The Journal of Neuroscience, July 1993, 13(7): 2829–2836

Glutamate Neurons in Hypothalamus Regulate Excitatory Transmission

Anthony N. van den Pol[1] and Paul Q. Trombley[2]

Sections of [1]Neurosurgery and [2]Neurobiology, Yale University Medical School, New Haven, Connecticut 06510

The hypothalamus is the crucial part of the brain that regulates homeostasis throughout the body. It governs the endocrine and autonomic nervous systems, temperature, heart rate, emotional and motivational states, reproduction, energy and water balances, and circadian rhythms. In contrast to the prevailing belief that hypothalamic neurons use peptides, neuromodulators, or other slow-acting agents as their principal neuroactive substances, we present data indicating that the primary excitatory transmitter *released* by medial hypothalamic neurons is glutamate. This surprising new evidence is based on three converging approaches: Immunogold cytochemistry revealed that some hypothalamic neurons and their processes *in vitro* contained high amounts of immunoreactive glutamate. Ca^{2+} digital video imaging showed that cytoplasmic Ca^{2+} levels of cultured neurons, elevated because of spontaneous presynaptic release of a hypothalamic transmitter, were reduced by perfusion with the selective glutamate receptor antagonists cyano-2,3-dihydroxy-7-nitroquinoxaline and 2-aminophosphonovaleric acid. Electrophysiological analysis of whole-cell patch-clamp recordings from single and pairs of monosynaptically coupled hypothalamic neurons in culture showed that virtually all spontaneous and evoked EPSPs appear to be mediated by synaptic secretion of glutamate.

[Key words: neuroendocrine, mediobasal hypothalamus, excitatory amino acid transmitter, NMDA]

The presence of a large number of neurotransmitters and neuromodulators has been reported in the hypothalamus. Some of these substances were first isolated from hypothalamic tissue. Putative transmitters identified in the hypothalamus include dozens of peptides, dopamine, ACh, GABA, and glycine; many of these are presumed to be slow-acting neuromodulators (Swanson and Sawchenko, 1983; Renaud et al., 1985; Ganong and Martini, 1990). Some of these substances, for instance, GABA, have inhibitory actions, and the actions of others have been reported to be mixed. A widespread excitatory transmitter in the hypothalamus has not previously been identified. Excitatory neurons from other regions of the brain, for instance, the hippocampus, cortex, and cerebellum, release the amino acid glutamate as an excitatory transmitter (Storm-Mathisen et al., 1983; Iversen, 1984; Somogyi et al., 1986).

We have undertaken a series of converging experiments to test the hypothesis that glutamate is an endogenous transmitter of the medial hypothalamus. This report indicates not only that glutamate is released by hypothalamic neurons, but also that it appears to account for almost all the excitatory synaptic activity between hypothalamic neurons *in vitro*.

Materials and Methods

Neuronal culture. Rat medial hypothalamic neurons were prepared as previously described (van den Pol et al., 1992a). The hypothalamic tissue used was demarcated rostrally by the optic chiasm and caudally by the mammillary region. The area of the fornix and mammillothalamic tract served as the lateral boundary, and the top of the third ventricle served as the dorsal boundary. The medial hypothalamic area used included the anterior medial hypothalamus, suprachiasmatic, ventromedial, dorsomedial, arcuate, paraventricular, and periventricular nuclei. Neurons were incubated at 37°C for 45–60 min in low-calcium medium containing papain, and dissociated by gentle trituration. For imaging experiments and experiments examining high-frequency spontaneous electrical activity, cells were plated at a high density (25,000–50,000 cells/cm^2). For experiments that examined synaptic pairs or isolated excitatory postsynaptic potentials, neurons were plated at a low density (100–5000 cells/cm^2), which reduced the formation of polysynaptic connections. Neurons were plated on a monolayer of hypothalamic astrocytes cultured several days before the neuron cultures. Astrocytes were prepared on glass substrates for immunocytochemistry and for Ca^{2+} imaging, and on plastic for whole-cell electrical recording. Cells were grown in minimal essential medium not containing added glutamate or glutamine (GIBCO), supplemented with 10% fetal bovine serum (Hyclone) and with Serum Extender (Collaborative Research) in a Napco 6100 incubator at 37°C with 5% CO_2. Tissue was obtained from rat hypothalami over a period ranging from embryonic day 16 (E16) to postnatal day 1 (P1).

Immunocytochemistry. Cultured cells fixed with 3% glutaraldehyde were immunostained with antiserum against glutamate (van den Pol, 1991), followed by 10 nm colloidal gold particles adsorbed to goat anti-rabbit immunoglobulins. The size and detectability of the gold particle were increased with silver intensification as described elsewhere (van den Pol, 1985). Glutamate antibody specificity was tested with ELISA assays, immunodot blot, Western blots, and solid-phase adsorption. The antibody against glutamate binds to free glutamate fixed by glutaraldehyde, but not to other amino acids similarly fixed or to glutamate as part of a protein (van den Pol, 1991).

Synapsin immunofluorescence staining was done with synapsin antiserum (gift of Dr. P. De Camilli) and a rhodamine-conjugated secondary antibody (Boehringer-Mannheim) after formaldehyde fixation.

To gain access to both intracellular antigens, cells were treated with 0.3% Triton X-100. No immunostaining was seen in the absence of this detergent.

Calcium imaging. Video recordings were made with a Hamamatsu 2400 silicon-intensified target video camera after loading cells with 5 μM fluo-3 acetoxymethyl ester as described (van den Pol et al., 1990, 1992a). HEPES buffer was used for the video experiments (10 mM HEPES, 25 mM glucose, 137 mM NaCl, 5.3 mM KCl, 3 mM $CaCl_2$, 2 μM glycine, pH 7.4). Neurons on coverslips were placed in a chamber allowing

Received Sept. 25, 1992; revised Dec. 8, 1992; accepted Jan. 20, 1993.

We thank Dr. G. Shepherd for his generous support (P.Q.T.) and manuscript suggestions, and Dr. Y. Yang for help with tissue culture. Research support was provided by NIH 10174 and 16296 and AFOSR.

Correspondence should be addressed to Anthony N. van den Pol, Section of Neurosurgery, Yale University Medical School, 333 Cedar Street, New Haven, CT 06510.

