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Ionotropic Histamine Receptors and H_2 Receptors Modulate Supraoptic Oxytocin Neuronal Excitability and Dye Coupling

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Histaminergic neurons of the tuberomammillary nucleus (TM) project monosynaptically to the supraoptic nucleus (SON). This projection remains intact in our hypothalamic slices and permits investigation of both brief synaptic responses and the effects of repetitively activating this pathway. SON oxytocin (OX) neurons respond to single TM stimuli with fast IPSPs, whose kinetics resemble those of $GABA_A$ or glycine receptors. IPSPs were blocked by the Cl^- channel blocker picrotoxin, but not by bicuculline or strychnine, and by histamine H_2, but not by H_1 or H_3 receptor antagonists, suggesting the presence of an ionotropic histamine receptor and the possible nonspecificity of currently used H_2 antagonists. G-protein mediation of the IPSPs was ruled out using guanosine 5′-*O*-(2-thiodiphosphate) (GDP-βS), pertussis toxin, and Rp-adenosine 3′,5′-cyclic monophosphothioate triethylamine (Rp-cAMPs), none of which blocked evoked IPSPs. We also investigated the effects of synaptically released histamine on dye coupling and neuronal excitability. One hundred seventy-three OX neurons were Lucifer yellow-injected in horizontal slices. Repetitive TM stimulation (10 Hz, 5–10 min) reduced coupling, an effect blocked by H_2, but not by H_1 or H_3, receptor antagonists. Because H_2 receptors are linked to activation of adenylyl cyclase, TM-stimulated reduction in coupling was blocked by GDP-βS, pertussis toxin, and Rp-cAMPs and was mimicked by 8-bromo-cAMP, 3-isobutyl-1-methylxanthine, and Sp-cAMP. Membrane potentials of OX and vasopressin neurons were hyperpolarized, accompanied by decreased conductances, in response to bath application of 8-bromo-cAMP but not the membrane-impermeable cAMP. These results suggest that synaptically released histamine, in addition to evoking fast IPSPs in OX cells, mediates a prolonged decrease in excitability and uncoupling of the neurons.

Key words: chloride channels; cAMP; G-protein blockade; histamine receptor antagonists; IPSPs; tuberomammillary nucleus

Histaminergic neurons in the mammalian brain reside in the tuberomammillary nuclear complex (TM) in the posterior hypothalamus. Because they project, via extensive axon collateralization, to virtually all brain regions (Wada et al., 1991), they are of quite general interest. Although exogenously applied histamine (HA) has been shown to have important modulatory effects on neurons in various areas, such as thalamus, hypothalamus, neocortex, and cerebellum, studies of the effects of synaptically released histamine on neurons are few. Without such studies, it is difficult or impossible to discern whether HA might mediate fast synaptic as well as the slower second messenger-mediated effects that are associated with the known H_1-, H_2-, and H_3-HA receptors. Development of a horizontally cut hypothalamic slice that keeps intact the monosynaptic connections (Inagaki et al., 1988; Panula et al., 1989; Yang and Hatton 1994) between the TM and the supraoptic nucleus (SON) has permitted such studies (Weiss et al., 1989; Yang and Hatton, 1989; Hatton and Yang, 1996).

In vasopressin (VP)-synthesizing SON neurons, single-pulsed TM stimulation evokes fast EPSPs and action potentials (Yang and Hatton, 1989; Hatton and Yang, 1996), suggesting the presence of an HA-operated ion channel. Trains of stimulation or direct application of HA result in depolarization and prolonged discharge (Armstrong and Sladek, 1985; Smith and Armstrong, 1993; Li and Hatton, 1996). This latter effect is mediated by H_1 receptors. H_1 receptor antagonists also block the stimulus-evoked EPSPs in VP neurons, and the receptor mediating this excitation remains unknown. In contrast, single-pulsed electrical stimulation of the TM evokes fast IPSPs in SON oxytocin (OX) neurons, activating a bicuculline-insensitive chloride conductance (Yang and Hatton, 1994). Here too, G-protein mediation was not ruled out, because H_2 receptor antagonists could block the IPSPs. Therefore, the receptor mediating this effect is unknown.

It is now well established that electrical and metabolic coupling exists among neurons in many areas of the mammalian CNS and that this coupling can be synaptically modulated (Hatton, 1998). Physiological activation, e.g., dehydration and lactation, inducing increased synthesis and release of OX and/or VP, also induces increased incidence of dye coupling among these neurons (Hatton, 1997). Such coupling is cell-type specific (Cobbett et al., 1985) and is a generally accepted indicator of electrical and/or metabolic coupling. Our previous study (Hatton and Yang, 1996) showed that brief repetitive TM stimulation produced a fourfold increase in dye coupling among VP neurons, an effect that was blocked by H_1 receptor antagonists and by a selective inhibitor of guanylyl cyclase, to the activation of which H_1 receptors are often linked (Hough, 1999). Similar increases in coupling were seen with bath application of 8-bromo-cGMP. These results suggested that neurotransmitter–modulators that were linked to the activa-

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tion of guanylyl cyclase could expand the network of coupled VP neurons.

Here we report analyses of HA-mediated fast IPSPs and that synaptically released HA has effects on OX neurons that are completely opposite to those it has on VP cells.

Parts of this work have been published previously in abstract form (Yang et al., 1998).

MATERIALS AND METHODS

Animals and procedures. Animals were 120 adult male Sprague Dawley rats (Holtzman, Madison, WI), 50- to 65-d-old, housed three rats per cage with *ad libitum* food and water on a 12 hr light/dark cycle. At ~2–5 hr into the light portion of the cycle, they were gently introduced to a guillotine and decapitated without anesthesia. Brains were quickly removed, mounted cortex down on the stage of a vibratome, cut in the horizontal plane at 400–500 μm thickness, and placed in room temperature medium. Slices were then hemisected along the third ventricle by severing the optic chiasm and the medial mammillary bodies and transferred to a recording chamber. Slices used in dye-coupling experiments were placed in a static bath (Hatton et al., 1980), whereas determinations of the effects of cyclic nucleotides on membrane conductance were made using a perifusion chamber (Hatton et al., 1983). In either case, slices were maintained at 34–36°C in medium gassed with 95% O_2–5%CO_2. Control medium composition was (in mM): NaCl 126, $NaHPO_4$ 1.3, $NaHCO_3$ 26, KCl 5, $CaCl_2$ 2.4, $MgSO_4$ 1.3, glucose 10, and 3[*N*-morpholino] propanesulfonic acid buffer 5, pH 7.4. The combination of this organic buffer and $NaHCO_3$ has been found in previous studies to better stabilize the pH over prolonged recording sessions than does the use of the bicarbonate buffer alone. Recording electrodes were glass micropipettes filled with 3% Lucifer yellow (LY) (Stewart, 1978) in 0.25 M Li acetate, pH 7.3, and had resistances of 80–180 MΩ. Extracellular electrical stimulation was delivered through concentric bipolar electrodes (MCE 100; Rhodes Medical Instruments, Woodland Hills, CA) using constant current. In some experiments aimed at defining the ionic nature of the fast IPSPs, recording electrodes were filled with guanosine 5′-*O*-(2-thiodiphosphate) (GDP-βS).

