT3; homologous to rat/human GAT-2, SLC6A13) (Chen et al., 2004). After cRNA injection, oocytes were maintained in Barth’s medium (in mM: 88 NaCl, 1 KCl, 0.33 Ca(NO₃)₂, 0.41 CaCl₂, 0.82 MgSO₄, 2.4 NaHCO₃, 10 4-[2-hydroxyethyl]-1-piperazineethanesulfonylic acid [HEPES], pH 7.4, and 50 µg/mL gentamicin, 100 µg/mL streptomycin, and 100 units/mL penicillin) at 18 °C for up to 14 days until used in experiments. Unless otherwise indicated, experiments were performed at 19 ± 1 °C.

Unless otherwise indicated, experiments were performed in a NaCl buffer containing (in mM): 100 NaCl, 2 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, pH 7.4. In Na⁺-free solutions, NaCl was isosmotically replaced with choline-Cl. Choline does not interact with the GABA transporters (Mager et al., 1993; Sacher et al., 2002). GABA or 1-(4,4-diphenyl-3-butenyl)-3-piperidinocarboxylic acid (SKF-89976A) was added to the above solutions as indicated.

The two-microelectrode voltage clamp technique was used to control the holding membrane potential (Vₘ) and to record the whole-cell transporter-mediated currents. For the thermodynamic measurements of the reversal potential (Vₜₘ), cells expressing GAT1 were preloaded with GABA for 20 minutes (exposure to 1 mM GABA at −50 mV), following which voltage pulses were applied in the presence of different extracellular concentrations of GABA or Na⁺. Voltage pulses included a series of 400-ms steps from 120 mV to −80 mV in 20-mV steps. The steady-state current was measured at the end of the voltage pulse. To obtain the currents specifically mediated by GAT1, the measurements were repeated in the presence of 30 µM SKF-
89976A (specific, high-affinity inhibitor of GAT1). The measured shifts in $V_{\text{rev}}$ were used to assess the stoichiometry of GATs (Roux and Supplisson, 2000; Owe et al., 2006; Coady et al., 2007).

To determine the net charge transported across the plasma member per GABA or per Cl$^-$ for every transport cycle, uptake under voltage clamp experiments were performed in cells expressing GAT3, and the GABA-evoked current was measured in the presence of $[^3]$H-GABA or $^{36}$Cl$^-$ in the bathing medium of cells (Sacher et al., 2002; Gonzales et al., 2007; Matthews et al., 2009). At the end of the incubation period, GABA and the isotope were removed from the perfusion solution until the holding current returned to baseline. Oocytes were removed from the experimental chamber, washed in ice-cold choline-Cl buffer and solubilized in 10% sodium dodecyl sulfate. Oocyte $[^3]$H-GABA content was determined in a liquid scintillation counter. Net inward charge was obtained from the time integral of the GABA-evoked inward current and correlated with GABA or Cl$^-$ influx in the same cell.

**Results and Discussion**

For the reversal potential ($V_{\text{rev}}$) measurements, all three transporter substrates must be present on both sides of the membrane (i.e., intracellular and extracellular compartments). In these experiments, the cells were preloaded with GABA (1 mM for 20 minutes at $-50$ mV) in order to allow for reliable measurements of $V_{\text{rev}}$ at different extracellular concentrations of GABA or Na$^+$ (Fig. 1).

To examine the effect of GABA, following loading the cells with GABA, $V_{\text{rev}}$ measurements were made at 5, 10, 25, 50, 100, and 500 µM extracellular GABA, while the extracellular concentrations of Na$^+$ (50 mM) and Cl$^-$ (106 mM) remained constant (Fig. 2). As the extracellular GABA concentration was increased, $V_{\text{rev}}$ shifted to more positive membrane potential values (Fig. 2A). The measurement at 5 µM GABA served as the reference value for determining the shift in $V_{\text{rev}}$ at higher GABA concentrations. The shifts in $V_{\text{rev}}$ were $10.0 \pm 1.1$
mV (5 → 10 µM), 22.4 ± 1.4 mV (5 → 25 µM), 32.4 ± 2.8 mV (5 → 50 µM), 47.8 ± 2.9 mV (5 → 100 µM), and 56.1 ± 5.3 mV (5 → 500 µM). The measured shift in $V_{rev}$ under all experimental conditions was smaller than that predicted by a 2 Na$^+$ : 1 Cl$^-$ : 1 GABA stoichiometry (Fig. 2C). Indeed, the values were consistent with a 3 Na$^+$ : 1 Cl$^-$ : 1 GABA stoichiometry (Fig. 2C). Moreover, the slope of the plot of $V_{rev}$ as a function of the logarithm of extracellular GABA concentration revealed a slope of 31 ± 2 mV per 10-fold change in [GABA]$_o$ which is consistent with the slope predicted by the 3 Na$^+$ : 1 Cl$^-$ : 1 GABA stoichiometry model (29 mV per 10-fold change in [GABA]$_o$) and not the 2 Na$^+$ : 1 Cl$^-$ : 1 GABA stoichiometry model (58 mV per 10-fold change in [GABA]$_o$) (Fig. 2B).

