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SYNAPTIC INTEGRATION IN HYPOTHALAMIC GONADOTROPIN RELEASING HORMONE (GnRH) NEURONS

C. B. ROBERTS,[a,b] P. HEMOND[a] AND K. J. SUTER[a]*

[a]*Department of Biology, University of Texas at San Antonio, 1 UTSA Circle, San Antonio, TX 78249, USA*

[b]*Department of Electrical and Computer Engineering, Boston University, 8 Saint Mary's Street, Boston, MA 02215, USA*

Abstract—The impact of the A-type GABA (GABA-A) receptor in gonadotropin releasing hormone (GnRH) neurons is controversial. In adult GnRH neurons, the GABA-A receptor conductance has been reported to either hyperpolarize or depolarize GnRH neurons. Regardless of whether GABA is inhibitory or excitatory in GnRH neurons, GABAergic input would be integrated with post-synaptic potentials generated by other synaptic inputs. We used dynamic current clamping and compartmental computer modeling to examine the integration of AMPA-type glutamatergic input and GABA-mediated input in both the hyperpolarizing (inhibitory) and depolarizing (excitatory) modes in GnRH neurons from transgenic mice (Mus Musculus) generated on a C57BL6 background. In both living and model neurons, action potentials were most likely a few ms after a maximum in AMPA conductance coincided with a minimum in inhibitory GABA. Excitatory GABA interacted differently with AMPA, with spikes most likely, in both dynamic clamping of living neurons and in model neurons, when a maximum in AMPA coincided with the decay from peak of a maximum in GABA. Distributing synapses along the dendrite maximized the temporal relationship between AMPA and GABA conductances and therefore, the potential for spiking. Thus, these two dominant neurotransmitters could interact in multiple frames to generate action potentials in GnRH neurons.

Key words: GnRH, synaptic integration, compartmental model, glutamate, GABA, hypothalamus.

Hypothalamic gonadotropin releasing hormone (GnRH) neurons form the final pathway for integration of signals regulating reproduction. The control of GnRH neurons has been proposed to reflect both their intrinsic electrical properties and the input they receive from presynaptic neurons (Kusano et al., 1995; reviewed by Lopez et al., 1998). Despite the relatively precise requirements in the timing of GnRH pulses for proper reproductive function (Pohl et al., 1983), GnRH neurons have heterogeneous electrophysiological properties (Sim et al., 2001; Spergel et al., 1999). Moreover, emerging evidence suggests that GnRH neurons may differ in their responses to at least one key hypothalamic neurotransmitter, GABA. Application of ligands for the GABA-A receptor indicates that GABA-A receptor-mediated events in adult GnRH neurons are hyperpolarizing in some studies (Han et al., 2002, 2004), whereas others indicate they are depolarizing (DeFazio et al., 2002). Likewise, electrophysiological recordings aimed at determining the impact of endogenous GABA have revealed differences in responses. Using perforated patch recording, blocking of endogenous GABAergic transmission caused membrane depolarization in most GnRH neurons (80%), indicating that endogenous GABA inhibits GnRH neurons (Han et al., 2004). A second study reported a suppression of firing rates in most *active* GnRH neurons following application of GABA receptor antagonists (Moenter and DeFazio, 2005) suggesting endogenous GABA is excitatory in GnRH neurons.

Discrepancies in the reported actions of GABA within a population of neurons are not unique to the GnRH system (Carter and Regehr 2002; Chavas and Marty 2003). Differences in the action of GABA are generally attributed to issues surrounding the regulation of chloride homeostasis. The reversal potential for chloride depends on the ratio of the external to internal concentration of the ion through the Nernst equation:

$$E_{Cl} = -\frac{RT}{nF}\ln\frac{[Cl^-]_{out}}{[Cl^-]_{in}}$$

Since the ideal gas constant R, the temperature T, the number of excess electrons on the chloride ion n, and the Faraday constant F are all positive, and since the natural logarithm of a number greater than 1 is always positive, then if the concentration of chloride outside the cell is greater than that inside the cell, then the ratio in the Nernst equation will be positive, and the reversal potential will be negative. Since chloride is the main ion carried by the GABA-A receptor channel, the relative values of the chloride reversal potential and the resting membrane potential of the cell will determine whether a GABA-A-mediated input will be depolarizing or hyperpolarizing. It is now clear that the reversal potential for chloride and therefore, the actions of GABA can be modulated (see Cupello, 2003 for review). Several physiological conditions have been reported to lead to differences in the response to GABA. For example, the reversal potential for chloride shifts in cortical neurons due to changes in intracellular chloride concentrations during times of high GABA input (Gulledge and Stuart, 2003). In active GnRH neurons, GABA may also be