As in our previous coupling studies (Andrew et al., 1981; Cobbett and Hatton 1984; Yang and Hatton 1988; Hatton and Yang, 1994, 1996), the following precautions were taken to prevent spurious coupling: (1) only one neuron per SON was recorded and injected, (2) brief pulses of positive current were used in making impalements, and (3) penetrations were terminated if the action potential amplitude fell below 40 mV during the LY injections. Intracellular impalements were not attempted until after an incubation period of 3 hr had elapsed. LY was injected using pulsed negative currents (200 msec pulses at −0.1 to −0.3 nA) for 2–3 min. In experiments involving electrical stimulation, an SON neuron was impaled, and its spontaneous activity and response to stimulation of the TM were determined. Only neurons displaying nonphasic spontaneous firing and in which TM stimulation evoked IPSPs, i.e., putative OX neurons, were included in the coupling portion of this study. These cells were either subjected to synaptic input arising from TM stimulation for ≥ 5 ≤ 10 min at 10 Hz and then LY injected for ~3 min or, in the case of controls, simply recorded for 10 min and then LY injected. TM stimulation parameters were 20–100 μA, 0.1 msec. In all experiments, one-half of the slice received the experimental treatment, and the contralateral half was subjected to control procedures. In coupling experiments involving manipulation of the medium composition, a cell in one-half of the slice was LY injected, and then that half was removed and stored in the same medium until the other half slice had been treated and injected. After an additional delay of ~30 min, both halves were placed in buffered 4% paraformaldehyde fixative for 2 hr and then transferred to Tris-buffered saline overnight. In our previous studies, no relationship has been found between incidence of coupling and time from the LY injection to the fixation of the slice. The slices were then ethanol-dehydrated, cleared in methyl salicylate, and mounted on glass slides. Incidence of dye coupling in cleared slices was determined under epifluorescence (see Fig. 1). Statistical analyses of coupling incidence were done using Fisher's exact probability test. To facilitate direct comparisons, all methods and procedures used in this study were made as similar as possible to those used in our investigation of synaptically released HA on VP neurons (Hatton and Yang, 1996).

After the determination of the incidence of LY coupling, a sampling of 28 slices was treated immunocytochemically for identification of the injected neurons. Of these, 23 slices contained cells that met our electrophysiological criteria for OX neurons. Cells in the remaining five slices, which did not meet these criteria, i.e., were putative VP neurons and were treated as immunocytochemical controls. Slices were removed from methyl salicylate, rehydrated, and stored at 4°C overnight in 0.1 M phosphate buffer with 30% sucrose. Frozen sections (16-μm-thick) were cut on a cryostat. Those sections with LY-filled cells were rinsed in 0.1 M PBS at pH 7.3 and then incubated in 10% normal horse serum in 0.01 M PBS with 0.02% Na azide and 0.3% Triton X-100 for 1 hr at room temperature. Sections were then incubated in primary antiserum in 0.1 M PBS at 4°C for 72 hr: mouse anti-oxytocin-neurophysin or mouse anti-vasopressin-neurophysin (PS38 or PS41, respectively; Dr. H. Gainer, National Institutes of Health, Bethesda, MD) at a dilution of 1:3000, containing 0.02% Na azide, 0.3% Triton X-100, and 1% normal horse serum. After a PBS wash, sections were treated with horse anti-mouse IgG at 1:200 in PBS with 0.02% Na azide for 3 hr at room temperature. Sections were then incubated in 1:200 Texas Red-conjugated streptavidin (Vector Laboratories, Burlingame, CA) with 0.1% Triton X-100 in 0.1 M PBS for 2 hr and rinsed three times in PBS. The sections containing injected cells were mounted on glass slides, cover glassed in Aqueous/Dry mounting medium GEL/MOUNT (Biomeda Corp, Foster City, CA), and visualized under epifluorescence microscopy.

Effects of cAMP and 8-bromo-cAMP on membrane potential and membrane conductance were studied using conventional pulsed hyperpolarizations. In slices from nine male rats, a total of 16 neurons, including both putative OX and VP neurons, was examined in these experiments. Measurements of input conductance were made by passing brief hyperpolarizing current pulses (100–250 pA, 100 msec) through the recording electrode at rates from 0.6 to 1 Hz. Recordings were made using the bridge circuit of a Neurodata (New York, NY) Dual Intracellular Amplifier. Resting potentials were determined both at the time of entering and exiting the cell.

Drugs and other compounds. Except where otherwise specified, compounds were dissolved in medium and bath-delivered to the recording chamber. Drugs used were as follows: Rp-adenosine 3′,5′-cyclic monophosphothioate triethylamine (Rp-cAMPs), an inhibitor of activation by cAMP of cAMP-dependent protein kinase A (PKA), and Sp-adenosine 3′,5′-cyclic monophosphothioate triethylamine (Sp-cAMPs), activator of PKA (both from Research Biochemicals, Natick, MA); cAMP; 8-bromo-cAMP; the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX); pertussis toxin; pyrilamine, an H_1 receptor antagonist; the H_2 antagonists cimetidine and famotidine; and clobenpropit dihydrobromide, an H_3 receptor antagonist (Tocris Cookson, Ballwin, MO). GDP-βS was from Research Biochemicals and Sigma (St. Louis, MO); LY-CH and all other compounds were from Sigma.

RESULTS

Neuronal characteristics

A total of 173 SON neurons from 99 male rats was recorded and injected with LY. The membrane characteristics of the cells included here were as follows: resting membrane potentials, −56.9 ± 1.3 mV; action potentials, 63.5 ± 2.4 mV; and input resistances, 134 ± 7.2 MΩ (means ± SEM for all measures). These values are similar to those reported in a host of studies on SON neurons.

LY-filled single and dye-coupled cells are shown in Figure 1. Only well filled neurons, such as those in Figure 1, *B* and *D*, were accepted as single, uncoupled cells. As has been observed repeatedly in studies of coupling among SON neurons, the dye transfer occurs through the dendrites. From the total of 28 immunocytochemically treated slices, 15 of 15 injected cells meeting the electrophysiological criteria were positively identified as OX neurons. Five injected cells not meeting these criteria (and not otherwise included in this study) were immunostained with VP-neurophysin antiserum, and the remaining eight cells were lost in histological processing.

