Glutamate and GABA mediate suprachiasmatic nucleus inputs to spinal-projecting paraventricular neurons

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Cui, Lu-Ning, Elaine Coderre, and Leo P. Renaud. Glutamate and GABA mediate suprachiasmatic nucleus inputs to spinal-projecting paraventricular neurons. Am J Physiol Regulatory Integrative Comp Physiol 281: R1283–R1289, 2001.—We used patch-clamp recordings in slice preparations from Sprague-Dawley rats to evaluate responses of 20 spinal-projecting neurons in the dorsal paraventricular nucleus (PVN) to electrical stimulation in suprachiasmatic nucleus (SCN). Neurons containing a retrograde label transported from the thoracic (T1-T4) intermediolateral column displayed three intrinsic properties that collectively allowed distinction from neighboring parvocellular or magnocellular cells: a low-input resistance, a hyperpolarization-activated time-dependent inward rectification, and a low-threshold calcium conductance. Twelve of fifteen cells tested responded to electrical stimulation in SCN. All of 10 cells tested in media containing 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[\(\text{f}\)]quinoline-7-sulfonamide disodium (5 \(\mu\)M) and \((-\rangle\)-2-amino-5-phosphono pentanoic acid (20 \(\mu\)M) responded with constant latency (11.4 ± 0.7 ms) inhibitory postsynaptic potentials, able to follow 20- to 50-Hz stimulation and blockable with biccuculline (20 \(\mu\)M). By contrast, all eight cells tested in the presence of biccuculline demonstrated constant latency (9.8 ± 0.6 ms) excitatory postsynaptic potentials that followed at 20–50 Hz and featured both non-N-methyl-\(\text{d}\)-aspartate (NMDA) and NMDA receptor-mediated components. We conclude that both GABAergic and glutamatergic neurons in SCN project directly to spinal-projecting neurons in the dorsal PVN.

Retrograde labeling; rhodamine microspheres; intrinsic conductances; hyperpolarization-activated inward rectification; T current

ADAPTATION TO DAILY CHANGES in the environment is reflected in circadian rhythmicity in a wide array of physiological and behavioral activities that occur over a period of ~24 h. In mammals, these fluctuations can be traced to the hypothalamic suprachiasmatic nucleus (SCN), a structure whose neurons contain “clock” genes that regulate an intrinsic oscillatory mechanism but whose activities can also be entrained to the light-dark cycle by inputs via retinohypothalamic and geniculo-hypothalamic afferents. Lesion and transplantation studies have clearly documented that circadian rhythmicity in any number of physiological functions depends on the integrity of the SCN and that SCN efferent pathways are largely responsible for subsequent entrainment of brain functions (17, 25). SCN projects heavily to areas of the hypothalamus (2, 17, 41), a brain region that contains the paraventricular nucleus (PVN), recognized as a critical center for homeostasis (34). PVN encompasses distinct populations of magnocellular and parvocellular neurons whose outputs include projections to the posterior pituitary for control of body fluid balance, the median eminence for adenohypophysial regulation, and brain stem and spinal cord for influencing autonomic output functions (35). The latter includes a population of parvocellular neurons located in the dorsal cap region of PVN, whose axons project to brain stem and spinal “autonomic” centers (4, 14, 19, 23, 27, 33, 34). Recent anatomic tracer studies reveal that the dorsal cap area of PVN receives an innervation from the SCN (2, 37, 39), a connection that may identify a neuronal circuitry that participates in the entrainment of a circadian rhythmicity in autonomic variables such as blood pressure (20), heart rate (26), and body temperature (10, 31). However, little is known at the cellular level about the functional features of such an innervation. To address this issue, we first used rhodamine latex microspheres to retrogradely label dorsal PVN neurons projecting axons to the thoracic spinal cord. We then used patch-clamp recording techniques to record from these identified neurons in hypothalamic rat brain slice preparations and characterize both their intrinsic properties and responses to focal electrical stimulation in SCN. We now report that these neurons have distinct electrical properties and that activation of SCN efferents monosynaptically evokes postsynaptic responses in a majority of tested dorsal PVN neurons, mediated via GABAergic and glutamatergic receptors.