*Corresponding author. Tel: +1-210-458-7243; fax: +1-210-458-5658.
E-mail address: kelly.suter@utsa.edu (K. J. Suter).

Abbreviations: ACSF, artificial cerebrospinal fluid; AP-5, D(−)-2-amino-5-phosphonopentanoic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; FWHM, full width at half maximum; GENESIS, General Neural Simulation System; GFP, green fluorescent protein; GnRH, gonadotropin releasing hormone; NMDA, *N*-methyl-D-aspartic acid; PSC, post-synaptic current; PSP, post-synaptic potential.

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excitatory through this mechanism (Moenter and DeFazio, 2005). Differences in chloride transporter expression between GnRH neurons have been reported (DeFazio et al., 2002; Leupen et al., 2003). Up-regulation of NKCC2 expression by testosterone and dihydrotestosterone has been reported in males and females in neurons of the substantia nigra (Galanopoulou and Moshé, 2003). The NKCC2 transporter acts to remove chloride ions from the cell. Thus, GABA may be excitatory or inhibitory in individual neurons based on NKCC2 expression, or potentially excitatory and inhibitory in the same neuron based on changes in activity and accumulation of intracellular chloride.

Independent of whether GABA is excitatory or inhibitory, GABAergic input would be integrated with that of other presynaptic neurons providing input to GnRH neurons. For example, glutamate is a second dominant neurotransmitter in the mammalian hypothalamus (Decavel and van den Pol, 1990; van den Pol et al., 1990). There is general agreement that glutamate excites GnRH neurons (Kuehl-Kovarik et al., 2002, 2005; Suter, 2004). However, it is unclear how synaptic integration of glutamatergic input in GnRH neurons would be affected when GABA is excitatory, as would be the case with a suppression of the NKCC2 transporter or chloride increase due to high GABA activity, or when GABA is inhibitory as would be the case in a situation with reduced intracellular chloride. In the present study, we used the techniques of dynamic current clamping and compartmental modeling, where we were able to set the reversal potential for simulated GABA inputs, to examine integration of glutamatergic input with both inhibitory and excitatory GABA.

EXPERIMENTAL PROCEDURES

Tissue preparation

Hypothalamic slices (200 μm) were prepared using a vibrating microtome (Slicer HR-2; Sigmann Elektronik, Hueffenhardt, Germany) from male GnRH-GFP mice in which GnRH neurons express green fluorescent protein (GFP; Spergel et al., 1999). The average age of mice used for whole-cell voltage clamp recordings of spontaneous post-synaptic currents (PSCs) was 92±2.6 d (n=19), and the average weight was 26.7±0.67 g. The average age of mice used for dynamic current clamp recordings was 63.3±5.2 d (n=13), and the average weight was 22.3±1.4 g. All experiments were approved by the IACUC committees at Emory University and the University of Texas at San Antonio and were in compliance with the NIH Guide for Care and Use of Animals. All efforts were made to reduce both the animal numbers and minimize pain. Mice were anesthetized with halothane and decapitated. Brains were quickly removed and placed in cold (1–2 °C), artificial cerebrospinal fluid (ACSF) solution containing (in mM): NaCl (125), $NaHCO_3$ (24), KCl (2.5), $CaCl_2$ (1), $MgCl_2$ (1), and glucose (10), equilibrated with 95% O_2/5% CO_2, pH 7.3–7.4. Slices were incubated in ACSF for 1–2 h at 32 °C, transferred to a recording chamber mounted on the stage of an upright microscope (Axioskop, Carl Zeiss Microimaging Inc., Thornwood, NY, USA), and then continuously perfused with ACSF (32 °C). GnRH neurons were identified through their GFP expression using epifluorescent excitation at 470 nm with a 60× water immersion objective. To prevent excessive exposure of the slices to the epifluorescent excitation, a shutter (Uniblitz, Vincent Associates, Rochester, NY, USA) was used between the light source (AttoArc, Carl Zeiss) and the objective. Slices were illuminated 100 ms every 1.5 s during identification.

