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DIFFERENTIAL EFFECTS OF GABA IN MODULATING NOCICEPTIVE VS. NON-NOCICEPTIVE SYNAPSES

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Abstract—GABA (γ-amino-butyric acid) -mediated signaling is normally associated with synaptic inhibition due to ionotropic GABA receptors that gate an inward Cl^- current, hyperpolarizing the membrane potential. However, there are also situations where ionotropic GABA receptors trigger a Cl^- efflux that results in depolarization. The well-characterized central nervous system of the medicinal leech was used to study the functional significance of opposing effects of GABA at the synaptic circuit level. Specifically, we focused on synapses made by the nociceptive N cell and the non-nociceptive P (pressure) cell that converge onto a common postsynaptic target. It is already known that GABA hyperpolarizes the P cell, but depolarizes the N cell and that inhibition of ionotropic GABA receptors by bicuculline (BIC) has opposing effects on the synapses made by these two inputs; enhancing P cell synaptic transmission, but depressing N cell synapses. The goal of the present study was to determine whether the opposing effects of GABA were due to differences in Cl^- homeostasis between the two presynaptic neurons. VU 0240551 (VU), an inhibitor of the Cl^- exporter K-Cl co-transporter isoform 2 (KCC2), attenuated GABA-mediated hyperpolarization of the non-nociceptive afferent while bumetanide (BUM), an inhibitor of the Cl^- importer Na-K-Cl co-transporter isoform 1 (NKCC1), reduced GABA-mediated depolarization of the nociceptive neuron. VU treatment also enhanced P cell synaptic signaling, similar to the previously observed effects of BIC and consistent with the idea that GABA inhibits synaptic signaling at the presynaptic level. BUM treatment depressed N cell synapses, again similar to what is observed following BIC treatment and suggests that GABA has an excitatory effect on these synapses. The opposing effects of GABA could also be observed at the behavioral level with BIC and VU increasing responsiveness to non-nociceptive stimulation while BIC and BUM decreased responsiveness to nociceptive stimulation. These findings demonstrate that distinct synaptic inputs within a shared neural circuit can be differentially modulated by GABA in a functionally relevant manner.
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Key words: GABA, chloride, nociception, synaptic transmission.

INTRODUCTION

GABA (γ-aminobutyric acid) is normally considered to be an inhibitory neurotransmitter due to its activation of Cl^- channels and the subsequent Cl^- influx that hyperpolarizes the membrane potential. These inhibitory effects are dependent on maintaining a concentration gradient in which the extracellular Cl^- levels are greater than intracellular Cl^-. Cl^- homeostasis is maintained by the K-Cl co-transporter isoform 2 (KCC2), which transports Cl^- along with K^+ out of the cell (Rivera et al., 1999; Haam et al., 2012). However, there are situations where the intracellular Cl^- levels are elevated relative to the outside of the cell due to the actions of a Na-K-Cl co-transporter isoform 1 (NKCC1), which imports Cl^-. In these situations, ionotropic GABA receptor activation results in a Cl^- efflux that depolarizes the membrane potential. Originally, GABA-mediated depolarization was thought to happen primarily in the immature/developing brain (Cherubini et al., 1991) or in the adult spinal cord following nerve injury (Coull et al., 2003). However, GABA-induced depolarization has been observed in the adult nervous systems under normal conditions, for example in the spinal cord (Duchen, 1986), cerebellum (Xu et al., 2012), striatum (Adermark et al., 2009) and hypothalamus (Haam et al., 2012).

How are differences in Cl^- homeostasis used to modify synaptic pathways at the microcircuit level? Are there neural circuits that possess both GABA-inhibited and GABA-excited synaptic connections as a result of differences in how intracellular Cl^- levels are regulated? To examine this possibility, differences in GABAergic modulation in nociceptive (N) neurons and non-nociceptive (pressure-sensitive or P) neurons and their synapses were examined in the central nervous system of the medicinal leech. Both N and P cells are readily identifiable afferent neurons that have glutamatergic

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Abbreviations: 5-HT, serotonin; ACh, Acetylcholine; ANOVA, analysis of variance; BIC, bicuculline; BUM, bumetanide; DMSO, dimethyl sulfoxide; E_{Cl}, chloride equilibrium potential; E_{GABA}, GABA reversal potential; EPSP, excitatory post-synaptic potential; GABA, γ-amino-butyric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IR, input resistance; KAc, potassium acetate; KCC2, K-Cl co-transporter isoform 2; NEO, neostigmine; NKCC1, Na-K-Cl co-transporter isoform 1; NMDG, *N*-methyl-D-glucamine; PPF, paired-pulse facilitation; PPR, paired-pulse ratio; VU, VU0240551.

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synaptic input onto a number of shared targets, including the longitudinal (L) motor neurons that innervate the longitudinal muscles and contribute to the defensive withdrawal reflex, whole-body shortening (Nicholls and Purves, 1970; Shaw and Kristan, 1995). Previous studies have shown that while the P cell is hyperpolarized by GABA, GABA depolarizes the N cell (Sargent et al., 1977; Higgins et al., 2013). Furthermore, inhibition of GABA-A receptors with bicuculline (BIC) has opposing effects on synaptic transmission; enhancing P cell synapses (presumably due to disinhibition), but depressing N cell synapses (see Fig. 1) (Higgins et al., 2013). Because it is possible to make intracellular recordings of the individual neurons in the N-to-L and P-to-L synaptic pathways, this provides a unique opportunity to directly examine the cellular basis for the differential effects of GABA on a synaptic circuit. Specifically, whether differences in Cl^- homeostasis at the presynaptic level mediate the opposing effects of GABA on these two converging synaptic pathways.