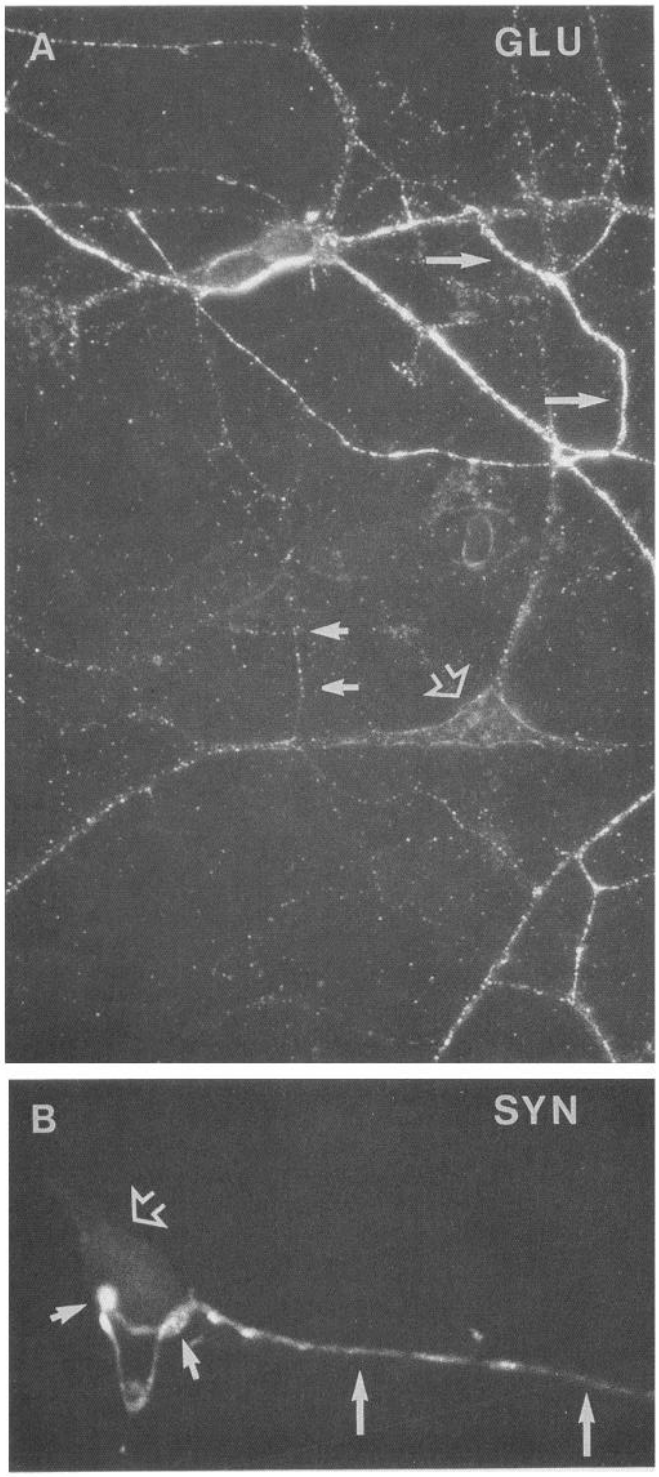

Figure 1. Immunocytochemistry. *A,* Some neurites in hypothalamic cultures show strong glutamate (*GLU*) immunoreactivity (*long arrows*) whereas other neurites (*short arrows*) and neuron perikarya (*open arrow*) show weak immunoreactivity. Underlying astrocytes show very little immunoreactivity. Dark-field micrograph of silver intensified immunogold staining. *B,* Fluorescent immunostaining was done with antiserum against synapsin (*SYN*), a protein associated with clear synaptic vesicles near synaptic specializations. After 13 d *in vitro,* a single immunoreactive axon (*long arrows*) terminates with several boutons (*short arrows*) on a very lightly labeled neuron (*open arrow*).

sequential perfusion of different substances on the stage of a Zeiss IM35 inverted microscope fitted with an Olympus 40× UV objective (numerical aperture, 0.85). Signals were averaged by an ITI 151 video processor interfaced with an IBM AT computer that also controlled a Uniblitz shutter to block light between video recordings. Images were saved on a Panasonic 2023 optical laser disk recorder. To reduce phototoxicity, fluorescent light was reduced to 1% with neutral density filters. To accommodate differences between cells related to gain and offset in the video field, loading with fluo-3, endogenous esterase activity, and cell thickness, data are represented as the change in fluorescence over the baseline fluorescence for each cell ($\Delta F/F$; vertical calibration in Fig. 2), thereby facilitating comparisons of cells in the same experiment (Cornell-Bell et al., 1990; van den Pol et al., 1990; Finkbeiner, 1991).

Whole-cell recordings. Electrophysiological recordings were made using an Axoclamp 2A amplifier as previously described (Trombley and Westbrook, 1990; Trombley and Shepherd, 1992). Patch electrodes were pulled from borosilicate glass and fire polished to a final resistance of 4–6 MΩ. Agonist-evoked currents were recorded under voltage clamp at a holding potential near −60 mV. Most postsynaptic potentials were recorded near −60 mV using small holding currents (<20 pA). The recording pipette contained 145 mM $KMeSO_4$ or CsCl, 5 mM $MgCl_2$, 10 mM HEPES, 4 mM Na-ATP, 0.5 mM Na-GTP, and 1.1 mM EGTA; pH 7.2, osmolarity 310. The recording chamber was perfused at 0.5–2.0 ml/min with a control solution containing 162.5 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose, and 1 μM glycine. Drugs were diluted in the recording solution and applied using a flow pipe perfusion system, consisting of an array of 400 μm internal diameter glass barrels aimed at the recorded cell. Neurons were continuously perfused from the barrel containing control solution except during drug delivery when a drug-containing barrel was activated. Agonist-evoked currents were filtered at 1–3 kHz, and digitized at 5–10 kHz. In current-clamped cells, voltages were filtered at 3 kHz, and digitized at 10 kHz.