Analysis of fast synaptic responses

The neurons selected for this study displayed the so-called "fast continuous" pattern of spontaneous activity (Fig. 2*A*) and re-

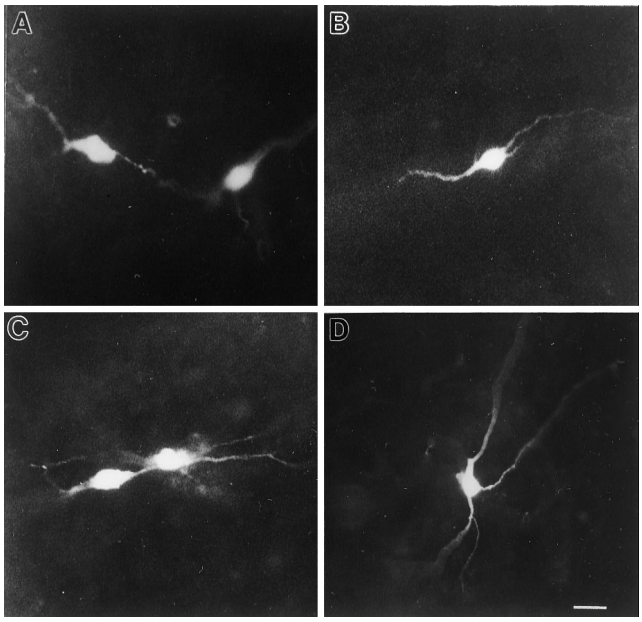

Figure 1. Fluorescence photomicrographs of LY-filled SON neurons. *A*, Coupled pair of neurons from a slice bathed in control medium and without TM stimulation. *B*, Single dye-filled neuron in a slice bathed in control medium and subjected to TM stimulation for 5 min at 10 Hz. *C*, Dye-coupled cells in a slice bathed for 30 min in medium containing 2 μm cimetidine (H_2 antagonist) with TM stimulation for 10 min at 10 Hz. *D*, Single dye-filled cell in SON after administration of 10 μM IBMX for 25 min. Scale bar, 45 μm.

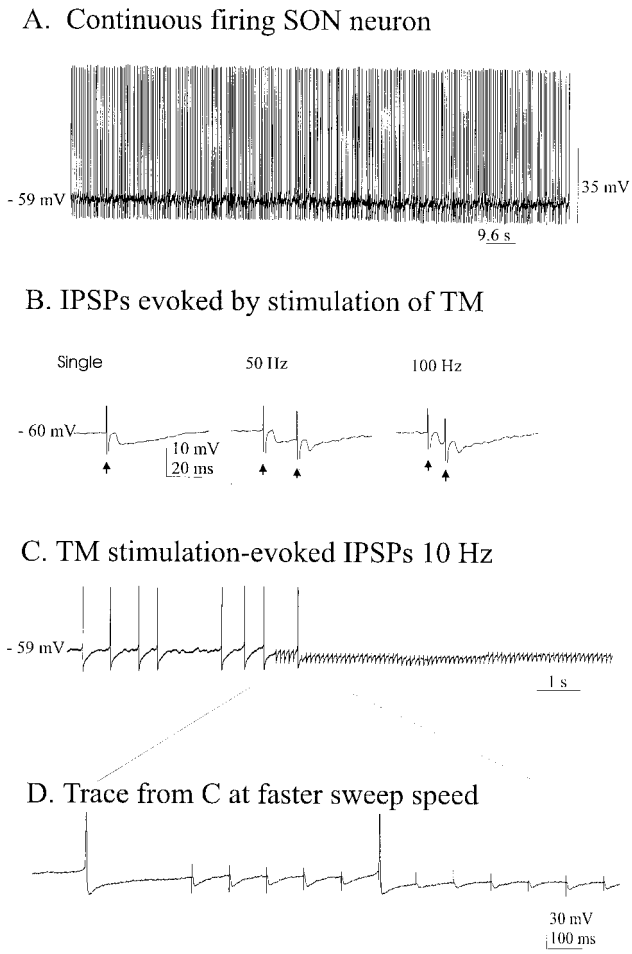

Figure 2. Effects of TM stimulation. *A*, Spontaneously occurring, continuous firing typical of oxytocin neurons included in this study. *B–D*, Synaptically evoked activity. Synaptic responses follow 50 and 100 Hz paired-pulse stimulation (*B*). *C*, Repetitive TM stimulation at 10 Hz. *D*, Segment of *trace* in *C* shown at faster sweep speed. Note that membrane potential hyperpolarized slightly and IPSPs became smaller after 600 msec of stimulation.

sponded to TM stimulation with IPSPs, after twin pulses delivered at 50 and 100 Hz ($n = 24$) (Fig. 2*B–D*). Stimulus-following at these frequencies has also been observed in response to longer trains (Yang and Hatton, 1994). These are characteristics that, in our hands, identify a large majority of OX neurons in the rat SON. In Figure 2, *C* and *D*, are shown typical responses ($n = 17$) to the repetitive 10 Hz stimulation used routinely in this study. As can be seen, ongoing spontaneous activity ceased, and the membrane potential hyperpolarized after six stimulus pulses. The sixth pulse immediately preceded the last spontaneous action potential, which seemed to have occurred during the synaptic delay period, obliterating the evoked IPSP (Fig. 2*D*). This ensuing prolonged hyperpolarization, perhaps revealing the onset of second-messenger effects, slightly reduced the sizes of the IPSPs that followed 600 msec of stimulation (Fig. 2*D*).

With hyperpolarizing current injection, the IPSPs evoked by TM stimulation reversed at approximately −70 mV, close to the chloride equilibrium potential (Fig. 3*A*,*C*). As shown in our previous study (Yang and Hatton, 1994) and not repeated here, lowering extracellular chloride concentration from 134 to 4.8 mM also reversed the IPSPs, and they were blocked by 20 μM picrotoxin but not by 10 μM bicuculline methiodide. These fast IPSPs could be blocked by the commonly used H_2 receptor antagonists cimetidine ($n = 10$) and famotidine ($n = 13$) (Fig. 3*B*,*D*), perhaps indicating nonspecific effects of these compounds.

That the TM stimulation-evoked IPSPs in the present study were also not $GABA_A$-mediated is shown in Figure 4*A3*, in which 10 μM bicuculline, a known blocking concentration in this system, was applied after testing in control medium (Fig. 4*A2*). Bicuculline failed to block the IPSPs. The possibility that OX cells in the SON express glycine receptors and that the synaptically released HA is effective in activating them was tested. IPSPs were evoked by TM stimulation in control medium and then with bicuculline, to which was then added 50 μM strychnine, a glycine receptor blocker (Fig. 4*A4*). All three of the cells tested in this way showed strychnine to be ineffective in blocking the evoked IPSPs. The responses of another of these cells are shown in Figure 4, *B1* and *B2*. These results indicate that synaptically released HA is not evoking chloride-mediated IPSPs by activating either $GABA_A$ or glycine receptors.