MATERIALS AND METHODS

Retrograde labeling. Sprague-Dawley rats (12–14 days) of either sex were initially anesthetized with methoxyflurane (Janssen). The spinal cord at the T1-T4 level was exposed by dorsal laminectomy, and 1 \(\mu\)l of a rhodamine-labeled fluorescent microspheres suspension (FluoSpheres, 0.04 \(\mu\)m, red fluorescence 580/605; Molecular Probes, Eugene, OR) was slowly injected into the region of the intermediolateral cell

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column. Animals were returned to their cages for 2–3 days, sufficient to permit retrograde transport to the hypothalamus.

Slice preparation. On the day of experiment, animals were killed by decapitation and the brain was rapidly removed and placed in ice-cold oxygenated (95% O2–5% CO2) artificial cerebrospinal fluid (ACSF) composed of (in mM) 124 NaCl, 3.2 KCl, 26.2 NaHCO3, 1.3 MgCl2, 1 Na2HPO4, 10 glucose, and 2.4 CaCl2 at pH of 7.4. A coronal section 250- to 300-μm thick containing the PVN and the SCN in the same plane (see Fig. 1D) was cut with a tissue slicer (Leica VT1000S, Leica), trimmed, preincubated in ACSF for 1 h, and then transferred to a submerged chamber for recording at room temperature.

Electrophysiology. Retrogradely labeled neurons were visualized with an upright microscope equipped with a fluorescent attachment and a port for a differential interference contrast camera (Hamamatsu C2400, Japan) and monitor unit. Recordings were obtained during the subjective dark period (of a 12:12-h light-dark cycle) using glass micropipettes (borosilicate glass; OD 1.5 mm; ID 1.1 mm) filled with a solution containing (in mM) 135 K gluconate, 10 KCl, 10 Tris, 1 M KCl, 10 HEPES, 1 EGTA, 2 Mg-ATP, and 0.2 GTP at pH 7.3 and osmolarity of 285 mosM. We obtained patch-clamp data from pettes (borosilicate glass; OD 1.5 mm; ID 1.1 mm) filled with a solution containing (in mM) 135 K gluconate, 10 KCl, 10 Tris, 1 M KCl, 10 HEPES, 1 EGTA, 2 Mg-ATP, and 0.2 GTP at pH 7.3 and osmolarity of 285 mosM. We obtained patch-clamp data from both labeled and unlabeled neurons. Lucifer yellow (1 mg/ml) was included in the pipette to verify the morphology of recorded cells. Recording pipettes had an open resistance of 3–7 MΩ and seal resistances >3 GΩ. The series resistance was estimated in brief voltage clamp sessions from the whole cell capacitive current in response to a voltage pulse; with whole cell recording, the estimated series resistance was generally <20 MΩ. A concentric bipolar electrode (outside diameter of 325 μm, tip-ring separation of 100 μm; FHC) positioned in the SCN was connected to a stimulus isolation unit that delivered electrical pulses (duration 0.5 ms; intensity 5–15 V) under software control. Data obtained with an Axopatch 1-D amplifier (Axon Instruments) were low-pass filtered at 2 kHz. pClamp 8.0 software (Axon Instruments) was used for data acquisition and analysis. Values of membrane potential were corrected for liquid junction potential. Results are expressed as means ± SE.

Histological identification. Slices were fixed in 4% paraformaldehyde in 0.1 M PBS overnight, reimmersed in PBS containing 20% sucrose (wt/vol), and double-labeled neurons were visualized with a BIORAD 1024 confocal microscope (excitation/emission settings of 568/555 and 488/515 for rhodamine and Lucifer yellow, respectively). Images of 20–40 1-μm sections were projected onto a single panel (Fig. 1, A-C).

Pharmacology. The following drugs from Tocris Cookson (Ballwin, MO) were bath applied at concentrations indicated in the text: (-)-bicuculline methochloride; D(-)-2-amino-5-phosphonopentanoic acid (D-AP5); 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium (NBQX). Lucifer yellow was from Sigma (St. Louis, MO).