Results

Glutamate immunoreactivity in vitro. Some cultured hypothalamic neurons showed strong glutamate immunoreactivity in the cell body and processes. Thin, nontapering processes with the appearance of axons were the most immunoreactive structures found. No specific immunostaining was seen with elimination of the primary antibody, or after solid-phase adsorption with glutamate conjugated by glutaraldehyde to a carrier protein not used for antiserum generation. On the basis of gold particles per unit area, these hypothalamic neurons showed 3-fold greater immunogold labeling per unit area than other neurons (Fig. 1*A*), and 11-fold greater labeling than underlying astrocytes. This relative difference in glutamate immunoreactivity is similar to that reported for putative glutamatergic neurons in other regions of the brain (Somogyi et al., 1986; Ottersen, 1989) and in the adult hypothalamus (van den Pol, 1991).

Calcium digital imaging. To determine whether an excitatory amino acid is secreted by cultured hypothalamic neurons, we used digital video imaging of intracellular Ca^{2+} with the dye fluo-3 to study hypothalamic neurons in high-density cultures. By 5 d in culture, initial synaptic contacts were made between cells in the culture dish, as detected with electron microscopy. Spontaneous release of hypothalamic transmitters set a baseline of intracellular Ca^{2+}. When the glutamate antagonists cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX; 5 μM) and 2-D,L-aminophosphonovaleric acid (AP5; 100 μM) were perfused over the cells, 18 of 23 neurons showed a reduction in Ca^{2+} levels as detected by the decrease in fluorescent intensity (Fig. 2*A*). When AP5 and CNQX were washed out, Ca^{2+} rose to the preantagonist baseline. The reduction and recovery to baseline levels of Ca^{2+} by the combination of CNQX and AP5 could be demonstrated during repeated perfusions of the antagonists.

