Internuclear coupling of hypothalamic magnocellular nuclei by glutamate synaptic circuits

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Boudaba, Cherif, and Jeffrey G. Tasker. Internuclear coupling of hypothalamic magnocellular nuclei by glutamate synaptic circuits. Am J Physiol Regul Integr Comp Physiol 291: R102–R111, 2006. First published February 9, 2006; doi:10.1152/ajpregu.00795.2005.—Magnocellular neurons of the supraoptic nucleus (SON) and paraventricular nucleus (PVN) display bursting activity that is synchronized under certain conditions. They receive excitatory synaptic inputs from intrahypothalamic glutamate circuits, some of which are activated by norepinephrine. Ascending noradrenergic afferents and intrahypothalamic glutamate circuits may be responsible for the generation of synchronous bursting among oxytocin neurons and/or asynchronous bursting among vasopressin neurons located in the bilateral supraoptic and paraventricular nuclei. Here, we tested whether magnocellular neurons of the PVN receive excitatory synaptic input from the contralateral PVN and the region of the retrochiasmatic SON (SONRX) via norepinephrine-sensitive internuclear glutamate circuits. Whole cell patch-clamp recordings were performed in PVN magnocellular neurons in coronal hypothalamic slices from male rats, and the ipsilateral SONRX region and contralateral PVN were stimulated using electrical and chemical stimulation. Electrical and glutamate microdrop stimulation of the ipsilateral SONRX region or contralateral PVN elicited excitatory postsynaptic potentials/currents (EPSP/Cs) in PVN magnocellular neurons mediated by glutamate release, revealing internuclear glutamatergic circuits. Microdrop application of norepinephrine also elicited EPSP/Cs, suggesting that these circuits could be activated by activation of noradrenergic receptors. Repetitive electrical stimulation and drop application of norepinephrine, in some cases, elicited bursts of action potentials. Our data reveal glutamatergic circuits that interconnect the magnocellular nuclei and that can be activated by norepinephrine. These internuclear glutamatergic circuits may provide the functional architecture to support burst generation and/or burst synchronization in hypothalamic magnocellular neurons under conditions of activation.

MAGNOCELLULAR NEURONS of the hypothalamic supraoptic nucleus (SON) and paraventricular nucleus (PVN) release either of the two hormones, vasopressin and oxytocin, directly into the bloodstream from their axon terminals located in the neurohypophysis. Under a variety of physiological conditions (e.g., dehydration, hypovolemia, lactation), these neurons display bursting activities that produce a large increase in the circulating concentrations of oxytocin and vasopressin. There is considerable evidence to suggest that glutamate plays an important role in the generation of bursts in these neurons. Glutamatergic terminals are abundant on hypothalamic magnocellular neurons (20) and glutamate is responsible for the fast excitatory synaptic input to the magnocellular neurons (54, 57). Glutamate appears to be essential for the onset and maintenance of bursting activity in both vasopressin and oxytocin neurons in vitro (18, 21) and in vivo (31, 35, 36). Glutamate circuits also carry information from perinuclear zones surrounding the SON and PVN (5, 6, 10, 21), as well as from circumventricular osmosensitive brain structures to the magnocellular neurons (12, 38).

In rat SON and PVN oxytocin neurons, the onset of bursts is synchronous during lactation to within about 700 ms (3, 4), suggesting a functional connection between neurons located in the different magnocellular nuclei. Oxytocin injected into one PVN or SON in vivo has been shown to induce bursting activity in the contralateral nucleus of the lactating rat (23, 30, 34), providing evidence for functional interactions among the nuclei. Further support for functional internuclear connections has been provided by several anatomical and electrophysiological studies showing projections between the bilateral paraventricular (42, 43) and supraoptic nuclei (46, 53) and between the ipsilateral supraoptic and paraventricular nuclei (39, 40, 52). Here, we used whole cell patch-clamp recordings in a hypothalamic slice preparation to determine whether the paired PVN and the PVN and ipsilateral SON are synthetically coupled via glutamatergic circuits and whether these circuits are sensitive to norepinephrine. We show that the two paraventricular nuclei and the PVN and ipsilateral retrochiasmatic SON (SONRX) area are connected by glutamatergic circuits and that these circuits are activated by norepinephrine and are capable of triggering bursts in magnocellular neurons. Part of this work has been published in abstract form (7).