Similarly, the measured shifts in the external Na$^+$ concentration, with a slope of 81 ± 5 mV per 10-fold change in [Na$^+$]$_o$, were not consistent with the 2 Na$^+$ : 1 Cl$^-$ : 1 GABA stoichiometry model (slope of 116.7 mV per 10-fold change in [Na$^+$]$_o$), but rather were closely predicted by the 3 Na$^+$ : 1 Cl$^-$ : 1 GABA stoichiometry model (slope of 87.5 mV per 10-fold change in [Na$^+$]$_o$) (Fig. 3).

GABA and Cl$^-$ fluxes under voltage clamp revealed that for every GABA molecule translocated across the plasma membrane, 2.1 ± 0.1 elementary charges enter the cell. Similarly, for every Cl$^-$ ion translocated across the plasma membrane, 2.0 ± 0.1 elementary charges enter the cell. These measurements are consistent with a 3 Na$^+$ : 1 Cl$^-$ : 1 GABA coupling stoichiometry (Fig. 4).

In summary, our results are not consistent with the currently accepted stoichiometric model of 2 Na$^+$ : 1 Cl$^-$ : 1 GABA, but rather point strongly to a 3 Na$^+$ : 1 Cl$^-$ : 1 GABA ion/substrate stoichiometric model (Fig. 5). These findings have important implications for the role of the GABA transporters in establishing the resting concentration of GABA in the brain, as well as for the contribution of the GABA transporters toward regulating GABAergic inhibitory neurotransmission in the brain.
References


Appendix

Fig. 1. Reversal potential of GAT1-mediated current.
The GABA-evoked current is plotted as a function of the membrane potential. The current-voltage (I-V) relationships show that the direction of current (i.e., direction of transport) depends on the presence of GABA in the extracellular compartment, intracellular compartment, or both compartments. (A) When GABA was present only in the extracellular medium of the oocyte, the current generated was primarily an inward current (forward transport). (B) When GABA was present only in the oocyte cytoplasm, the current generated was primarily an outward current (reverse transport). This cell had been preloaded with GABA and was then washed in a solution without external GABA. (C) When GABA was present in both the intracellular and extracellular compartments, robust inward (negative voltages) and outward (positive voltages) currents were measured. The reversal potential ($V_{\text{rev}}$) is the voltage at which the current is zero. This cell had been preloaded with GABA and was then washed in a solution containing external GABA (50 µM).
Fig. 2. Effect of external GABA concentration on the reversal potential. See figure on previous page. 
(A) Current-voltage relationships are shown at different external GABA concentrations for a GABA-loaded GAT1-expressing cell. The reversal potential ($V_{rev}$) was measured at each external GABA concentration and plotted as a function of the log of the external GABA concentration (see panel B). (B) $V_{rev}$ plotted as function of log[GABA]o revealed a linear relationship with a slope of 31 mV for a 10-fold change in [GABA]o. The dashed lines are predictions of the 2 Na$^+$ : 1 Cl$^-$ :1 GABA (red) and 3 Na$^+$ : 1 Cl$^-$ :1 GABA (blue) stoichiometry models. (C) The measured shifts in $V_{rev}$ are compared with the predictions of the 2 Na$^+$ : 1 Cl$^-$ :1 GABA and 3 Na$^+$ : 1 Cl$^-$ :1 GABA stoichiometry models.

Fig. 3. Effect of external Na$^+$ concentration on the reversal potential.
(A) Current-voltage relationships are shown at different external Na$^+$ concentrations for a GABA-loaded GAT1-expressing cell. The reversal potential ($V_{rev}$) was measured at each external Na$^+$ concentration and plotted as a function of the log of the external Na$^+$ concentration (see panel B). (B) $V_{rev}$ plotted as function of log[Na$^+$]o revealed a linear relationship with a slope of 81 mV for a 10-fold change in [Na$^+$]o. The dashed lines are predictions of the 2 Na$^+$ : 1 Cl$^-$ :1 GABA (red) and 3 Na$^+$ : 1 Cl$^-$ :1 GABA (blue) stoichiometry models. (C) The measured shifts in $V_{rev}$ are compared with the predictions of the 2 Na$^+$ : 1 Cl$^-$ :1 GABA and 3 Na$^+$ : 1 Cl$^-$ :1 GABA stoichiometry models.
Currently-accepted and proposed revised stoichiometry models for GAT1.

The results obtained in the present study are consistent with a 3 Na⁺ : 1 Cl⁻ : 1 GABA stoichiometry model for the GABA transporters. This revised model has significant implications for GABA concentrations at synaptic and extrasynaptic regions in the brain.