Neurons in culture for 13 d showed a greater degree of spon-

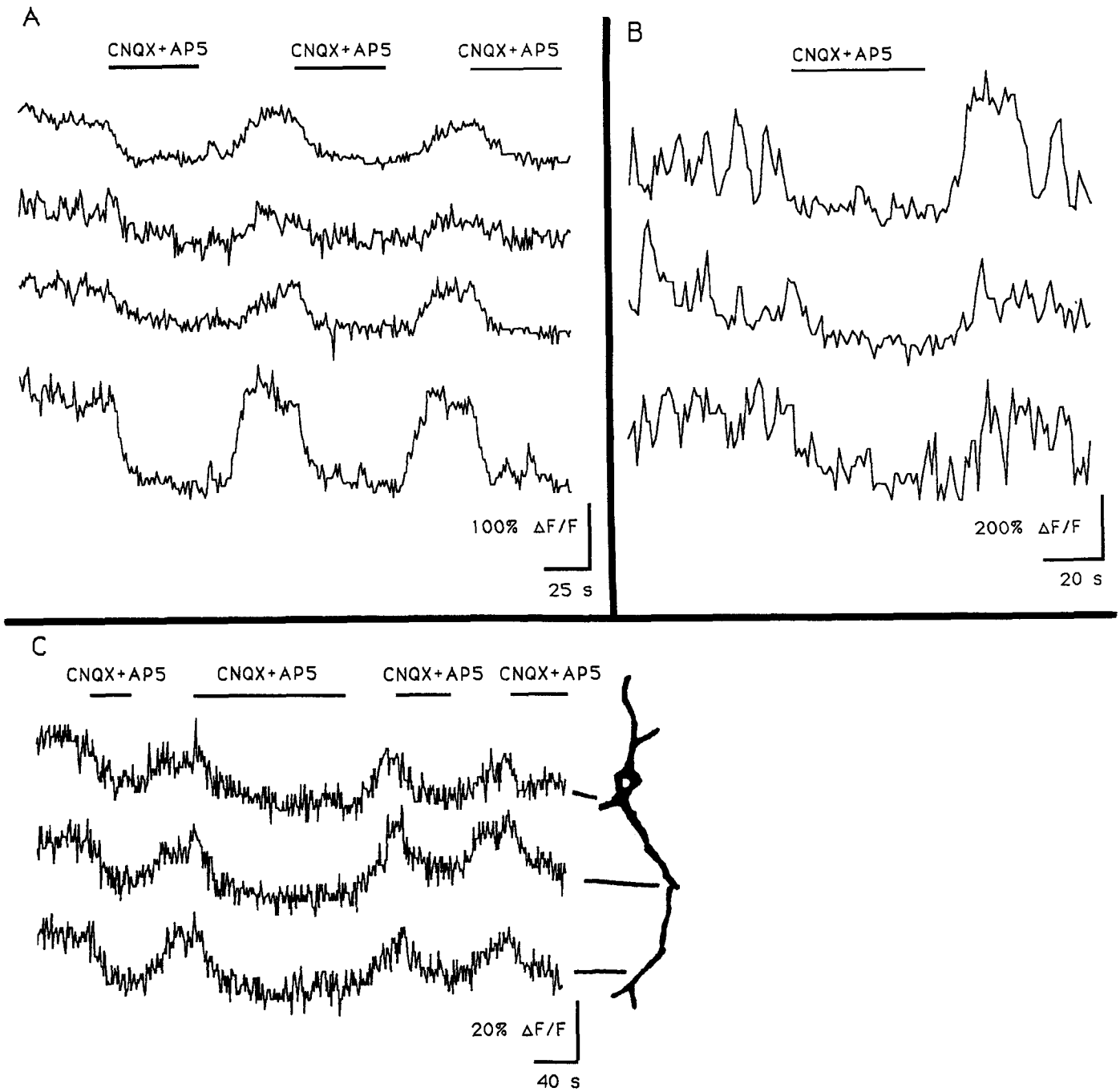

Figure 2. Ca^{2+} imaging. *A,* Repetitive addition of 3 μM CNQX and 100 μM AP5 to four medial hypothalamic neurons, each represented by a single trace, after 5 d *in vitro,* caused a reduction in intracellular Ca^{2+} as detected with fluo-3 fluorescence. Cellular fluorescence increased in the absence of the glutamate receptor blockers. The *bottom trace* shows the most dramatic response; the others show a smaller response. The calibration shows the time period in which recordings were made (*horizontal bar*) and the change in fluorescence over baseline fluorescence (*vertical bar*), as described in detail elsewhere (Cornell-Bell et al., 1990; van den Pol et al., 1990; Finkbeiner, 1991). In this experiment the long recording period resulted in photobleaching of the dye used, seen in the raw data as a slight decline in fluorescence over time in both cells that responded to CNQX and AP5 and those that did not. Data are shown after the average level of photobleaching over the entire experiment was assessed in responsive and nonresponsive cells and then subtracted from the traces shown here. *B,* Addition of 3 μM CNQX and 100 μM AP5 caused a decrease in three medial hypothalamic neurons cultured for 13 d. In many of the cells, the most dramatic effect of the CNQX + AP5 addition was the loss of some of the greater Ca^{2+} transients seen in these cells, for instance, in the top *trace* here. *C,* Three traces were taken from different regions of the dendritic tree of a single cell at successive distances from the cell body (see *drawing* of neuron at *right*). All regions of the dendrites showed a reduction of intracellular Ca^{2+} in response to bath-applied CNQX + AP5. The *top* and *bottom traces* were taken from regions on the dendrite 120 μm apart. Six additional traces from other regions of the same dendritic tree and cell body showed parallel responses to glutamate receptor blockers. All experiments were done in the presence of 3 μM glycine and the absence of Mg^{2+}.

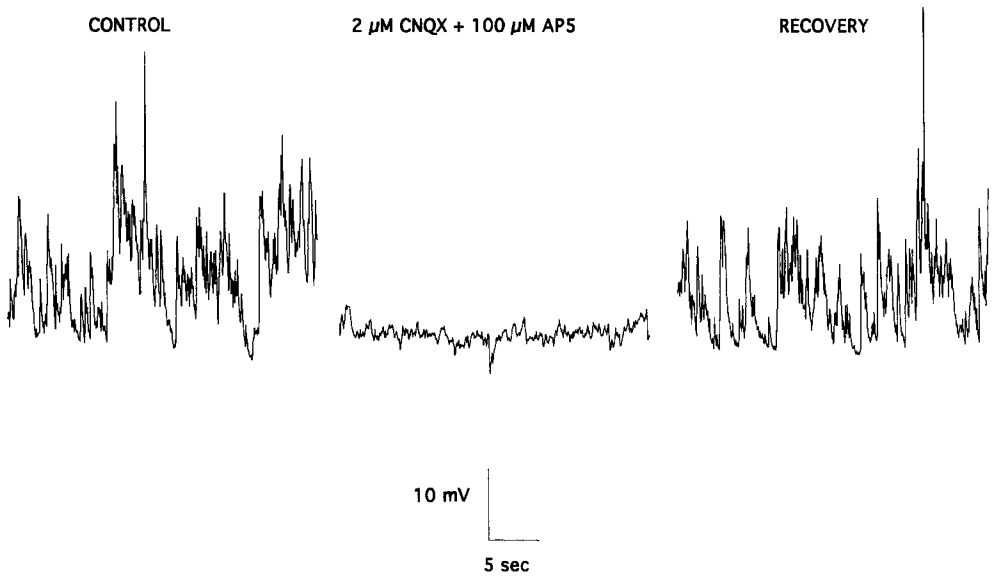

Figure 3. Glutamate antagonists block spontaneous EPSPs. Whole-cell recording from a hypothalamic neuron in high-density primary culture showed a high frequency of spontaneous EPSPs, which was reversibly blocked by application of CNQX (3 μM) and AP5 (100 μM). These spontaneous EPSPs often overlapped to generate large depolarizations (up to 30 mV), sometimes resulting in action potentials.

taneous irregular transients of Ca^{2+} than did the cells after 5 d *in vitro*. Perfusion with AP5 and CNQX reduced the amplitude of these Ca^{2+} oscillations, and the mean Ca^{2+} levels were reduced in 14 of 19 cells (Fig. 2*B*). Ca^{2+} responses to glutamate antagonists were found not only in the cell body, but also throughout the dendritic tree (Fig. 2*C*). Ca^{2+} increases and decreases in dendritic trees paralleled those of the parent cell body.

Whole-cell patch electrical recording. We further defined the identity of the excitatory hypothalamic transmitter using whole-cell electrical recording from medial hypothalamic neurons in cultures prepared from brains ranging in age from E16 to P1. Resting potentials varied from −58 to −72 mV. Lower resting potentials in neurons were likely caused by damage during attempts to make a seal, and were not used for further analysis. Spontaneous EPSPs were observed in 35 of 45 neurons examined. To test for the possibility of both slow and fast neurotransmission, time periods of 50 msec to 5 min were studied. In all cells tested (n = 12) in high-density cultures, nearly all the spontaneous EPSPs were blocked by flow pipe application of the non-NMDA glutamate receptor antagonist CNQX. The selective NMDA receptor antagonist AP5 also reduced spontaneous EPSPs but was much less effective. EPSPs were eliminated when the neurons were perfused with AP5 and CNQX together (Fig. 3).

The differential effects of AP5 and CNQX were further explored using isolated spontaneous EPSPs in very low-density cultures. Whole-cell recordings were made from neurons that had only one other neuron within a 400 μm radius. These spontaneous EPSPs were consistent in amplitude, duration, and shape, suggesting they were mediated by a single presynaptic neuron. Immunocytochemical examination of sister cultures, as described below, was consistent with monosynaptically coupling of pairs of neurons. Flow pipe application of AP5 blocked only a low-amplitude slow component of isolated spontaneous EPSPs consistent with the activation of an NMDA receptor–mediated event. In contrast CNQX blocked most of the EPSP, leaving only a low-amplitude, long-lasting potential in six of six cells examined (Fig. 4*A*). AP5 and CNQX together eliminated all the spontaneous EPSPs (not shown), similar to their effects on high frequency EPSPs (Fig. 3) and monosynaptically evoked EPSPs (Fig. 5). Although EPSPs were mediated primarily by non-NMDA receptors, flow pipe application of 100 μM NMDA evoked an inward current in 9 of 10 cells studied, suggesting that most hypothalamic neurons in culture do express NMDA receptors (Fig. 4*B*). These results suggest that hypothalamic neurons release a transmitter that produces a multiple-component EPSP through activation of both NMDA and non-NMDA receptors in the same neuron.