Although the kinetics of these IPSPs appear to be too fast to result from a second-messenger cascade, we further investigated this possibility. To more definitely rule out the possibility that these chloride channels were being opened via G-protein-mediated mechanisms, we first intracellularly injected the nonhydrolyzable GDP analog GDP-βS, which blocks G-protein pathways. To be certain that the GDP-βS was being delivered into the recorded cell, LY, a compound that also has a negative charge and a similar molecular weight (M_r 457 for LY vs 477 for GDPβS), was coinjected. Thus, electrodes were filled with 500 μM GDP-βS and 3% LY, and these were simultaneously injected with pulsed negative current. Results of separate dye-coupling experiments (given below) indicate that such GDP-βS injections were

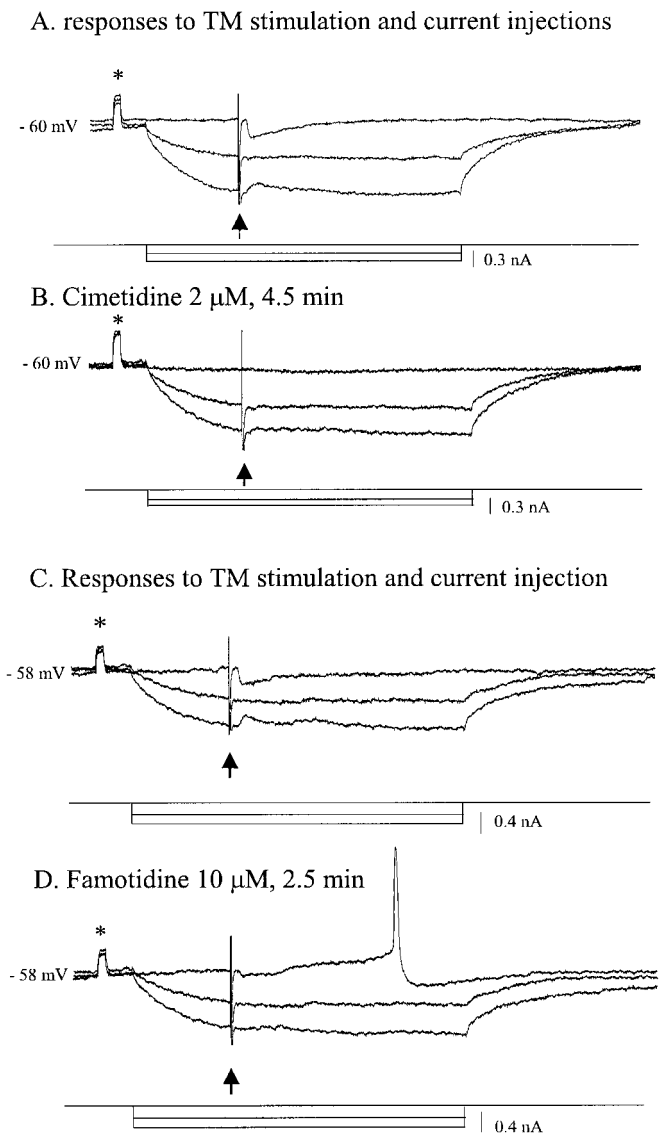

Figure 3. Reversal potentials and effects of histamine antagonists. *A*, Voltage traces from two different SON neurons (*A*, *B*, same cell as in Fig. 2; and *C*, *D*) showing IPSPs evoked by TM stimulation (*A*, *C*), their reversal by hyperpolarizing current injections, and their blockade by two different H_2 receptor antagonists (*B*, *D*). IPSPs reverse with membrane hyperpolarization (*A*, *C*). Reversal potential is close to E_{Cl-} (approximately −70 mV). *Asterisks* indicate calibration pulses: 10 mV, 5 msec.

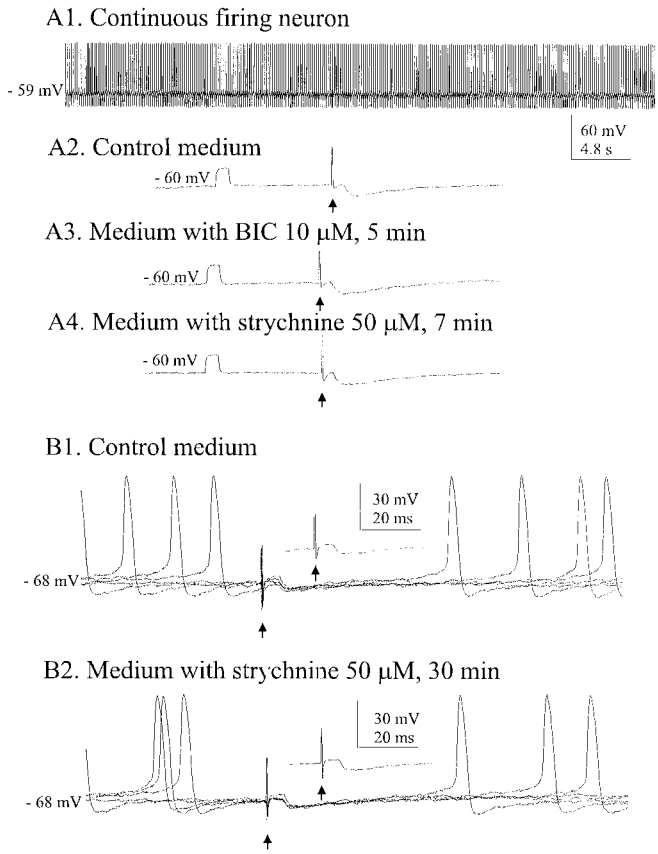

Figure 4. Effects of $GABA_A$ and glycine receptor blockade. *A1*, Spontaneous firing. *A2*, Synaptically evoked responses to TM stimulation in control medium. *A3*, IPSPs remain after 5 min bath application of bicuculline ($GABA_A$ receptor antagonist). *A4*, Addition of strychnine (glycine receptor antagonist) to the bath did not affect the evoked IPSPs. *A2–A4*, Five traces averaged in each case. *B1*, IPSPs evoked in another cell in control medium. *B2*, Prolonged bath application of strychnine affected neither the spontaneous nor the synaptically evoked activity.

effective in blocking the G-protein-mediated inhibition of coupling. After a control recording period during which IPSPs were evoked by TM stimulation, cells were injected for 3 min with GDP-βS–LY, and recording continued for at least 5 min. In Figure 5, the *gap* in the *top trace* represents the 3 min injection period. Although in each case these neurons were subsequently found to be LY-filled, in five of five injected cells, there was no effect on the IPSPs (Fig. 5, *insets below top trace*). HA delivered to the bath slowed and eventually inhibited spontaneous firing (*top trace*) but had no effect on the synaptic potentials (*left inset below middle trace*). Addition of the H_2 antagonist famotidine (10 μM), however, almost completely blocked the stimulus-evoked IPSPs (*right inset below middle trace*) and reinstated spontaneous firing. Washout reversed these effects (Fig. 5, *bottom trace* and *insets*).