In the present study, we confirmed that differences in responses to GABA by the P and N cells were mediated by differences in Cl^- homeostasis. Inhibition of Cl^- transport also influenced synaptic transmission, enhancing P cell synapses when an inhibitor of KCC2 was applied and depressing N cell synapses when an inhibitor of NKCC1 was used. These differential effects of GABA influenced synapses at the presynaptic level and appeared to be due to tonic modulation by GABA. Consistent with these electrophysiology findings, treatments that interfered with GABAergic signaling or Cl^- homeostasis had opposing effects on behavioral responses elicited by nociceptive or non-nociceptive stimuli. That is, decreases in putatively tonic GABAergic signaling reduced responses to nociceptive stimuli, but enhanced responses to non-nociceptive stimuli.

EXPERIMENTAL PROCEDURES

Animal preparation

Leeches (*Hirudo verbena*; *3g*) were obtained from commercial suppliers (Leeches USA, Westbury, NY, USA and Niagara Leeches, Cheyenne, WY, USA) and maintained in artificial pond water [0.52 g/L H_2O Hirudo salt (Leeches USA Ltd.)] on a 12-h light/dark cycle at 15 °C. Ganglia were dissected and pinned in a recording chamber with constant perfusion of normal leech saline (110 mM NaCl, 5 mM NaOH, 4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH = 7.4) at a rate of approximately 1.5 mL/min. For experiments using low Na^+ saline, Na^+ was substituted with *N*-methyl-D-glucamine (NMDG) to reduce Na^+ content to 11.5 mM. Drugs used for each experiment were kept as frozen aliquot solutions and then diluted to their final concentration in normal saline just before respective experiments. GABA, acetylcholine (ACh), neostigmine (NEO) and bicuculline methiodide (BIC) stocks were made in normal saline. Serotonin (5-HT), bumetanide (BUM) and VU 0240551 (VU) stocks were made in dimethyl sulfoxide (DMSO). BIC, BUM and VU were obtained from Tocris (Ellisville, MO, USA), while GABA, DMSO, NEO, NMDG and 5-HT were obtained from Sigma-Aldrich (St. Louis, MO, USA).

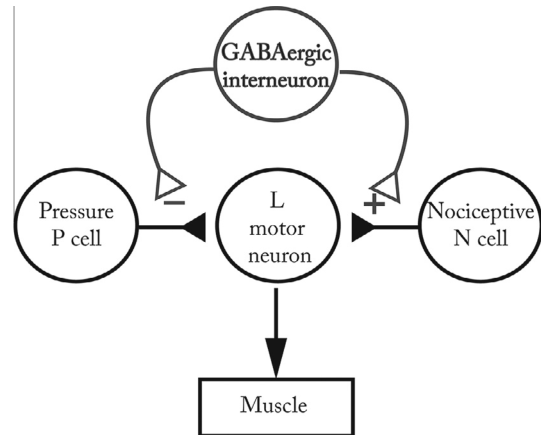

Fig. 1. Simplified circuit of the leech sensorimotor connections and the GABAergic input they receive. Both the N and P cells have excitatory, glutamatergic input onto the L motor neuron (which innervates the longitudinal muscles of the leech). Putative GABAergic inputs have also been included and are thought to have inhibitory vs. excitatory effects on the P and N synapses, respectively.

Electrophysiology

Techniques used in this study have been described in detail in (Yuan and Burrell, 2010). Briefly, current clamp (bridge balanced) intracellular recordings were carried out using sharp glass microelectrodes (tip resistance 20–30 MΩ) made from borosilicate capillary tubing (1.0 mm OD, 0.75 mm ID; FHC, Bowdoinham, ME, USA) using a horizontal puller (Sutter Instruments P-97; Novato, CA, USA). Microelectrodes were filled with 3 M potassium acetate (KAc). Manual micropositioners (Model 1480; Siskiyou Inc., Grants Pass, OR, USA) were used to impale individual neurons during experiments. Current was delivered to electrodes using a multi-channel programmable stimulator (STG 1004; Multi-Channel Systems; Reutlingen, Germany) and the signal was recorded using a bridge amplifier (BA-1S; NPI, Tamm, Germany) and digitally converted for analysis (Axoscope; Molecular Devices, Sunnyvale, CA, USA).

The presynaptic lateral nociceptive (N) and pressure (P) cells and the postsynaptic longitudinal (L) motor neuron and anterior pagoda (AP) neuron were identified based on their position within the ganglion, size, and characteristic electrophysiological properties (size and shape of action potential). Each ganglion contains bilateral pairs of N cells. The lateral N cells are polymodal nociceptors and the medial N cells are mechano-only nociceptors (Nicholls and Baylor, 1968; Blackshaw et al., 1982; Pastor et al., 1996). All experiments in this study were carried out using the lateral N cell. There are also two bilateral pairs of P cells in each ganglion, with the medial P cells innervating the dorsal skin and the lateral P cells innervating the ventral skin (Nicholls and Baylor, 1968). L motor neuron identification could be confirmed by recording from the electrically coupled contralateral L motor neuron and observing the synchronous activity in both neurons (Stuart, 1970). For

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