METHODS

Experiments were performed according to a protocol approved by the Tulane University Institutional Animal Care and Use Committee and in conformance with U.S. Public Health Service guidelines. Male Sprague-Dawley rats (60–150 g) were deeply anesthetized with in-
traperitoneal pentobarbital sodium (50 mg/kg body wt) and decapitated with a guillotine, and the brain was quickly removed from the cranial cavity and placed in ice cold (1–2°C), oxygenated artificial cerebrospinal fluid (aCSF). The aCSF contained (in mM): 140 NaCl, 3 KCl, 2.4 CaCl₂, 1.3 MgSO₄, 1.4 NaH₂PO₄, 11 glucose, 5 HEPES; pH was adjusted to 7.2–7.4 with NaOH. Hypothalamic slices (400 μm) containing the PVN were sectioned in the coronal plane with a vibrating microtome (Campden Instruments). Slices were stored submerged in a holding chamber in oxygenated ACSF at room temperature and allowed to recover for 1 1/2 to 2 h before their use. One slice at a time was transferred to an interface recording chamber and continuously superfused with ACSF at 34°C.

Patch pipettes were pulled from borosilicate glass (1.65 mm OD, 1.2 mm I.D., KG-33, Garner Glass) using a horizontal puller (P-97, Sutter Instruments) and had a tip resistance of 3–6 MΩ. The pipette solution contained (in mM): 120 β-glucosic acid, 10 HEPES, 1 NaCl, 1 CaCl₂, 1 MgCl₂, 2 Mg-ATP, 0.3 Na-guanosine-5’-triphosphate, and 10 EGTA; pH was adjusted to 7.2–7.4 with KOH. The osmolarity of the internal solution was adjusted to 290–300 mOsm/l with 20 mM d-sorbitol.

Excitatory postsynaptic potentials (EPSPs) were recorded in PVN magnocellular neurons at resting potential (~50 to ~70 mV) with an Axoclamp-2B, Axopatch-1D, or Multiclamp 700A amplifier using the “blind” whole cell patch-clamp technique. The amplitude of the spikes was ±50 mV measured from threshold. Excitatory postsynaptic currents (EPSCs) were recorded at a holding potential of ~70 mV in the presence of bicuculline methiodide (30 μM). Magnocellular neurons were identified in the PVN, according to electrophysiological criteria (26, 48) and recorded while the ipsilateral SON-retrochiasmatic area or contralateral PVN was stimulated electrically with a bipolar platinum-iridium electrode (0.3–0.8 mA, 0.5 ms, 0.08–3 Hz) or chemically with glutamate (10–20 mM) or norepinephrine (1–2 mM) microdrop application on the surface of the slice (Fig. 1).

All data were converted to digital video format (Neuro-Corder, NeuroData Instruments) and stored on videotape for off-line analysis. Episodes of selected synaptic activity were played from videotape, digitized, and recorded on a computer using the Digitdata 1200 interface and PCLAMP 6 or 7 software (Axon Instruments). Episodes were analyzed off line using the Mini Analysis Program (v. 3.0.1 and 4.0, Synaptosoft). Mean frequencies of EPSP/EPSCs during 3-min epochs from control periods and during electrical or chemical stimulation were compared statistically using the Student’s paired t-test; P < 0.05 was considered significant.

Oxytocin (10 μM), the GABA_A receptor antagonist bicuculline methiodide (30 μM), the ionotropic glutamate receptor antagonists d,L-2-amino-5-phosphonovalerate (AP5, 100 μM) and 5,6-dinitroquinoxaline-2,3-dione (DNQX, 50 μM), and TTX (1.5–3 μM) were dissolved in aCSF and applied in the bath perfusion. A patch pipette with a broken tip was used to apply microdrops of glutamate and norepinephrine by pressure on the surface of the slice using a picospritzer (General Valve). The dye Janus green was added to the glutamate and norepinephrine solutions to visualize the spread of the microdrops in the slice. Microdrops were ~250 μm in diameter and were always positioned downstream from the recorded neurons to avoid diffusion of the drugs to the PVN in which the recorded neurons were located.