In a separate series of six experiments, cells were first recorded in control medium for at least 5 min to establish the continuous firing pattern and the synaptic efficacy of TM stimulation (Fig. 6, *top trace* and *A*). Slices were then incubated in pertussis toxin (500 ng/ml) for 5 min, during which the IPSPs remained unchanged (Fig. 6*B*,*E*). Addition of the H_2 antagonists famotidine (three cells) or cimetidine (three cells) (Fig. 6*C*,*F*) blocked the IPSPs reversibly (Fig. 6*D*,*G*). Such a brief incubation with pertussis toxin might be expected to be ineffective in blocking G-proteins, but similar incubation times were found to be effective in eliminating the H_2-mediated effects on dye coupling (see below).

Finally, we tested the remote possibility that the IPSPs were somehow mediated by a second-messenger cascade that involved activation of PKA and that had escaped our other attempts to block the G-protein pathways. In five separate experiments, TM stimulation-evoked IPSPs were recorded in control medium. Then, 1 mM Rp-cAMPs, a membrane-permeant inhibitor of activation by cAMP of cAMP-dependent PKA, was added to the bath. Rp-cAMPs failed to block these IPSPs in any of the five cells tested. A typical example is shown in Figure 7. Although each of these three independent experimental approaches is imperfect, taken together with the dye-coupling data (below), the results strongly suggest that the IPSPs recorded in response to TM stimulation are not G-protein-mediated.

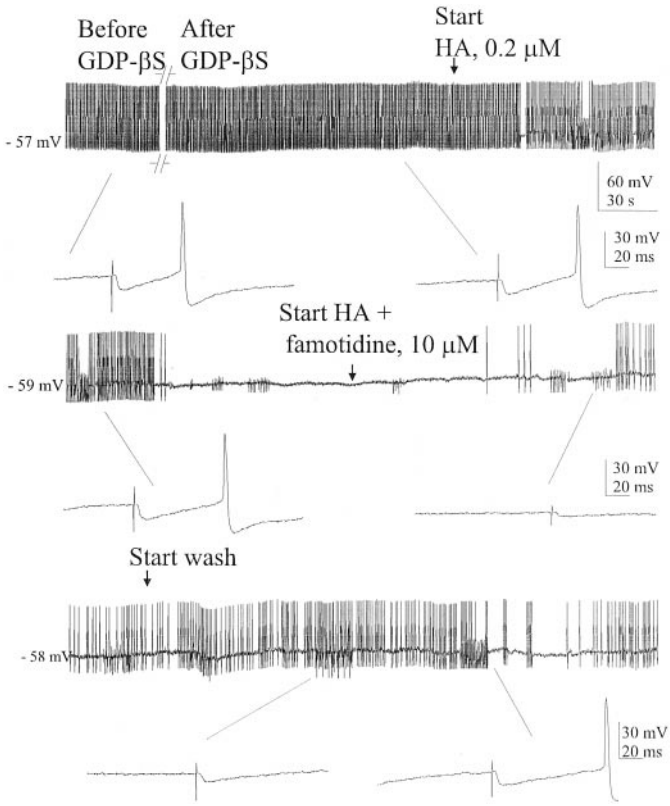

Figure 5. GDP-βS blockade of G-protein pathways. *Top trace*, Spontaneous activity before and after intracellular 3 min injection (-//-) of GDP-βS. No effect was observed on the IPSPs (*insets*), although HA inhibited firing. *Middle trace*, The H_2 receptor antagonist famotidine reinstated firing and blocked the IPSPs. *Bottom trace*, Washout reversed the effects.

TM stimulation effects on coupling

LY injections into 11 cells (one per SON) in unstimulated slices bathed in control medium yielded eight single and seven coupled cells, i.e., 0.64 coupled cells/injection (Fig. 8). In slices in which TM was stimulated for 5–10 min at 10 Hz, 15 injections yielded 14 single and two coupled cells, a greater than fourfold decrease from control in number of coupled cells per injection (i.e., 0.13). Both of the H_2 receptor antagonists used, cimetidine (2 μM) and famotidine (10 μM), prevented the effects of repetitive TM stimulation on OX cell coupling (23 injections yielded 16 single and 17 coupled neurons). These H_2 receptor antagonists did not, by themselves, exert any measurable effect on coupling in the absence of stimulation (Fig. 8). Figure 8 also shows that TM stimulation-induced coupling decreases were not affected by bathing slices in medium containing 5 μM pyrilamine, an H_1 antagonist (16 injections producing 15 single and two coupled cells), or 10 μM clobenpropit dihydrobromide, an H_3 antagonist (18 injections yielding 17 single and two coupled cells). Thus, coupling decreases appeared to be mediated by HAergic activation of H_2 receptors. In addition to the effects on the overall incidence of coupling, activation of H_2 receptors consistently limited the extent of the coupled network, as indicated by the numbers of coupled triplets. The 34 injections in control and H_2 antagonist-treated slices resulted in 12 cells coupled as triplets. In sharp contrast, no triplet coupling was observed after 49 injections in cells under stimulation conditions in which the H_2 receptors were not blocked. This result for triplets is significant at $p < 0.005$ by χ^2 analysis.

