Glutamate, the Dominant Excitatory Transmitter in Neuroendocrine Regulation

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Glutamate has been found to play an unexpectedly important role in neuroendocrine regulation in the hypothalamus, as revealed in converging experiments with ultrastructural immunocytochemistry, optical physiology with a calcium-sensitive dye, and intracellular electrical recording. There were large amounts of glutamate in boutons making synaptic contact with neuroendocrine neurons in the arcuate, paraventricular, and supraoptic nuclei. Almost all medial hypothalamic neurons responded to glutamate and to the glutamate agonists quisqualate and kainate with a consistent increase in intracellular calcium. In all magnocellular and parvocellular neurons of the paraventricular and arcuate nuclei tested, the non-NMDA (non–*N*-methyl-D-aspartate) glutamate antagonist CNQX (cyano-2,3-dihydroxy-7-nitroquinoxaline) reduced electrically stimulated and spontaneous excitatory postsynaptic potentials, suggesting that the endogenous neurotransmitter is an excitatory amino acid acting primarily on non-NMDA receptors. These results indicate that glutamate plays a major, widespread role in the control of neuroendocrine neurons.

THE NEUROENDOCRINE HYPOTHALAMUS has been a fertile ground for the discovery of neuroactive substances. In the search for new transmitters one important classical neurotransmitter, glutamate, has practically been ignored with respect to its contribution to neuroendocrine regulation (*1, 2*). However, our data suggest that glutamate accounts for the majority of excitatory synapses in the neuroendocrine hypothalamus. We tested whether glutamate occurs in high concentrations in a subset of presynaptic axons; whether hypothalamic neurons respond to glutamate, to what extent, and through which receptor; and whether the postsynaptic response to the naturally released neurotransmitter can be blocked by glutamate antagonists.

Characterized rabbit antisera raised against glutamate (*3*) were used to label hypothalamic ultrathin sections with postembedding silver-intensified gold particles (*4, 5*). Immunoreactive axons were found in synaptic contact with dendrites and cell bodies in all regions of the neuroendocrine mediobasal hypothalamus examined, including the magnocellular and parvocellular paraventricular (Fig. 1), supraoptic, and arcuate nuclei. These regions represent the final common neuronal pathway regulating endocrine secretions. Serial ultrathin sections revealed that all cells (n = 14) studied in the paraventricular and arcuate nuclei received synaptic contact from axons exhibiting glutamate immunoreactivity (*6*). The postsynaptic neurons (n = 9) in the paraventricular nucleus were large (28 to 35 μm in diameter) magnocellular neurons containing large (200 nm) dense-core vesicles immunoreactive for oxytocin and vasopressin-neurophysin. Glutamate-immunoreactive boutons contained small, clear, round vesicles apposed to an asymmetrical synaptic specialization; dense-core vesicles, suggestive of peptide colocalization, were also found in the presynaptic boutons. The ratio of immunogold particles over axons to their postsynaptic dendrites was 5:1, a ratio similar to that found with immunogold staining in glutamatergic axons in other brain regions (*7–9*).

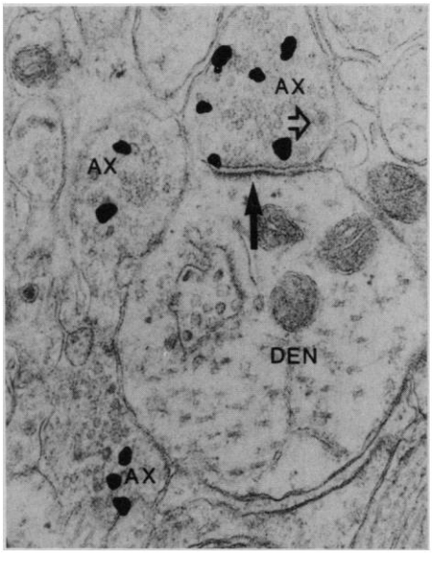

Fig. 1. Electron micrograph of glutamate-immunoreactive presynaptic bouton in the paraventricular nucleus making an asymmetrical synapse (black arrow) with a dendrite (DEN). The axon is filled with small round vesicles and with a few dense-core vesicles (open arrow). Two other axons (AX) also contact the same central dendrite in the paraventricular nucleus. The black dots are silver-intensified gold particles, which identify the three axons as containing high levels of glutamate. Width of micrograph, 0.95 μm.