Biocytin was added to patch pipettes (0.3–0.5%). After experiments, slices were fixed overnight in 4% formaldehyde in 0.1 M PBS. Slices were sectioned at 20–25 μm on a freezing microtome. Slices were incubated for 4 h in streptavidin-conjugated 7-amino-4-methyl coumarin 3-acetic acid (AMCA) diluted (1:300) in 0.1 M PBS containing 0.5% Triton X-100. To detect biocytin-filled and AMCA-labeled neurons, sections were examined under the microscope with epifluorescence using a UV/420 K filter combination.

Sections containing AMCA-labeled neurons were incubated for 36 h in a mixture of rabbit polyclonal antibody to oxytocin-associated neurophysin (VA-10) diluted 1:2,000 and a mouse monoclonal antibody to vasopressin-associated neurophysin (PS-41) diluted 1:4,000 in PBS with 1% normal sheep serum and 0.2% sodium azide (antibodies were kindly provided by Dr. H. Gainer, National Institutes of Health). The sections were then rinsed in PBS and incubated for an hour in anti-rabbit secondary IgG conjugated with FITC and goat anti-mouse secondary IgG conjugated with rhodamine diluted 1:200 in PBS with 1% normal sheep serum and 0.2% sodium azide. The sections were mounted in elvanol, coverslipped, and the coverslips were sealed with nail polish. The slides were examined under 515- to 560-nm excitation/580-nm barrier filters or under 450- to 490-nm excitation/560-nm excitation/580-nm barrier filters to detect the rhodamine-labeled, vasopressin-positive neurons, or under 450- to 490-nm excitation/515-nm barrier filters to detect FITC-labeled, oxytocin-positive neurons. Biocytin-labeled neurons were identified as oxytocinergic or vasopressinergic only if they were found to be positive for one antibody and negative for the other.

RESULTS

To determine whether the PVN receives excitatory synaptic inputs from ipsilateral and contralateral magnocellular nuclei via intrahypothalamic glutamate circuits, we recorded synaptic responses in PVN magnocellular neurons to electrical stimulation of the contralateral PVN and ipsilateral SONrx area in coronal hypothalamic slices (Fig. 1). Magnocellular neurons were distinguished from parvocellular neurons of the hypothalamic paraventricular nucleus (PVN). The contralateral PVN or the ipsilateral supraoptic nucleus-retrochiasmatic area (SONrx) was electrically stimulated or chemically activated by microdrop application of glutamate or norepinephrine (NE). 3V, third ventricle; Fx, fornix; OT, optic tract.

Fig. 1. Whole cell recordings were performed in magnocellular neurons of the hypothalamic paraventricular nucleus (PVN). The contralateral PVN or the ipsilateral supraoptic nucleus-retrochiasmatic area (SONrx) were electrically stimulated or chemically activated by microdrop application of glutamate or norepinephrine (NE). 3V, third ventricle; Fx, fornix; OT, optic tract.
neurons in the PVN on the basis of their lateral location within the nucleus and their characteristic electrical properties (48).

Electrical stimulation. In the coronal slice preparation, electrical stimulation of the contralateral PVN elicited EPSCs and EPSPs with a latency of 1.1 to 9.5 ms in 20 of 26 (77%) PVN magnocellular neurons (Fig. 2). Electrical stimulation of ipsilateral SON$_{rx}$ evoked EPSCs and EPSPs with a latency of 3.1 to 10.5 ms in 10 of 19 (52.6%) PVN magnocellular neurons (Fig. 2). Nineteen of the thirty responsive neurons were tested to 10.5 ms in 10 of 19 (52.6%) PVN magnocellular neurons lateral SON$_{rx}$ evoked EPSCs and EPSPs with a latency of 3.1 to 9.5 ms in 20 of 26 (77%) PVN magnocellular neurons.

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EPSPs with a latency of 1.1 to 9.5 ms in 20 of 26 (77%) PVN magnocellular neurons were often evoked with a single stimulus, suggesting that they were monosynaptic. Multiple EPSP/EPSCs were often evoked with a single stimulus, suggesting a possible polysynaptic component to the response (6).