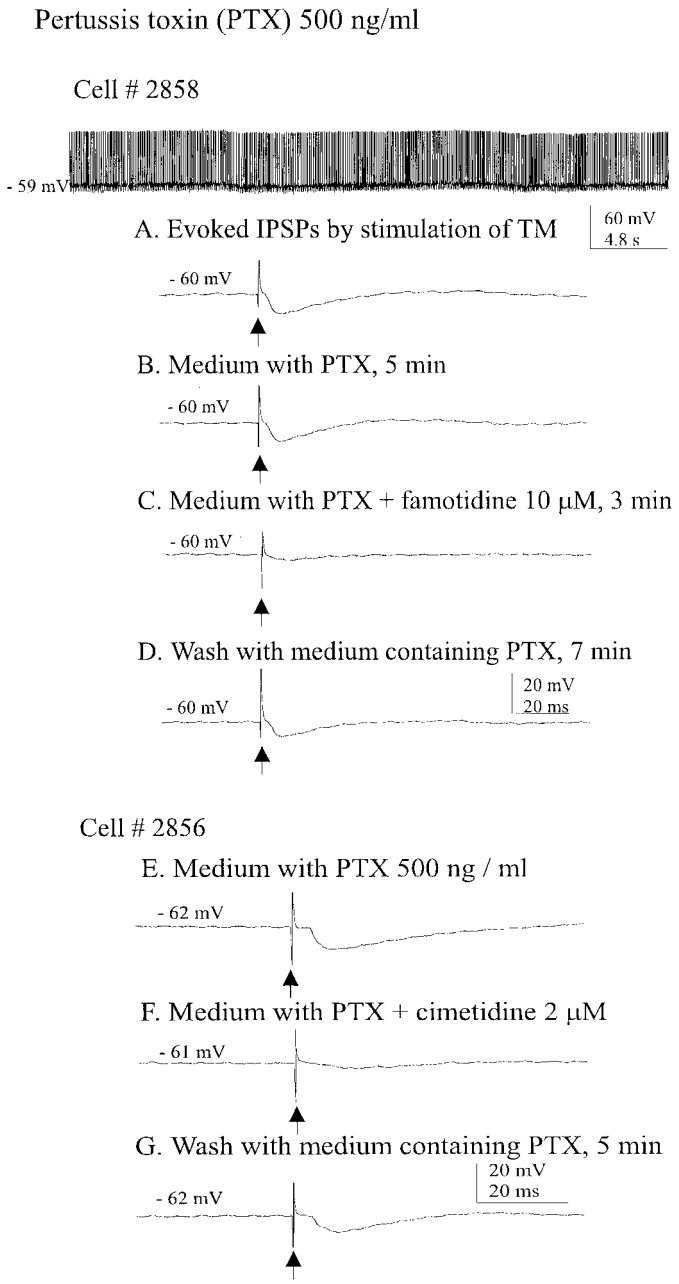

Figure 6. Pertussis toxin blockade of G-protein pathways. *Top trace*, Cell #2858. Spontaneous continuous firing. *A*, *B*, IPSPs in control medium and with pertussis toxin (*PTX*) after a 5 min incubation. *C*, IPSP blockade by the H_2 receptor antagonist famotidine. *D*, Washout of antagonist with medium containing PTX. *E–G*, Cell #2856. *E*, Evoked IPSPs in medium with PTX. *F*, Blockade of IPSPs by cimetidine. *G*, Reinstated upon washout with PTX-containing medium. *A–G*, Five traces averaged in each case.

Second-messenger effects on coupling

Because H_2 receptors are commonly linked to activation of adenylyl cyclase (AC), we tested the hypothesis that the observed uncoupling effects of HA released by repetitive synaptic activation on OX cell coupling were cAMP-dependent. On the *left side* of Figure 9, the *filled bars* present a comparison of nonstimulated slices maintained in control medium versus slices in medium containing 1 mM 8-bromo-cAMP. This membrane-permeant cAMP analog reduced coupling to one-third of the control value.

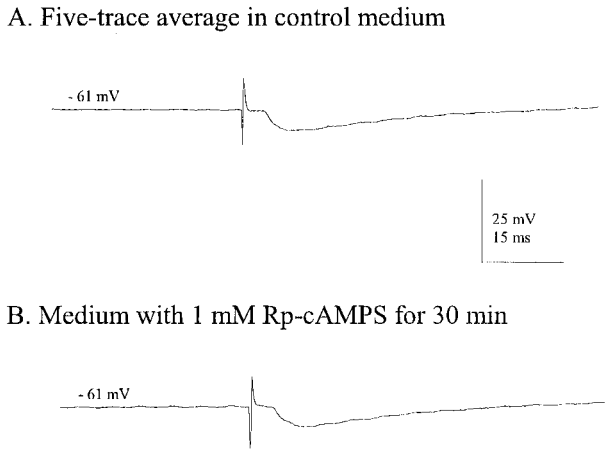

Figure 7. Effects of Rp-cAMPs inhibition of protein kinase A activation. *A*, TM stimulation-evoked IPSPs in control medium. *B*, After 30 min in medium containing Rp-cAMPs.

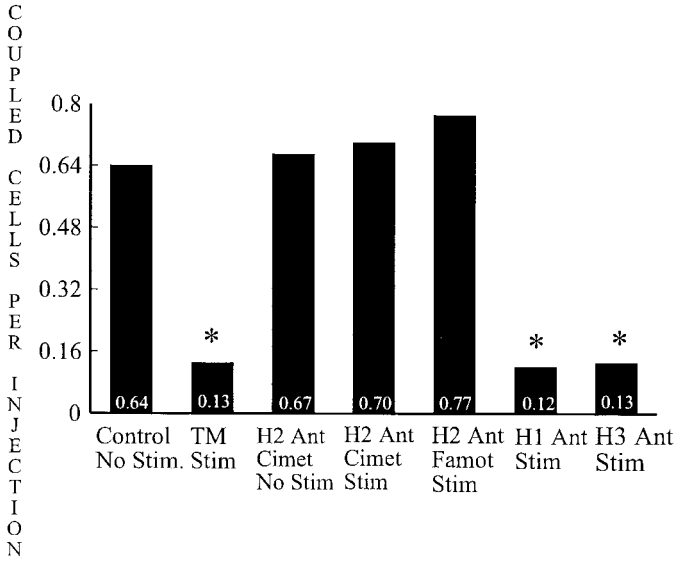

Figure 8. Comparisons of the incidence of coupling under a variety of experimental conditions. TM stimulation (*TM Stim*; $n = 15$ injections) reduced coupling to 20% of unstimulated control (*Control No Stim*; $n = 11$), an effect abolished by two different H_2 receptor antagonists, cimetidine (*Cimet*; $n = 10$), which had no effect by itself (*Cimet No Stim*; $n = 9$), or famotidine (*Famot*; $n = 13$). Neither pyrilamine (H_1 *Ant*; $n = 16$) nor clobenpropit (H_3 *Ant*; $n = 18$) was effective in preventing the effects of TM stimulation. * indicates significantly different at $p < 0.05$ to $p > 0.03$ from *Control No Stim*.

Also from nonstimulated slices, the data shown in the *center two bars* illustrate the effects on OX cell coupling of 100 μM Sp-cAMPs (activator of PKA) or 10 μM IBMX (a phosphodiesterase inhibitor that prevents the metabolism of cAMP). It can be seen that these two compounds caused decreases in coupling equal in magnitude to that induced by TM stimulation. For further comparison, the *bars* on the *right side* of Figure 9 present the data obtained when an inhibitor of PKA, Rp-cAMPs (100 μM), was added to the medium and the TM was stimulated as in controls. The uncoupling effects of stimulation and, therefore, of activating the H_2 receptors, was abolished by this treatment. Moreover, as shown in Figure 7, addition of Rp-cAMPs did not block the efficacy of synaptically released HA to evoke IPSPs, as did the H_2 receptor antagonists.