Dual-neuron electrical recording from synaptic pairs. Simultaneous whole-cell recordings between pairs of monosynaptically coupled hypothalamic neurons were used to identify further the endogenous excitatory transmitter (Fig. 5*A*). Cultures were grown at low densities to reduce the probability of polysynaptic connections between neurons. Immunostaining with antisera against synapsin, which has been shown to label presynaptic boutons and boutons en passant (De Camilli and Jahn, 1990), indicated that most neurons were contacted by only one (Fig. 1*B*), or sometimes two, presynaptic axons. Of 21 pairs examined electrophysiologically, only six were monosynaptically coupled. Intracellular stimulation of the presynaptic neuron evoked an EPSP with a fixed latency in the postsynaptic neuron (Fig. 5*B*). In all six coupled pairs the EPSP was eliminated by the combined application of AP5 and CNQX (Fig. 5*C*), parallel to the dramatic effects of these antagonists on spontaneous EPSPs. Subsequent recovery of the EPSP was found after removal of AP5 and CNQX from the perfusion buffer (Fig. 5*D*). To ensure that we were not recording at the reversal potential for an unidentified transmitter, we varied the holding potential from −80 mV to +10 mV. Regardless of the holding potential used, we did not observe excitatory synaptic potentials that could not be blocked by CNQX and AP5.

Glutamate regulation of inhibitory interneurons. Although not the primary focus of the present study, spontaneous IPSPs were frequently observed in high-density cultures. Similarly, evoked IPSPs were detected in an analysis of synaptically coupled neurons. In current-clamped cells these IPSPs reversed near the chloride equilibrium potential of −72 mV. We examined the possibility that glutamate-secreting neurons might regulate activity of inhibitory interneurons. In a probable synaptic triad, IPSPs with latencies varying between 4 and 8 msec were elicited

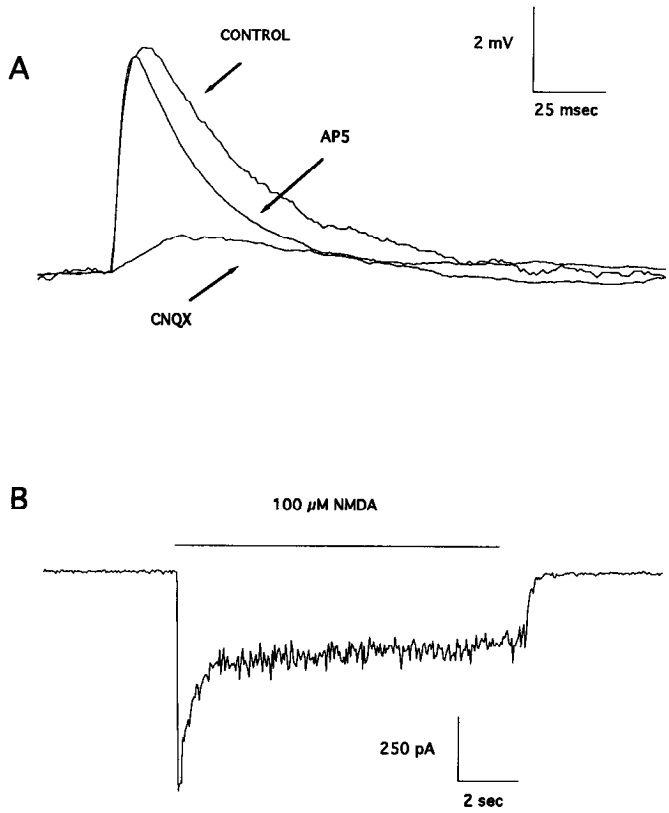

Figure 4. Relative contribution of NMDA and non-NMDA glutamate receptors. *A*, In isolated spontaneous EPSPs, AP5 reduced the duration of the EPSP with only a small reduction in amplitude. CNQX revealed a slow component consistent with the activation of NMDA receptors. EPSPs were superimposed, and reflect the average of three sweeps each. *B*, NMDA at 100 μM evoked a densensitizing inward current followed by a sustained component, and an increase in current noise.

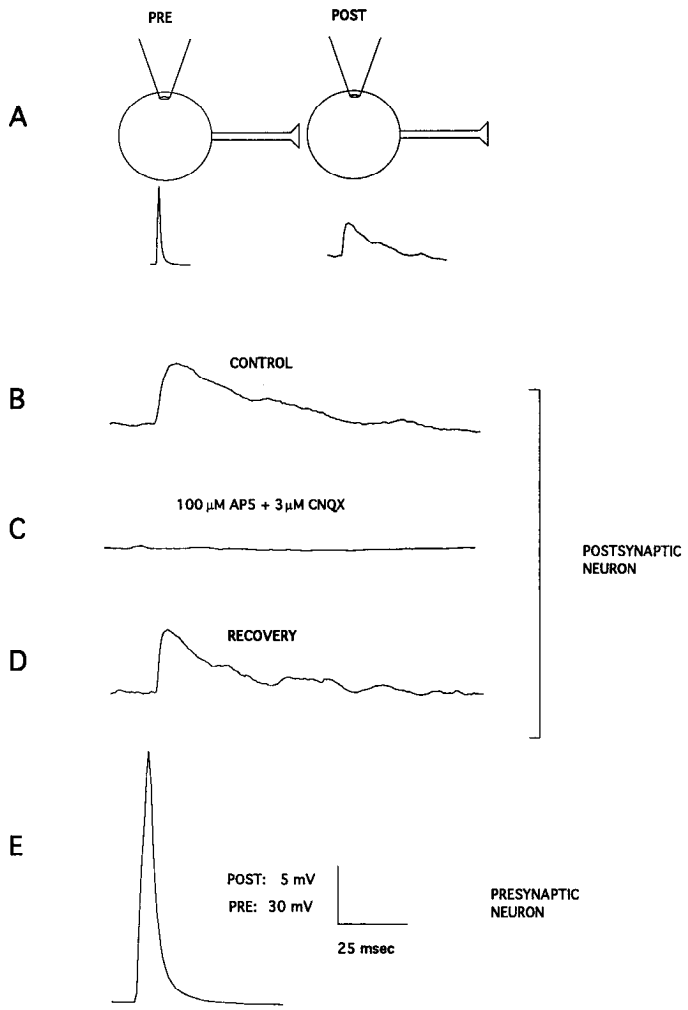

Figure 5. Glutamate mediates evoked EPSPs between monosynaptically coupled neurons. *A*, Recording configuration. *B*, Intracellular stimulation of the presynaptic neuron evokes an EPSP of fixed latency in the postsynaptic neuron. *C*, AP5 and CNQX blocked the EPSP. *D*, Washout and recovery. *E*, Induced presynaptic action potential. These EPSPs represent single traces from the recorded cell.

from the recorded cell (neuron 3) upon intracellular excitation of the stimulated cell (neuron 1). Addition of the glutamate antagonists CNQX and AP5 prevented excitation of an interneuron (neuron 2) as revealed by the block of the evoked IPSP (Fig. 6*A–E*).