RESULTS

Properties of spinal-projecting PVN neurons. Data were obtained from 20 retrogradely labeled cells in the dorsal PVN. These cells displayed oblong, bipolar, or tripolar neuronal somata measuring 15–20 μm in the long axis, with two to three main dendrites that were oriented along the mediolateral axis. Some cells had dendrites that branched extensively, occasionally extending into the magnocellular or medial periventricular part of the nucleus. An axon could often be visualized arising from a main dendrite. Cells displayed a resting membrane potential of −59.7 ± 0.9 mV and time constant of 53.2 ± 5.1 ms. Action potentials had an amplitude, width (at half of amplitude), and threshold of 69.5 ± 2.3 mV, 2.3 ± 0.2 ms, and −34.0 ± 0.6 mV, respectively, and were followed by a prominent afterhyperpolarization (AHP) of 18.5 ± 1.5 mV (measured between threshold and peak of negativity).

Three properties common to these spinal-projecting PVN neurons permitted their distinction from neighboring neurons. First, their input resistance of 509.1 ± 38.6 MΩ was significantly lower (P < 0.05) than that obtained among a population of magnocellular neurons (864 ± 51.4 MΩ; n = 40 cells; see Ref. 6) or parvocellular neurons located in the periventricular or ventrolateral PVN (772 ± 48 MΩ; n = 49 cells). Second, a

Fig. 1. A-C: laser scan confocal microscope images of a spinal-projecting neuron in the dorsal paraventricular nucleus (PVN). A: retrograde label (rhodamine-fluorescent microspheres) in a neuron. Image is reconstructed by projecting 40 images scanned at 1-μm intervals into a panel. B: intracellular tracer Lucifer yellow outline of the same neuron. C: superimposed image of A and B. D: schematic of a coronal brain section to illustrate the position of the stimulating electrode in the suprachiasmatic nucleus (SCN) located above the optic chiasm (OX) and the recording pipette in the dorsal PVN. Symbols indicate the approximate locations of retrogradely labeled neurons. III, third cerebral ventricle.
majority (16/20) of these neurons displayed a slow time-dependent hyperpolarization-activated inward rectification (Fig. 2, Aa and c), blockable with bath applications of cesium (Fig. 2Ab) or ZD-7288 (Fig. 2B), identifying this current as an I_{H} conductance. I_{H} is a feature of many central neurons, including those in the hypothalamic supraoptic nucleus (11, 12). Third, these cells displayed a prominent low-threshold spike (LTS) when depolarized from a hyperpolarized holding potential (Fig. 3A) or on return from a hyperpolarized potential (Fig. 3B). The activation curve (Fig. 3B, inset) and sensitivity of this LTS to nickel (Fig. 3C) are features consistent with the existence of a low-threshold calcium conductance, a property previously noted in an unidentified population of parvocellular PVN neurons (15, 36).

Response to SCN stimulation. In 15 of 20 retrogradely labeled neurons, orientation of the slice permitted observations on their response to SCN stimulation. Of these, 12 neurons displayed an evoked response and 3 did not respond to SCN stimulation. When recorded in ACSF, this featured an inhibitory postsynaptic potential (IPSP) as the dominant response in four neurons, whereas a mixed inhibitory and excitatory response was seen in eight cells. To minimize possible competing responses, IPSPs in 10 cells were characterized in the presence of glutamate receptor antagonists D-AP5 (20 μM) and NBQX (5 μM). At suprathreshold stimulation strengths, latencies of evoked IPSPs ranged from 9.2 to 15.2 ms (mean 11.4 ± 0.7 ms). In individual cells, latencies remained constant over a range of stimulation intensities and followed stimulation frequencies of 20–50 Hz (Fig. 4). Mean IPSP rise rate and decay times were 0.85 ± 0.33 mV/ms and 87 ± 15 ms, respectively, when measured at membrane potentials held at −75 to −80 mV. SCN-evoked IPSPs reversed at −65 mV, close to the chloride equilibrium potential under current conditions and were reversibly blocked with 20 μM bicuculline, indicating mediation via GABA_{A} receptors (Fig. 4A).