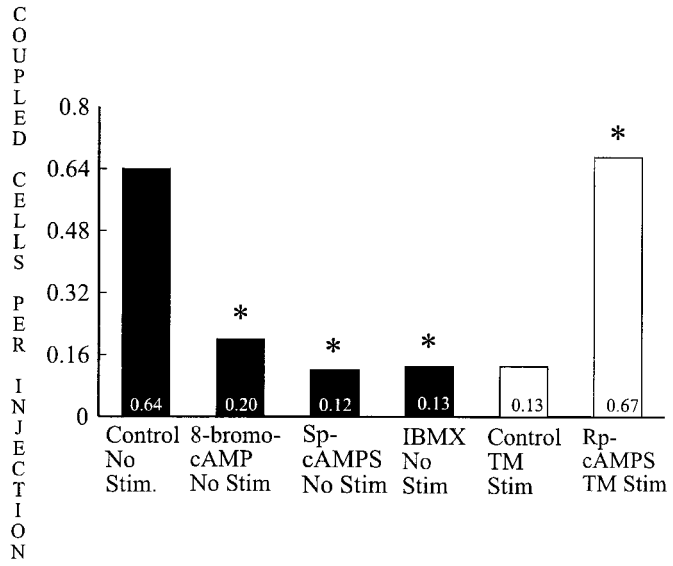

Figure 9. Comparison of the incidence of coupling for several groups of slices in which there was no TM stimulation (*No Stim*; *filled bars*) and for two stimulated groups (*TM Stim*; *open bars*). Bath application of the membrane-permeant cAMP analog 8-bromo-cAMP ($n = 20$), the activator of PKA, Sp-cAMPs ($n = 16$), or the phosphodiesterase inhibitor IBMX ($n = 15$) all reduced coupling to significantly below nonstimulated control levels. The effect of TM stimulation (*Control TM Stim*; $n = 15$) was abolished by bath application of Rp-cAMPs ($n = 9$), an inhibitor of activation by cAMP of cAMP-dependent PKA. * indicates significantly different at $p < 0.05$ to $p < 0.02$ from *Control No Stim* (for *filled bars*) or at $p < 0.04$ from *Control TM Stim* (for *open bars*).

Injection of 11 TM-stimulated cells with both GDP-βS and LY resulted in eight singles and seven coupled cells (two coupled pairs and one triplet) or 0.64 coupled cells per injection. This indicates that the injected GDP-βS was successful in blocking the G-protein-mediated coupling decrease caused by TM stimulation. A similar result was obtained for TM-stimulated cells treated with pertussis toxin. Ten LY injections resulted in seven singles and six coupled cells. Thus, both of these treatments were effective in blocking the second-messenger cascade but not the HA-mediated IPSPs. As in all other brain areas for which there are adequate data, coupling modulation is accomplished via G-protein–second-messenger actions (Hatton, 1998).

Cyclic nucleotide effects on membrane conductance

Eight continuously firing, putative OX neurons and eight phasically firing, putative VP neurons were exposed to bath applications of cAMP, 8-bromo-cAMP, or first one and then the other of these nucleotides under current-clamp conditions. Examples of the effects obtained are shown in Figure 10. No effects of the membrane-impermeant cAMP (Fig. 10*A*) were observed in any of the cells so tested ($n = 4$). All SON cells, regardless of peptide type, showed decreased membrane conductances with slight (1–3 mV) hyperpolarization of the membrane potential in response to the membrane-permeant compound 8-bromo-cAMP (Fig. 10*A*,*C*). As can be seen in Figure 10, these conductance changes were consistent but not large. When a steady positive current sufficient to hold the membrane at or near resting potential was injected through the recording electrode during 8-bromo-cAMP application, thereby preventing hyperpolarization, the decrease in membrane conductance was still observed in six of six cells tested (Fig. 10*D*). This indicates that the conductance change is not a simple consequence of membrane hyperpolarization.

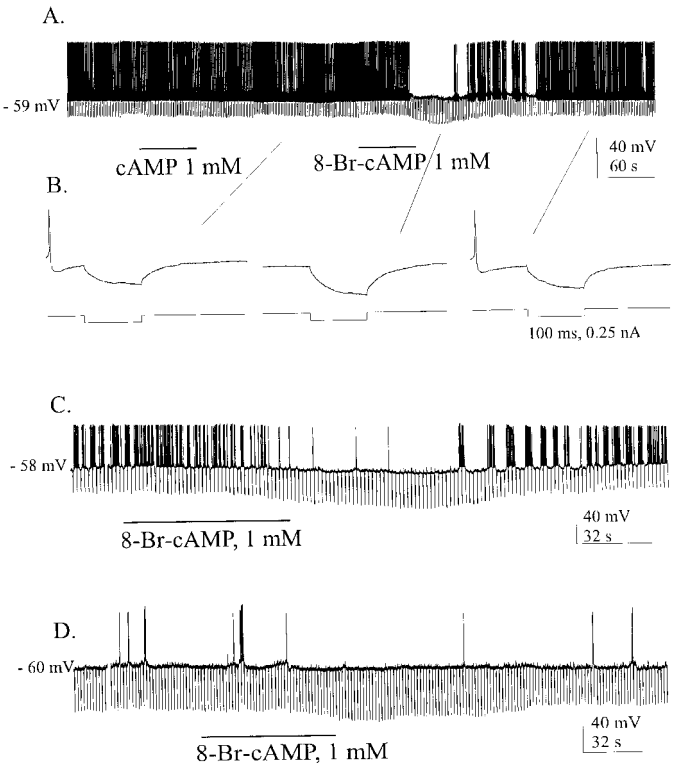

Figure 10. Effects of cAMP and 8-bromo-cAMP (*8-Br-cAMP*) on membrane potential and conductance in continuously (*A*, *B*; $n = 8$) and phasically (*C*, *D*; $n = 8$) firing neurons. Pulses of hyperpolarizing current (detailed in *B*) were injected at 1 Hz to monitor conductance changes. *A–C*, Bath application of 8-bromo-cAMP, but not cAMP, resulted in membrane hyperpolarization and decreased conductance. *D*, Decreased conductance was also observed when the membrane was current clamped at ~1 mV below resting potential.

DISCUSSION

An ionotropic HA receptor?

Our results suggest that HA released from the terminals of TM neurons has two distinct effects on OX neurons of the SON: one ionotropically and one metabotropically mediated. The first effect, corroborating our previous suggestive findings, is that TM terminal depolarizations evoke fast IPSPs by opening chloride channels (Yang and Hatton, 1994; this study). The receptor mediating this effect is unknown, because the only established HA receptors are metabotropic (Hough, 1999). It could be argued that, because TM neurons express glutamic acid decarboxylase (GAD), they might release GABA from their axon terminals and that this observed, bicuculline-insensitive ionotropic effect might then be mediated by GABA acting on $GABA_C$ receptors or that they may release glycine, which could act on the recently demonstrated glycine receptors (Hussy et al., 1997), because picrotoxin but not bicuculline blocks these responses. Several factors rule out such explanations. The IPSPs recorded in this study all had kinetics similar to those of $GABA_A$ or glycine receptors (e.g., decay times of 50–60 msec) (Fig. 2), but they cannot be blocked by bicuculline or strychnine. $GABA_C$ receptors have prolonged decay times in comparison (150 msec) (Bormann and Feigenspan, 1995), so the kinetics argue against the $GABA_C$ receptor hypothesis. Moreover, electron microscopic immunocytochemical data showed that GAD is not located in the HA terminals and that the terminals immunoreactive for the HA synthetic enzyme histidine decarboxylase all formed asymmetrical synapses (Ericson et al., 1991). It was concluded therefore that GABA, if released at all by TM neurons, is unlikely to be released from terminals. Also, because the presence of $GABA_A$ receptors on both OX and VP neurons is well established (Randle et al., 1986; Fenelon and Herbison, 1995), any GABA released from HA neuronal terminals should activate those receptors, resulting in similar responses to TM stimulation in the two neuronal types, when in fact the responses are opposite. Furthermore, those responses would be bicuculline-sensitive. If the TM terminals released glycine, IPSPs instead of the observed EPSPs would be evoked in response to stimulation in VP neurons. This is not the case (Yang and Hatton, 1994; Hatton and Yang, 1996), and strychnine failed to block the IPSPs. It is also unlikely that the H_2 antagonists that blocked the IPSPs were acting presynaptically because these compounds have been shown to be ineffective in interfering with the EPSPs produced in VP neurons by synaptically released HA (Yang and Hatton, 1994). Even relatively fast metabotropically mediated responses, such as HA evoking Ca^{2+} release leading to Ca^{2+}-activated Cl^- conductances, that could result from H_2 receptor activation occur via G-protein pathways. SON neurons have not been shown to express such Cl^- channels (Hatton and Li, 1998). Finally, our accumulated evidence strongly suggests that these IPSPs are not G-protein-dependent. Our working hypothesis, then, is that synaptically released HA binds to an as yet uncharacterized receptor (maybe H_5) capable of activating a fast chloride conductance.