Discussion

Immunocytochemical detection of a neuroactive substance in a neuron is one prerequisite for establishing that the substance is a transmitter in that cell. The immunocytochemical results with glutamate antisera suggest that some hypothalamic neurons in culture have high relative levels of glutamate. Given the high number of local axons and local collaterals of longer axons within the hypothalamus, these data suggest that the source of at least some of the axons in the adult hypothalamus that show strong glutamate immunoreactivity (Meeker et al., 1989; van den Pol et al., 1990; van den Pol, 1991) may originate from other neurons in the hypothalamus. Because there are no reliable immunocytochemical markers for neurons that release glutamate as a transmitter (Storm-Mathisen et al., 1983), it is difficult to determine which cells secrete glutamate, and which are immunoreactive for glutamate that is part of the metabolic pool not destined for release (Ottersen, 1989). To address the question of glutamate release, additional experiments were based on Ca^{2+} imaging and whole-cell electrical recording.

The Ca^{2+} imaging experiments suggest that normal excitatory synaptic interaction between cultured hypothalamic neurons is mediated, at least in part, by glutamate. Blocking glutamate action reduces the intracellular Ca^{2+} levels. Ca^{2+} transients seen in some neurons maintained in culture for about 2 weeks were reduced or eliminated with CNQX and AP5, suggesting that part of the episodic Ca^{2+} transient was due to intermittent release of glutamate from other hypothalamic neurons.

Glutamate-mediated intracellular Ca^{2+} levels could be regulated by several cellular mechanisms. Direct movement of Ca^{2+} could occur through ionotropic receptors such as NMDA-gated channels (Ascher and Nowak, 1986). Recent evidence suggests that Ca^{2+} may also enter neurons through certain specific combinations of AMPA- and kainate-type glutamate receptors (Hollmann et al., 1991; Hume et al., 1991; Verdoorn et al., 1991). Glutamate receptor–initiated depolarization could result in calcium influx through plasmalemma voltage-regulated calcium channels. Glutamate-mediated release of Ca^{2+} into the cytoplasm from intracellular stores could also be controlled by secondary messengers via a hypothalamic metabotropic receptor (Sortino et al., 1991).

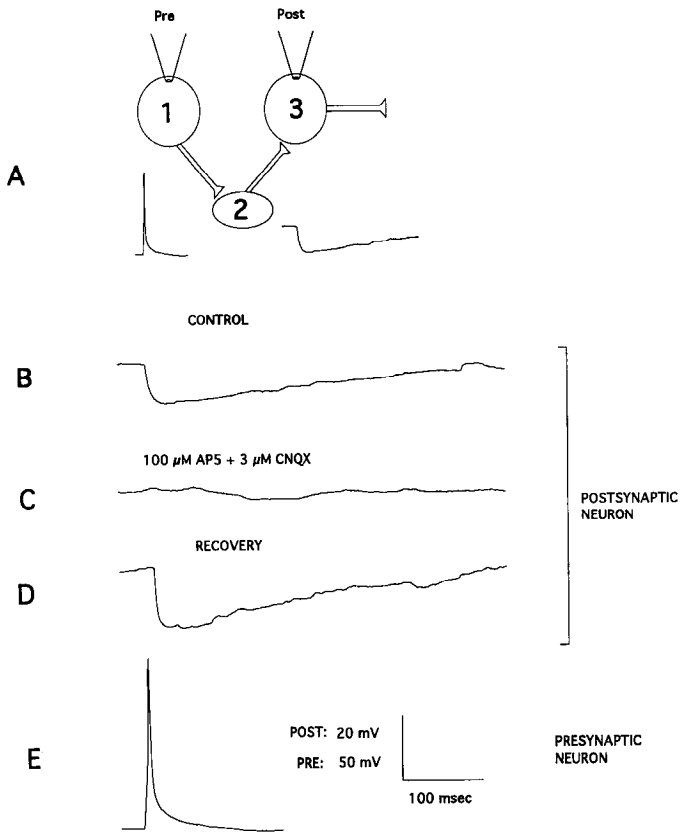

Figure 6. Glutamate mediates excitation of inhibitory interneurons. *A*, Recording configuration of a synaptic triad in culture consisting of an excitatory presynaptic neuron (*1*), inhibitory interneuron (*2*), and a neuron postsynaptic to the interneuron (*3*). *B*, Intracellular stimulation of an action potential in the presynaptic neuron (*1*) evoked a disynaptic IPSP of variable latency in the postsynaptic neurons (*3*) via the interneuron. *C*, Excitation of the interneuron was blocked by 100 μM AP5 and 3 μM CNQX, thereby preventing the IPSP in the postsynaptic cell. *D*, Washout and recovery. *E*, Induced presynaptic action potential.

The postsynaptic receptor physiology of spontaneous and evoked EPSPs indicates that the endogenous excitatory hypothalamic transmitter activates both non-NMDA and NMDA receptors. Among known excitatory transmitters, only glutamate mimics this response, as it alone activates non-NMDA receptors that can be blocked by CNQX (Mayer and Westbrook, 1984). Most EPSPs were due primarily to non-NMDA glutamate receptor activation. However, our results suggest that most hypothalamic neurons also express NMDA receptors. The large contribution of the non-NMDA component relative to the NMDA component in culture is similar to that found in slices of adult hypothalamus (van den Pol et al., 1990). This contrasts with the relatively large NMDA component of EPSPs in cultured hippocampus or olfactory bulb neurons (Forsythe and Westbrook, 1988; Trombley and Westbrook, 1990).