The evoked response was completely blocked by AP5 (100 μM) and DNQX (50 μM) in 4 of 4 neurons tested, indicating that it was mediated by synaptic glutamate release (Fig. 3A). TTX was applied to block action potential-dependent synaptic transmission in two neurons to confirm that the synaptic response was mediated by activation of synaptic circuits; TTX blocked the response in both neurons tested.

PVN magnocellular neurons responded to electrical stimulation of the contralateral PVN or ipsilateral SON$_{rx}$ with an increase in the frequency of EPSCs when the stimulation was applied tonically at a frequency of 0.1–3 Hz (Fig. 3, A and B). The response was blocked by prior application of glutamate receptor antagonists in the bath perfusion, indicating that it was mediated by activation of glutamate circuits. In three PVN magnocellular neurons that did not show any response during tonic stimulation of the ipsilateral SON$_{rx}$ area, a second stimulation in the presence of oxytocin (10 μM, 10 min) induced an increase in the frequency of EPSCs in one of the cells (Fig. 3, C and D). These responses were suggestive of a windup phenomenon with repetitive stimulation. Two of the PVN magnocellular neurons responsive to single electrical stimuli of the contralateral PVN in current clamp mode responded to repeated stimulation (0.08–2 Hz) applied to the contralateral PVN with bursts of EPSPs and action potentials (Fig. 4).

Chemical stimulation. To determine whether the excitatory synaptic responses evoked in PVN magnocellular neurons by stimulation of the contralateral PVN and ipsilateral SON$_{rx}$ were mediated by local synaptic circuits and not by fibers of passage, we applied glutamate microdrop stimulation to the same sites (Fig. 1). Glutamate microdrops (10–20 mM) applied in the contralateral PVN induced a 130% increase in the frequency of EPSPs (from 1.79 ± 0.67 Hz to 3.57 ± 1.35; P < 0.05) with a latency of 4 to 36 s in 7 of 25 PVN magnocellular neurons (24%). Glutamate microdrops applied in the ipsilateral SON$_{rx}$ induced a 62% increase in the frequency of EPSPs (from 1.74 ± 0.33 Hz to 2.62 ± 0.42 Hz; P < 0.05) with a latency of 2 to 30 s in 8 of 44 PVN magnocellular neurons (18%) (Fig. 5). In 5 of the neurons that responded to glutamate microdrops, the stimulation triggered repetitive bursts of EPSPs, which also generated repetitive bursts of action potentials in three of the neurons. In eight PVN magnocellular neurons, stimulation of the contralateral PVN (n = 2) or the ipsilateral SON$_{rx}$ (n = 6) elicited an increase in the frequency of inhibitory postsynaptic potentials. This inhibitory response was not analyzed further in this study.

To determine whether the internuclear glutamate circuits stimulated by electrical and chemical stimulation were sensitive to norepinephrine, microdrops of norepinephrine (1–2 mM) were also applied in the same presynaptic sites. Norepinephrine microdrops applied in the contralateral PVN induced a 50% increase in the frequency of EPSPs (from 2.03 ± 0.68 Hz to 3.05 ± 1.1 Hz; P < 0.05) in 10 of 30 PVN magnocellular neurons (33%); norepinephrine microdrops applied in the ipsilateral SON$_{rx}$ elicited a 34% increase in the frequency of EPSPs (from 2.28 ± 0.66 Hz to 3.07 ± 0.6; P < 0.05) in six of eighteen PVN magnocellular neurons (33%) (Fig. 6). Norepinephrine often elicited compound EPSPs that generated bursts of action potentials (Fig. 6).

Cell identification. Biocytin injection and immunohistochemical analyses were performed on a subset of PVN magnocellular neurons that responded to internuclear stimulation. Among the neurons responding to electrical stimulation, three were identified as vasopressinergic (two responded to contralateral PVN stimulation, one to SON$_{rx}$ stimulation) and one
Fig. 3. Repetitive electrical stimulation of the ipsilateral retrochiasmatic SON elicits windup activation of glutamate synaptic inputs to a PVN magnocellular neuron. 

A: tonic electrical stimulation (3 Hz) of the ipsilateral SON, for 2 min induced an increase in the frequency of EPSCs (Control) in the same neuron as that shown in Fig. 2B. This effect was blocked by ionotropic glutamate receptor antagonists (AP5/DNQX). Repetitive stimulation begins and ends at arrows Stim On and Off, respectively; stimulus artifacts are blanked. This neuron was recorded at a holding potential of −60 mV in the presence of bicuculline (30 μM). 