Electrophysiology

Recordings were made with an Axoclamp 2B amplifier (Axon Instruments; Union City, CA, USA). Pipettes (9–12 MΩ) were fabricated from borosilicate glass (AM Systems, Carlsborg, WA, USA) using a pipette puller (PP-83; Narishige, Tokyo, Japan) and coated with Sylgard 184 (Dow Corning, Midland, MI, USA) to minimize pipette capacitance. Electrodes were positioned using piezoelectric micromanipulators (Luigs and Neumann, Ratingen, Germany). Data were digitized at 10 kHz using custom software. When studying activity of GnRH neurons in response to simulated GABAergic inputs, picrotoxin (40 μM; Tocris, Ellisville, MO, USA), a GABA-A receptor antagonist, was added to the bath solution to block endogenous GABA receptor activation. Likewise, when studying both AMPA and GABA effects, the AMPA/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM; Tocris) and the *N*-methyl-D-aspartic acid (NMDA) receptor antagonist D(−)-2-amino-5-phosphonopentanoic acid (AP-5, 100 μM; Tocris) in addition to picrotoxin (40 μM) were included in the bath solution to block non-NMDA and NMDA glutamatergic receptors, and GABA-A receptors respectively.

Dynamic current clamp recording

Three basic parameters that characterize the electrical activity of neurons are voltage or electrical potential, current, or flow of charge, and conductance, or its inverse, resistance. In a living synapse, the conductance is proportional to the number of ion channels that are open at any given time. Thus, the value of conductance is never negative. The relation between voltage, current and conductance is described mathematically by Ohm's law: $I_i = g_i(V_m - E_i)$. In this formula, I_i is an ionic current, g_i is the conductance for this current, V_m is the membrane potential, and E_i is the reversal potential for the ionic species that carries the current. The reversal potential for a given ion will depend on the relative concentration of that type of ion in the exterior and interior of the cell. As indicated in Ohm's law above, if the conductance g_i increases, then there will be a proportional increase in the magnitude of the current I_i, as long as there is a non-zero driving force $V_m - E_i$. Similarly, if the conductance is unchanged, but the reversal potential E_i changes, then a larger or smaller current will flow depending on whether the change in E_i causes an increase or a decrease in the magnitude of the driving force. The sign of the current that flows is determined by the sign of the driving force.

Dynamic current clamping is used to apply the electrical equivalents of synaptic currents to neurons. In this technique, the injected current mimics biophysical currents. The currents that flow in living cells, such as the GABA-A synaptic current, are characterized by a time-varying conductance and a reversal potential. Thus, one can construct simulated synapses in which one defines both the magnitude and time course of the conductance and the reversal potential. For dynamic clamping experiments, one simulates a pre-defined pattern of synaptic input by storing a conductance waveform that is the sum of many individual post-synaptic conductance events. This waveform can then be applied multiple times to the same cell, or to different cells. Since the membrane potential changes in a living neuron, the dynamic current clamp uses feedback from a neuron to a computer, where V_m is updated online (in our experimental configuration at 10 kHz). The computer program adjusts the injected current to reflect changes in driving force that occur with alterations in V_m (Sharp et al., 1993). Thus, the magnitude of inputs applied using the dynamic current clamp depends on the activity of the recorded neuron.

Fig. 1A shows application of simulated AMPA and inhibitory (reversal potential −70 mV, simulating reduced intracellular chloride) GABA inputs to a living GnRH neuron via dynamic clamping. The bottom traces are the simulated AMPA and GABA conductances. In this case, there were three simulated GABAergic syn-