The physiological responses recorded in this study are unlikely to arise from slow release of glutamate from non-neuronal cells since the recorded neurons were always perfused with a fast flow from a barrel containing control solution or glutamate antagonists dissolved in the control solution. The control buffer contained no added glutamate, serum, or medium. In addition, evoked responses could arise only from synaptic release of a transmitter because they were phase locked with the presynaptic action potential with a fixed latency of about 4 msec.

The results in the present experiments suggesting glutamate is released from hypothalamic neurons are complementary to previous work examining glutamate receptors in medial hypothalamic cells. *In situ* hybridization studies show a widespread expression of NMDA (Moriyoshi et al., 1991) and non-NMDA glutamate receptor subtypes including glutamate receptors preferring both kainate and AMPA (van den Pol et al., 1992b). Using intracellular electrophysiology, we previously found that all adult hypothalamic neurons tested in the arcuate and paraventricular nucleus in an acute hypothalamic slice preparation had glutamate receptors (van den Pol et al., 1990). Supraoptic neurons also had glutamate receptors (Arnauld et al., 1983; Renaud and Bourque, 1991). In parallel, in hundreds of hypothalamic neurons tested in culture, almost all showed a Ca^{2+} rise in response to glutamate (van den Pol et al., 1990, 1992a; van den Pol, 1991).

Converging evidence supports the idea that glutamate is released not just from cultured hypothalamic neurons but also from presynaptic axons in the adult hypothalamus *in vivo*. Almost all of the excitatory response of hypothalamic neuroendocrine neurons to electrical stimulation of their afferent axons of undetermined origin could be blocked with the broad-spectrum glutamate receptor blocker kynurenic acid and with CNQX (Gribkoff and Dudek, 1990; van den Pol et al., 1990; Wuarin and Dudek, 1991). This was true of all cells tested with intracellular recording in hypothalamic slices ($n = 26$) in the paraventricular and arcuate nuclei (van den Pol et al., 1990).

Together, these data provide strong evidence that the primary excitatory transmitter released by medial hypothalamic neurons is glutamate. Whether release of additional neurotransmitters may be induced by conditions not found in our cultures remains to be determined. Glutamate, however, appeared to account for almost all excitatory activity examined in the millisecond to minute range, irrespective of hypothalamic age at dissection (E16–P1), period in culture (5–14 d), or cell density. Although peptides and other substances may be coreleased with glutamate, glutamate alone can account for most of the excitatory response. We found little evidence that any other fast-acting excitatory transmitter was operating in our medial hypothalamic tissue cultures.

In addition to fast excitatory transmission, fast inhibitory transmission was found in the hypothalamic cultures. IPSPs were frequently observed with a reversal potential suggestive of a GABA response. The adult hypothalamus has a high density of neurons (Tappaz et al., 1982) and presynaptic axons (Decavel and van den Pol, 1990) that are immunoreactive for GABA and the GABA-synthesizing enzyme glutamate decarboxylase. The elimination of IPSPs with glutamate blockers as described in this study indicates that inhibitory neurons are innervated and stimulated by glutamate-releasing excitatory neurons, in keeping with immunocytochemical data showing strong glutamate immunoreactivity in axons making synaptic contact with GABA-immunoreactive neuroendocrine neurons in the mediobasal hypothalamus (Decavel and van den Pol, 1992). The widespread distributions of GABA and glutamate provide further support for these amino acids having a multiplicity of roles in hypothalamic systems.

Many different neuroactive substances have been identified in the hypothalamus (see reviews by Swanson and Sawchenko, 1983; Ganong and Martini, 1990). Previous experiments have demonstrated that the neurotransmitters, neuropeptides, and receptors found in specific hypothalamic loci and in nonhy-

pothalamic loci *in vivo* are also found in neurons cultured from those regions (van den Pol et al., 1986; Wray et al., 1988; Murakami et al., 1991), suggesting that neurons retain their transmitter related phenotype *in vitro*. One possibility for the lack of demonstrable nonglutamate excitatory transmission may be that many of the putative neuroactive substances, rather than exerting a direct effect on electrical activity, may serve to modulate the action of other transmitters, particularly the amino acids. A clear example of this is found in the retina, where dopamine had no direct effect on membrane conductance of bipolar cells. However, when the glutamate agonist kainate was added in the presence of dopamine, the electrical response was several times greater than found in the absence of dopamine (Knapp and Dowling, 1987). A modulatory role has also been reported for neuropeptide Y (NPY) in the hippocampus. NPY had no direct effect on CA1 pyramidal cells. On the other hand, NPY produced a long-lasting reduction in the amplitude of monosynaptic EPSPs by modulating presynaptic glutamate release (Colmers et al., 1988). Other peptides have been shown to enhance release of glutamate, as described for tachykinins and calcitonin gene–related peptide (Kangrga and Randic, 1990).

The medial hypothalamus is generally perceived as operating in a relatively slow mode, governing homeostatic systems that exhibit relatively slow responses, as in the case of hormone release from endocrine glands. However, our results suggest that the hypothalamus is similar to other regions of the brain in that a fast-acting excitatory amino acid transmitter, probably glutamate, may be the primary neurotransmitter released by excitatory neurons. These results underline the probable importance of rapid excitatory neuronal communication in hypothalamic homeostatic and regulatory information processing.

References

Arnauld E, Cirino M, Layton BS, Renaud LP (1983) Contrasting actions of amino acids, acetylcholine, noradrenaline, and leucine enkephalin on the excitability of supraoptic vasopressin-secreting neurons. Neuroendocrinology 36:187–196.

Ascher P, Nowak L (1986) Calcium permeability of the channel activated by *N*-methyl-D-aspartate (NMDA) in isolated mouse central neurones. J Physiol (Lond) 377:35P.

Colmers WF, Lukowiak K, Pittman QJ (1988) Neuropeptide Y action in the rat hippocampal slice: site and mechanism of presynaptic inhibition. J Neurosci 8:3827–3837.

Cornell-Bell AH, Finkbeiner SM, Cooper MS, Smith SJ (1990) Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. Science 247:470–473.

De Camilli P, Jahn R (1990) Pathways to regulated exocytosis in neurons. Annu Rev Physiol 52:625–645.

Decavel C, van den Pol AN (1990) GABA: a dominant transmitter in the hypothalamus. J Comp Neurol 302:1019–1037.