B: cumulative fraction plot of EPSC interevent intervals before (Basal) and during tonic stimulation (Stim). The shift to the left of the EPSC interval distribution during stimulation indicates an increase in EPSC frequency. 

C: tonic electrical stimulation (4 Hz) of the ipsilateral SON, for 2 min had very little effect on the excitatory synaptic activity in a different PVN magnocellular neuron (Control). After 10 min of bath application of oxytocin (10 μM), however, the same electrical stimulation elicited a robust increase in the frequency of EPSCs (Oxytocin). 

D: cumulative fraction plot of EPSC interevent intervals before (Basal) and during tonic stimulation in the absence (Stim) and in the presence of oxytocin (Stim w/oxytocin). The shift of the response to the left during tonic stimulation in the presence of oxytocin indicates an increase in the frequency of EPSCs. This neuron was recorded at a holding potential of −60 mV in the presence of bicuculline (30 μM).
was identified as oxytocinergic (responsive to contralateral PVN stimulation) after biocytin injection and immunohistochemical processing (Fig. 7). Of the neurons that responded to glutamate microdrop stimulation, four were oxytocinergic (two responded to stimulation of contralateral PVN, two to stimulation of SONrx); none of the responsive neurons recovered were positive for vasopressin. Two of the neurons that responded to norepinephrine microdrop stimulation were identified as vasopressinergic (responsive to stimulation of contralateral PVN) and three were identified as oxytocinergic (one responsive to contralateral PVN stimulation, two to SONrx stimulation).

Fig. 5. Local stimulation of the ipsilateral SONrx elicited an increase in excitatory synaptic inputs. A PVN magnocellular neuron responded to a glutamate microdrop (Glu) applied to the ipsilateral SONrx (arrow) with an increase in the frequency of EPSPs. This neuron was recorded at −66 mV in the absence of bicuculline.
DISCUSSION

Here, we describe intrahypothalamic glutamate circuits that synaptically couple the contralateral PVN and the ipsilateral SONrx area with PVN magnocellular neurons and that are activated by norepinephrine. We stimulated the SONrx area because it is the part of the SON that is present in coronal slices containing the PVN. Because of the restricted size of the SON in its retrochiasmatic portion, we can say only that we stimulated the region of the SONrx, and not the SON magnocellular neurons directly. Our findings with stimulation in the PVN and SONrx corroborate data from our laboratory and others that provide increasing evidence (1) that magnocellular neurons are regulated by intrahypothalamic glutamatergic circuits (10, 18, 21, 22), 2) that these circuits are activated by norepinephrine (5, 10), and 3) that these circuits may play a role in the generation of synchronous bursting activity in oxytocin neurons (19). Brainstem catecholaminergic neurons are activated during suckling (25) and parturition (1, 28). There are increased numbers of glutamatergic and noradrenergic terminals in the SON during lactation (13, 29), and both the glutamatergic and noradrenergic inputs to oxytocinergic neurons appear to be critical for parturition and lactation (1, 2, 11, 25, 28, 51). Norepinephrine is released in the SON before and during a rise in SON glutamate levels, which correlates temporally with the activation of oxytocin neurons and oxytocin release associated with parturition and milk ejection (2, 17).

Thus a possible mechanism for the synchronous burst generation among oxytocin neurons during parturition and/or milk ejection is by sensory stimulation, via uterine contraction or suckling, of ascending brainstem noradrenergic projections to hypothalamic glutamate neurons and the subsequent activation of intrahypothalamic glutamate circuits to drive synchronous firing. NE activates internuclear excitatory synaptic inputs to PVN magnocellular neurons. A: a PVN magnocellular neuron responded to microdrop application of NE (1 mM) in the contralateral PVN (arrow) with an increased frequency of EPSPs after a latency of \( \sim 5 \) s. B: another PVN magnocellular neuron responded to NE microdrop application (1 mM) in the contralateral PVN (arrow) with large compound EPSPs. Responses to three successive NE microdrop applications are shown. The third NE application elicited two compound EPSPs, the second one arising after a delay. C: another PVN magnocellular neuron responded to a NE microdrop application (1 mM) in the ipsilateral SONrx (arrow) with a burst of action potentials after a prolonged delay. D: a second application of NE in the ipsilateral SONrx elicited an increase in the frequency of EPSPs in the same cell, which reversed after 1 min (Wash).