In ACSF containing bicuculline to block GABA_{A}-mediated responses, SCN stimulation was seen to evoke excitatory postsynaptic potentials (EPSPs) in all eight neurons tested. When examined in the same neurons, thresholds (range 5–20 V) and latencies (range 7.8–11.0 ms; mean 9.8 ± 0.6 ms) for evoked EPSPs were not significantly different than for IPSPs. EPSP latencies were constant over a range of stimulation intensities and followed stimulation frequencies of 20–50 Hz (Fig. 4B). EPSPs displayed a rise rate of 0.55 ± 0.11 mV/ms and a decay time constant of 70.3 ± 12.9 ms. While data from six neurons displayed complete blockade after addition of 5 μM NBQX, a D-AP5-sensitive component could be demonstrated in two of eight neurons (Fig. 5A), indicating that both non-NMDA and NMDA subtype of receptors participated in the SCN-evoked responses. Further tests in magnesium-free solutions were not conducted.

To ensure that results of SCN stimulation were not due to current spread to lateral hypothalamic structures, in two slices that provided data mentioned above we placed a knife cut along the lateral edge of the SCN. We then compared the responses of dorsal PVN neurons to a stimulation electrode located within the SCN vs. another stimulation electrode placed lateral to the knife cut. In each of two unidentified (unlabeled) PVN neurons recorded, intra-SCN stimulation evoked a monosynaptic EPSP (n = 1) or a mixed EPSP-IPSP response (n = 1), whereas no response was recordable.
from the laterally positioned electrode even at double the stimulus intensities.

In magnocellular neurons, we recently reported the presence of paired-pulse depression in SCN-evoked IPSPs and data consistent with their attenuation by presynaptic GABA_B receptors (6). In the present study, we employed latex microspheres to identify these neurons for patch-clamp analyses. Previous reports have indicated that this marker remains localized to the site of injection, transports readily to the somata, and remains intact for prolonged periods without degeneration or diffusion to extracellular structures and, importantly, is apparently devoid of toxicity to neurons (9, 38). An added benefit is the relative ease with which doubly labeled neurons can be revealed with laser scan confocal microscopy without further resectioning.

Although this is not the first report where electrophysiology has been applied to spinal-projecting PVN neurons (13, 18), the data presented here illustrate some intrinsic electrical properties that are distinct from either neighboring magnocellular or parvocellular cells. In particular, magnocellular PVN neurons, previously classified as type I (36), demonstrate a frequency-dependent action potential broadening and a transient outward conductance but lack a low-threshold calcium conductance; these properties can influence their spike patterning and the magnitude of secretion of neurohypophysial hormones (24). Heterogeneity among parvocellular PVN neurons has also been recognized in earlier investigations leading to the designation of type II (nonburst LTS) and type III (burst LTS) neurons; both types show a time-independent inward rectification, with the latter showing a large LTS that triggers a burst of action potentials (15, 23).

Spinal-projecting PVN neurons feature low-threshold calcium spikes. A: Top voltage traces are the response to depolarizing current pulses (below) in a neuron held at a hyperpolarized membrane potential (−80 mV). The uppermost trace illustrates an LTS that triggers an action potential. B: voltage traces from another neuron exhibit rebound membrane depolarization on return from a hyperpolarized potential. Inset: LTS activation curve. C: sample voltage traces illustrate that the LTS elicited after return of the membrane potential to resting levels from a hyperpolarized level of −110 mV is blockable in ACSF containing 0.5 mM nickel.

Fig. 3. Spinal-projecting neurons feature low-threshold calcium spikes. A: top voltage traces are the response to depolarizing current pulses (below) in a neuron held at a hyperpolarized membrane potential (−80 mV). The uppermost trace illustrates an LTS that triggers an action potential. B: voltage traces from another neuron exhibit rebound membrane depolarization on return from a hyperpolarized potential. Inset: LTS activation curve. C: sample voltage traces illustrate that the LTS elicited after return of the membrane potential to resting levels from a hyperpolarized level of −110 mV is blockable in ACSF containing 0.5 mM nickel.