H_2 receptor effects

Because the HAergic neurons of the TM send axonal projections to most if not all brain regions, metabotropically mediated effects of HA on neuronal excitability have been studied in neurons of many brain areas. Examples are the thalamus (McCormick and Williamson, 1991), septum (Gorelova and Reiner, 1996), hippocampus (Selbach et al., 1998; Yanovsky and Haas, 1998), dentate gyrus (Manahan-Vaughan et al., 1998), and the hypothalamus (Armstrong and Sladek, 1985; Smith and Armstrong, 1996; Li and Hatton, 1996; Li et al., 1999). All of those studies used exogenous applications of HA to ensure receptor activation. In the present study and several previous ones (Yang and Hatton, 1989; 1994; Hatton and Yang, 1996), these metabotropically mediated effects were observed with repeated activation of the TM input, during which HA concentrations probably build up in the tissue to levels not dissimilar to those reached with exogenous applications of HA.

In the present study, repetitive TM stimulation at 10 Hz for 5–10 min resulted in a dramatic uncoupling of OX neurons. Identical stimulation protocols increase coupling among the VP neurons of the SON via H_1 receptors linked to guanylyl cyclase (Hatton and Yang, 1996). Our present findings indicate that this uncoupling of OX neurons is mediated by activation of H_2 receptors positively linked to AC, accumulation of cAMP, and activation of PKA. Blocking any one of these steps in this cascade prevented the stimulation-induced uncoupling of OX neurons. It is noteworthy that cAMP-related actions have been shown to decrease gap junctional coupling among neurons in many other brain areas. Examples are found in the nucleus accumbens, in which dopamine via D_1 receptors activates AC and uncouples the neurons, whereas D_2 receptor-mediated inhibition of AC increases coupling (O'Donnell and Grace, 1993). Similar effects of activating these dopamine receptor subtypes have been reported for coupling among neurons in the islands of Calleja (Halliwell and Horne, 1998), neocortical layers II/III (Rörig et al., 1995),

and retinal horizontal cells (Dong and McReynolds, 1991). The common finding in these studies was that increases in cAMP accumulation were associated with decreased coupling and the inverse, i.e., inhibition of AC, was found to increase coupling. Therefore, our present results strongly suggest that those transmitter inputs that effect AC activation would reduce the extent of the coupled network of SON neurons, in this case OX neurons. Conversely, the coupled network would be expanded by repetitive depolarization of terminals whose transmitters activate receptors linked to inhibition of AC. There are several known inputs to the SON, in addition to the HAergic one from the TM, that are capable of affecting cAMP-dependent cascades (Hatton, 1990), including norepinephrine, dopamine, serotonin, and GABA, via $GABA_B$ receptors. It was perhaps the activity of some of these inputs in our slices that may have provided a counterbalance for the known tonic activity of the TM neurons *in vitro* and thereby precluded our observing any increases in coupling in unstimulated slices treated with H_2 receptor antagonists. Finally, these differential effects on coupling appear to be achievable on a time scale of a few minutes. Such a short time course suggests that coupling and uncoupling are accomplished by gating junctional conductances (e.g., channel opening frequencies and/or durations) rather than by adding or subtracting connexons.

Cyclic nucleotide effects on excitability

In addition to the effects observed on cell–cell coupling, 8-bromo-cAMP, but not cAMP, in the medium led to membrane hyperpolarization and decreased membrane conductance in both OX and VP cell types. Thus, HA binding to H_2 receptors leading to activation of the AC–cAMP–PKA cascade would effect a prolonged reduction in OX cell excitability. It is of interest here that activation of the guanylyl cyclase–cGMP cascade (which is linked to H_1 receptors in VP cells) increases coupling, depolarizes membrane potentials, and increases membrane conductance in both types of SON neurons (Yang and Hatton, 1999). Thus, these two second-messenger pathways have opposing effects on neuronal coupling and excitability, and in each case the effects on coupling and excitability are in the same direction. On the other hand, synaptically released HA, which activates H_1 receptors on VP and H_2 receptors on OX neurons, has prolonged opposite consequences for these two subpopulations of SON neurons.

Possible functional roles

TM neurons have generally been found to be tonically active both *in vitro* (Haas and Reiner, 1988; Weiss et al., 1989; Llinas and Alonso, 1992; Yang and Hatton, 1997) and *in vivo* (Sherin et al., 1998), although they are apparently inhibited during sleep. GABAergic inputs from the lateral preoptic area appear to be the chief source of sleep-related inhibition (Sherin et al., 1996; Yang and Hatton, 1997). Their observed tonic activity, along with the data of the present study, suggest that the TM inputs to OX neurons of the SON are a source of tonic inhibition. Furthermore, this inhibition is expressed on both a brief time scale, by evoking IPSPs, and on a somewhat longer one attributable to the reduced excitability conferred by the activation of the cAMP cascade. One functional consequence emerging from tonic activity of TM neurons, then, would be differential excitation of VP and inhibition of OX neurons. There are well documented instances of physiological conditions in which these two peptides are differentially released, although it is not yet known exactly what role(s) HA might play. For example, VP is selectively released during hemorrhage (Wakerley et al., 1975). Conversely, during the milk ejection reflex associated with lactation, OX but not VP is released (Wakerley et al., 1973). It is interesting to note, in this regard, that the OX-mediated milk ejection reflex normally occurs, and is greatly facilitated, when the dam's EEG patterns indicate that she is in slow-wave sleep (Lincoln, 1969). This would be a time when TM neurons are inhibited, thus releasing a brake on OX neuronal activity while also reducing excitatory drive to VP cells. Our studies are so far unique in that we have been able to observe separately both the rapid effects of synaptically released HA and its second-messenger-mediated effects. Our data lead us to conclude that there are ionotropic as well as metabotropic HA receptors.

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