Fig. 6. NE activates internuclear excitatory synaptic inputs to PVN magnocellular neurons. A: a PVN magnocellular neuron responded to microdrop application of NE (1 mM) in the contralateral PVN (arrow) with an increased frequency of EPSPs after a latency of \( \sim 5 \) s. B: another PVN magnocellular neuron responded to NE microdrop application (1 mM) in the contralateral PVN (arrow) with large compound EPSPs. Responses to three successive NE microdrop applications are shown. The third NE application elicited two compound EPSPs, the second one arising after a delay. C: another PVN magnocellular neuron responded to a NE microdrop application (1 mM) in the ipsilateral SONrx (arrow) with a burst of action potentials after a prolonged delay. D: a second application of NE in the ipsilateral SONrx elicited an increase in the frequency of EPSPs in the same cell, which reversed after 1 min (Wash).

Fig. 7. Immunohistochemical identification of responsive PVN magnocellular neurons. A: an oxytocinergic PVN magnocellular neuron in which an excitatory synaptic response was recorded in response to electrical stimulation and NE drop stimulation of the contralateral PVN. The biocytin-labeled cell (A1) was double labeled with the FITC oxytocin label (A2) and was negative for the rhodamine vasopressin label (A3), indicating that it was oxytocinergic. B: a vasopressinergic PVN magnocellular neuron that responded to electrical stimulation of the contralateral PVN with an excitatory synaptic response. The biocytin-labeled cell (B1) was negative for the FITC oxytocin label (B2) and was positive for the rhodamine vasopressin label (B3), indicating that it was vasopressinergic. 3V, third ventricle.
bursting among oxytocin neurons. This model would require that norepinephrine drive local glutamate circuits that interconnect magnocellular neurons within the individual magnocellular nuclei, as well as internuclear circuits that connect the ipsilateral and bilateral supraoptic and paraventricular nuclei. We and others have found evidence for each of these elements of the model. Thus Douglas et al. (11) have reported that ascending brainstem noradrenergic projections to the SON are activated by uterine contractions and stimulation oxytocin neurons during parturition. We reported in a previous study that norepinephrine activates local glutamate circuits within the PVN, providing evidence for an intra-PVN, norepinephrine-sensitive glutamate circuit that controls PVN magnocellular neuron activity (10). We also found evidence previously for local glutamate inputs to SON magnocellular neurons from the perinuclear region of the SON (6). Poulain and colleagues (19, 21) reported synchronous bursting activity in hypothalamic slice cultures that is generated and synchronized by excitatory synaptic inputs from local glutamate circuits. Finally, here we provide evidence for norepinephrine-sensitive intrahypothalamic glutamate circuits that connect the contralateral PVN and the ipsilateral SONrx area with magnocellular neurons in the PVN. Together, these observations support a model of norepinephrine activation of inter- and intranuclear glutamate circuits that may provide the functional synaptic infrastructure to support the coordinated activation of magnocellular neurons under stimulated conditions, whether it be synchronized burst generation in oxytocin neurons or simultaneous, asynchronous burst generation in vasopressin neurons (Fig. 8).

Electrical stimulation of the contralateral PVN or ipsilateral SONrx elicited EPSPs/EPSCs in PVN magnocellular neurons with latencies ranging from 1.1 to 10.5 ms. As in our previous study (6), we often observed multiple EPSPs/EPSCs to a single electrical stimulus (77% and 52.6% of the time, respectively, with contralateral PVN and SONrx stimulation), all of which were blocked by glutamate receptor antagonists. The short and constant latencies of some evoked EPSPs/EPSCs in our experiments suggest that monosynaptic pathways were probably activated by stimulation of the contralateral PVN and ipsilateral SONrx. In contrast, the longer latency and multiple EPSPs/EPSCs in response to electrical stimulation are consistent with activation of polysynaptic circuits. Our findings suggest, therefore, that these internuclear glutamate circuits may, on the one hand, provide a direct, monosynaptic excitatory projection to PVN magnocellular neurons, yet they may also drive other excitatory circuits, presumably local glutamatergic circuits, which could be involved in synchronizing magnocellular neuronal activity under stimulated conditions, as proposed in the model in Fig. 8. The range of latencies seen in our experiments was narrower than the range of latencies reported in vivo (39), suggesting that some polysynaptic circuits were severed in our slices, as one might expect. We obtained synaptic responses from glutamate microdrops applied to the same loci as our electrical stimulation, the contralateral PVN and ipsilateral SONrx area, suggesting that the excitatory synaptic afferents activated by glutamate microdrops were among the afferents activated by the electrical stimulation, and that these were therefore probably also glutamatergic in nature.