Fig. 4. SCN-evoked inhibitory postsynaptic potentials (IPSPs). A: averaged (n = 6) voltage traces obtained from a spinal-projecting PVN neuron recorded in ACSF containing glutamate receptor antagonists 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoline-7-sulfonamide disodium (NBQX; 5 μM) and D(-)-2-amino-5-phosphonovaleric acid (D-AP5; 20 μM). Under these conditions and at this holding potential (−75 mV), an SCN stimulus (arrow) evokes a depolarizing IPSP (top trace) that is blockable in the presence of 20 μM bicuculline (middle trace) and recoverable (bottom trace). B: trace to illustrate that SCN-evoked IPSPs follow stimulation frequencies of 20 Hz.
apposition to spinal-projecting neurons (37, 39). An analysis at the ultrastructural level to verify that these SCN efferents project directly to dorsal PVN neurons has not yet been reported. However, observations reported here implying a monosynaptic pattern in SCN-evoked postsynaptic responses are consistent with a direct innervation of spinal-projecting PVN neurons.

Some concerns arise owing to the apparent duality in these evoked responses, in large measure because of immunocytochemical observations that most, but not all, SCN neurons contain glutamic acid decarboxylase and/or GABA (3, 8, 21, 22). This would imply that the output of SCN is largely GABAergic and therefore inhibitory in nature (21). Indeed, the electrophysiology supports this notion for the SCN innervation to magnocellular PVN neurons (6, 16). Although there is strong support for the GABAergic nature of most SCN efferents, it remains to be explained how an “inhibitory” output system might exert opposing (facilitatory vs. suppressant) influences on target neurons. One theory, yet to be confirmed, advanced by Wagner et al. (40) is that SCN neurons might undergo a diurnal shift in their chloride reversal potential, thereby permitting activated GABA_A receptors to depolarize postsynaptic neurons. An alternative possibility arising from our current and earlier electrophysiological studies (16) is the existence of a glutamatergic projection from SCN, most easily demonstrable in slice preparations after pharmacological blockade of GABA_A receptors. The unpredictability of glutamate-like immunoreactivity combined with the undisputed evidence for GABA in most SCN neurons argues against this postulate. Indeed, with electrical stimulation there is always the concern that the glutamate-mediated excitation we have reported in PVN does not come from SCN but rather is due to current spread to axons in passage or adjacent (non-SCN) neurons. Although this may be possible, we have noted that electrical stimulation in the hypothalamus lateral to a knife cut placed at the edge of the SCN was ineffective, contrasting with monosynaptic EPSCs and IPSCs evocable from an intra-SCN stimulation site. We also reported earlier that intra-SCN chemical (glutamate) microstimulation (to depolarize somata but not axons) in the presence of bicuculline can increase the frequency of EPSPs and evokes membrane depolarization in parvocellular PVN neurons (16), implying that a component of the SCN output is glutamatergic. A more reliable indication can be found in a recently reported study revealing the presence in SCN of a (relatively small) subpopulation of glutamatergic/aspartate neurons, identified through their ability to transport [³H]aspartate retrogradely from PVN (5). Perhaps the projections from this subpopulation of SCN neurons can explain the electrophysiological observations of SCN-evoked glutamatergic responses in neurons in PVN (6, 16), supraoptic nucleus (7), and ventromedial preoptic area (32).
Perspectives

The presence of diurnal rhythms in various physiological functions affects our ability to cope with environmental challenges such as shift work and jet lag, situations that may impair sleep-wake cycles and cognitive functions. While this topic has obvious practical and clinical interest, our understanding of the detailed cellular mechanisms whereby the biological clock in our brain entrains systems of neurons regulating homeostasis and various autonomic activities is far from complete. The present study adds to a growing body of evidence that supports a direct influence, via axonal projections, of SCN on the excitability of neurons engaged in homeostasis and the regulation of autonomic functions. Our data support the presence of both GABA and glutamate as mediators of rapid inhibitory and excitatory neurotransmission, respectively, from SCN to these target neurons. This concurs not only with earlier demonstrations of a prominent GABAergic cell composition within SCN (8, 21), but also recent evidence that a subpopulation of PVN-projecting SCN neurons is glutamatergic (5). Among the next challenges will be an understanding of the relative contributions of these subpopulations of SCN neurons in the entrainment of target cells at various stages of the circadian cycle.

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