Decavel C, van den Pol AN (1992) Converging GABA- and glutamate-immunoreactive axons make synaptic contact with identified hypothalamic neurosecretory neurons. J Comp Neurol 316:104–116.

Finkbeiner SM (1991) Calcium-mediated glutamate responses in cultured hippocampus. PhD thesis, Yale University.

Forsythe ID, Westbrook GL (1988) Slow excitatory postsynaptic currents mediated by *N*-methyl-D-aspartate receptors on cultured mouse central neurones. J Physiol (Lond) 396:515–533.

Ganong WF, Martini L (1990) Neuroendocrinology, 1990. Front Neuroendocrinol 11:1–5.

Gribkoff V, Dudek FE (1990) The effects of excitatory amino acid antagonists on synaptic responses of supraoptic neurons in slices of rat hypothalamus. J Neurophysiol 63:60–71.

Hollmann M, Hartley M, Heinemann S (1991) Calcium permeability of KA-AMPA-gated glutamate receptor channels depends on subunit composition. Science 252:851–853.

Hume RI, Dingledine R, Heinemann SF (1991) Identification of a site in glutamate receptor subunits that controls calcium permeability. Science 253:1028–1030.

Iversen LL (1984) Amino acids and peptides, fast and slow chemical signals in the nervous system? Proc R Soc Lond [Biol] 221:245–260.

Kangrga I, Randic M (1990) Tachykinins and calcitonin gene-related peptide enhance release of endogenous glutamate and aspartate from rat spinal dorsal horn slice. J Neurosci 10:2026–2038.

Knapp AG, Dowling JE (1987) Dopamine enhances excitatory amino acid–gated conductances in retinal horizontal cells. Nature 325:437–439.

Mayer ML, Westbrook GL (1984) Mixed-agonist action of excitatory amino acids on mouse spinal cord neurones under voltage clamp. J Physiol (Lond) 354:29–53.

Meeker RB, Swanson DJ, Hayward JN (1989) Light and electron microscopic localization of glutamate immunoreactivity in the supraoptic nucleus of the rat hypothalamus. Neuroscience 33:157–167.

Moriyoshi K, Masu M, Ishii T, Shigemoto R, Mizuno N, Nakanishi S (1991) Molecular cloning and characterization of the rat NMDA receptor. Nature 354:31–37.

Murakami N, Takamure M, Takahashi K, Utunomiya K, Kuroda H, Etoh T (1991) Long-term cultured neurons from rat suprachiasmatic nucleus retain the capacity for circadian oscillation of vasopressin release. Brain Res 545:347–350.

Ottersen OP (1989) Quantitative electron microscopic immunocytochemistry of neuroactive amino acids. Anat Embryol (Berl) 180:1–15.

Renaud LP, Bourque CW (1991) Neurophysiology and neuropharmacology of hypothalamic magnocellular neurons secreting vasopressin and oxytocin. Prog Neurobiol 36:131–169.

Renaud LP, Bourque CW, Day TA, Ferguson AV, Randle JC (1985) Electrophysiology of mammalian hypothalamic supraoptic and paraventricular neurosecretory cells. In: The electrophysiology of the secretory cell (Poisner AM, Trifaro J, eds), pp 165–194 Amsterdam: Elsevier.

Somogyi P, Halasy K, Somogyi J, Storm-Mathisen J, Ottersen OP (1986) Quantification of immunogold labelling reveals enrichment of glutamate in mossy and parallel fibre terminals in cat cerebellum. Neuroscience 19:1045–1050.

Sortino MA, Nicoletti F, Canonico PL (1991) Metabotropic glutamate receptors in the hypothalamus: characterization and developmental profile. Dev Brain Res 61:169–172.

Storm-Mathisen J, Leknes AK, Bore AT, Vaaland JL, Edminson P, Haug FM, Ottersen OP (1983) First visualization of glutamate and GABA in neurones by immunocytochemistry. Nature 301:517–520.

Swanson LW, Sawchenko PE (1983) Hypothalamic integration: organization of the paraventricular and supraoptic nuclei. Annu Rev Neurosci 6:269–324.

Tappaz ML, Oertel WH, Wassef M, Mugnaini E (1982) Central GABAergic neuroendocrine regulation: pharmacological and morphological evidence. Prog Brain Res 55:77–96.

Trombley PQ, Shepherd GM (1992) Noradrenergic inhibition of synaptic transmission between mitral and granule cells in mammalian olfactory bulb cultures. J Neurosci 12:3985–3991.

Trombley PQ, Westbrook GL (1990) Excitatory synaptic transmission in primary cultures of rat olfactory bulb. J Neurophysiol 64:598–606.

van den Pol AN (1985) Silver-intensified gold and peroxidase as dual ultrastructural immunolabels for pre- and postsynaptic neurotransmitters. Science 228:332–335.

van den Pol AN (1991) Glutamate and aspartate immunoreactivity in hypothalamic presynaptic axons. J Neurosci 11:2087–2101.

van den Pol AN, di Porzio U, Rutishauser U (1986) Growth cone localization of NCAM on central nervous system neurons *in vitro*. J Cell Biol 102:2281–2294.

van den Pol AN, Wuarin JP, Dudek FE (1990) Glutamate, the dominant excitatory transmitter in neuroendocrine regulation. Science 250:1276–1278.

van den Pol AN, Finkbeiner SM, Cornell-Bell AH (1992a) Calcium excitability and oscillations in suprachiasmatic nucleus neurons and glia *in vitro*. J Neurosci 12:2648–2664.

van den Pol AN, Hermans-Borgmeyer I, Heinemann S (1992b) Glutamate-receptor gene expression in hypothalamus: *in situ* hybridization with 7 receptor subtypes. Soc Neurosci Abstr 18:90.

Verdoorn TA, Burnashev N, Monyer H, Seeburg PH, Sakmann B (1991)

Structural determinants of ion flow through recombinant glutamate receptor channels. Science 252:1715–1718.

Wray S, Gahwiler BH, Gainer H (1988) Slice cultures of LHRH neurons in the presence and absence of brainstem and pituitary. Peptides 9:1151–1175.

Wuarin JP, Dudek FE (1991) Excitatory amino acid antagonists inhibit synaptic responses in the guinea pig hypothalamic paraventricular nucleus. J Neurophysiol 65:946–951.