Electrical stimulation is capable of activating local neurons, as well as fibers of passage, making it difficult to rule out the activation by electrical stimulation of afferents originating from elsewhere in the brain. We used microdrop application of glutamate to circumvent this problem. Ionotropic glutamate receptors are not present on axons (9, 14), such that the excitatory effects of glutamate microdrops can be interpreted as activation of the somata/dendrites of presynaptic neurons. Metabotropic glutamate receptors are present at presynaptic glutamatergic terminals on magnocellular neurons, but the activation of these receptors causes a reduction in glutamate release that is insensitive to TTX (41). The response seen here to glutamate microdrops, i.e., a synaptic excitation, was blocked by TTX and was opposite to that seen with activation of presynaptic metabotropic glutamate receptors, indicating that it was not caused by the activation of presynaptic glutamate receptors, but rather to excitatory glutamate receptors located on upstream neurons that were part of intact local glutamate circuits. Whether our microdrop stimulation of the PVN extended beyond its boundaries to the perinuclear region is unlikely. Although it is not possible to assess the area covered by the electrical stimulation, the glutamate and norepinephrine microdrop stimulation was monitored visually with a dye, and the ~250-μm diameter of the drops did not reach beyond the limits of the PVN based on visual monitoring. That the electrical stimulation or the microdrop stimulation of the SONrx area activated only SON magnocellular neurons, on the other hand, is not likely, because of the restricted size of the SONrx in the coronal plane. Therefore, it is not known whether the glutamate projections to PVN magnocellular neurons activated in these experiments originated in the SONrx or in the perinuclear region dorsal to the SONrx.

Fig. 8. Hypothetical model of NE-glutamate circuit regulation of magnocellular neurons. Intrahypothalamic glutamate circuits link contralateral PVN and ipsilateral SONrx with magnocellular neurons in the PVN via NE-sensitive monosynaptic and polysynaptic circuits. The model is based on the internuclear glutamate circuits presented here, and on NE-sensitive intrahypothalamic and intranuclear glutamate inputs to SON and PVN neurons reported previously (5, 10). Noradrenergic projections to presynaptic glutamate terminals and directly to magnocellular neurons have been left out for the sake of clarity.
Whether originating in the SON_{rx} or in the area surrounding the SON, evidence presented here and in previous studies suggests that this region may provide common projections to both the SON and the PVN (6, 49), making it a potentially important area for the simultaneous activation of magnocellular neurons in both nuclei.

The SON and PVN undergo dramatic structural (15, 50) and functional changes (37, 44) at late pregnancy, which include enhanced glutamatergic innervation, as indicated by increased glutamate synapses (13) and glutamatergic synaptic inputs (45). Although the theory has been challenged recently (8), these changes are thought to provide a structural substrate that supports burst generation and synchronization among oxytocin-secreting neurons in parturient and lactating female rats. To what extent these anatomical changes influence bursting activity in oxytocin neurons is not known. If they are not involved at all, as suggested by the recent report by Catheline et al. (8), it is then difficult to explain why they occur only during parturition and lactation, widely assumed to be the only physiological states in the female rat during which oxytocin neurons display bursting activity. Nevertheless, as proposed by Catheline et al. (8), these morphological changes may serve not to facilitate bursting activity, but rather to filter out other afferent inputs not relevant in the context of parturition and lactation, such as low-grade stress or osmotic inputs (33). Although we cannot interpret our results from male rats to bear directly on conditions in which synchronous bursting is observed in female rats, we can interpret our findings to represent a basic circuit structure present in the hypothalamus, subject to confirmation, in the female rat. We found sporadic burst generation in response to tonic electrical stimulation and to activation of local circuits in slices from male rats. Although burst generation has been reported under certain conditions in magnocellular neurons from male rats (55), and bolus release of oxytocin, similar to that seen during parturition and milk ejection, occurs in males during copulation (32), it is not known whether oxytocin neurons in male rats generate a synchronous bursting activity similar to that seen in lactating females. Nevertheless, if we assume that the local synaptic circuitry present in the male hypothalamus represents a baseline synaptic organization, and if the enhanced glutamatergic innervation of oxytocin neurons seen at parturition in females is facilitatory for burst generation/synchronization among the oxytocin neurons of parturient/lactating female rats, and if burst generation depends on local glutamate circuits, as shown in organotypic slice cultures (19) and proposed in our model (Fig. 8), then we might expect to find a greater density of local glutamate circuits and a greater incidence of bursting in response to activation of these circuits in the parturient/lactating female rat, a subject for further study.

There are two hypothalamic regions that, from previous reports, are in a good position to be involved in the afferent activation of oxytocinergic neurons during the milk ejection reflex, the hypothalamic dorsochiasmatic area and the dorso-medial hypothalamic nucleus (DMH). Neurons in the dorsochiasmatic area send axonal projections to both the SON and the PVN (49). The coronal slices we used contained the dorsochiasmatic area, and our ipsilateral SON_{rx} stimulation that resulted in the activation of glutamatergic synaptic inputs to PVN magnocellular neurons was likely to include cells in this area, although it is impossible to say from our data whether these cells were recruited into the stimulation. In a previous study, glutamate microstimulation of a region near here—dorsal to the SON and ~400 μm rostral to the SON_{rx} site—elicited an excitatory synaptic response in magnocellular neurons of the principal SON (6). It was also reported that this perinuclear zone receives collateral projections from SON magnocellular neurons (24, 27) and that this area may provide recurrent feedback (16) via glutamatergic inputs to the SON (6, 56). If the perinuclear zone is contiguous with the SON_{rx}, as appears likely, then those findings combined with the findings presented here suggest that the SON_{rx}-dorsochiasmatic area may provide glutamatergic synaptic inputs to magnocellular neurons of both the SON and PVN. Another potential upstream relay, the DMH, contains neurons that are antidromically activated by stimulation of the SON and that generate bursts that are coincident with oxytocin neuron bursts, oxytocin release, and milk ejection (47). In our previous study, we reported that glutamate microdrops placed in the region of the DMH elicited an increase in EPSPs in PVN magnocellular neurons (6), suggesting a local glutamatergic synaptic circuit linking the DMH with the magnocellular PVN. The positioning of the PVN, SON, and DMH in different planes of the hypothalamus makes it difficult to get all three structures in the same slice, so that we were not able to study the DMH here in the context of internuclear circuits that regulate PVN magnocellular neurons. However, on the basis of these previous findings, the DMH appears to be another region of the hypothalamus in a position to regulate magnocellular neurons in both the PVN and SON, suggesting that the model presented in Fig. 8 is probably an oversimplification of the actual local excitatory synaptic circuitry that regulates magnocellular neuron activity.

The latency of the glutamatergic response to electrical stimulation of the contralateral PVN was shorter than that to ipsilateral SON_{rx} stimulation. This is probably because of the close proximity of the two paraventricular nuclei to each other but may also be due to processes from each PVN that bridge the distance between the nuclei (43). These processes originate in each PVN and cross dorsally over the third ventricle. The nature of these processes and whether they emerge from the PVN magnocellular or parvocellular neurons is not known; however, the lesion of the midline area containing these processes deregulates bursting activity among oxytocin neurons in lactating rats (30).

In conclusion, we present evidence in acute hypothalamic slices for internuclear PVN-to-PVN and SON_{rx}-to-PVN synaptic coupling via norepinephrine-sensitive glutamate circuits. These intrahypothalamic excitatory synaptic circuits are capable of driving bursting activity in PVN magnocellular neurons, and may, therefore, play a key role in the generation and synchronization of bursting activity in oxytocin neurons and in the coordinated activation of vasopressin neurons under stimulated conditions.

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