Neurons responding to glutamate show an increase in intracellular Ca^{2+} (*10*), which mediates a wide variety of biochemical events in the cell. To study the Ca^{2+} response of neurons from the medial hypothalamus, we grew cells in monolayer primary tissue culture on glass cover slips (*11*); after the development of synaptic contacts, the response of neurons to glutamate was studied with the Ca^{2+} indicator dye fluo-3 by digital video microscopy (*12*). We examined the response of neurons to glutamate and related substances in a microscope perfusion chamber where glutamate and its agonists could be serially added to the perfusion medium. Nearly all neurons (93.1% of 522 cells) showed an increase in Ca^{2+} in response to glutamate (1 to 100 μM) (Fig. 2). Similarly, the glutamate agonists kainate and quisqualate induced a Ca^{2+} rise in almost all the cells (kainate: 90.1% of 121 cells; quisqualate: 92.8% of 168 cells). Cells that responded to one agonist generally responded to all three. A minor response to the glutamate agonist *N*-methyl-D-aspartate (NMDA) was seen in some cells.

To investigate the nature of the endogenous neurotransmitter, we used intracellular recording from coronal hypothalamic slices (500 μm) and tested whether the non-NMDA glutamate antagonist cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX) blocked the excitatory postsynaptic potentials (EPSPs) in the arcuate and paraventricular nuclei. If glutamate is an important endogenous transmitter in neuroendocrine circuits, EPSPs should be blocked by agents that block activation of the glutamate receptor. Bath application of 3 μM CNQX reduced, and 30 μM CNQX blocked almost completely, spontaneous EPSPs. Electrical stimulation lateral to the paraventricular nucleus or dorsal to the arcuate nucleus elicited EPSPs in the respective areas. Although many axons and pathways were electrically stimulated in these experiments, CNQX blocked EPSPs in a dose-dependent manner (Fig. 3) (*13*). This effect was observed in every cell of the 26 tested (20 in the paraventricular nucleus; 6 in the arcuate nucleus); at higher doses (30 μM) the EPSPs were virtually eliminated. Of the 20 cells in the paraventricular nucleus, 13 were determined to be neurosecretory on the basis of their electrophysiological properties (*14*). Kynurenic acid, a broad-spectrum glutamate antagonist, also reduced the amplitude of the EPSPs in the paraventricular nucleus, as had been previously reported in

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the supraoptic nucleus (*15*). Together, these data support the suggestion that most of the fast excitatory input to the arcuate and paraventricular nuclei is mediated by glutamate.

The widespread response to glutamate in almost all hypothalamic neurons examined with both optical and intracellular physiological techniques is in seeming contrast to the apparent low level of binding found in autoradiographic analysis of hypothalamic receptors (*16*). Similarly, a number of reports have shown that hypothalamic neurons appear relatively resistant to excitatory amino acid toxicity (*17*). We suggest that these results are caused not by the absence of glutamate receptors in the hypothalamus but rather by the lower number of receptors or to differences in second messenger systems. Although the hypothalamus has a lower density of glutamate-binding sites than cortical areas such as the hippocampus, neurotransmission mediated by glutamate may be equally important in the hypothalamus because neurons there tend to be less extensively branched (*18*), with smaller surface areas and higher input resistances; therefore, equal single-channel currents through a glutamate-activated channel would have a proportionately larger effect in a hypothalamic neuron than in a cortical one.

Although glutamate has been recognized as a major neurotransmitter in other brain systems such as the neocortex and hippocampus (*8, 9, 19*), investigations of the neural control of the endocrine system have focused on other possible neuroactive substances. On the basis of data from ultrastructural immunocytochemistry, intracellular electrophysiology, and optical digital physiology, we suggest that the major excitatory neurotransmitter regulating neuroendocrine neurons is glutamate. Our data argue that, not only does glutamate play an important role in neuroendocrine regulation, but also that it is probably the major excitatory neurotransmitter inducing release of neuroendocrine hormones from axons terminating in both the median eminence and the posterior pituitary. Combining our results on glutamate with work on the inhibitory transmitter γ-aminobutyrate acid (GABA) (*4, 20, 21*), we suggest that amino acid neurotransmitters account for the majority of all presynaptic axons involved in neuroendocrine regulation, greatly outnumbering amines, peptides, or other neuroactive substances. Since peptide colocalization may be found in most if not all cells with amino acid transmitters (*22*), this conclusion would suggest that glutamate and GABA would be responsible for the majority of fast excitatory and inhibitory synaptic potentials, while peptides and other neuroactive substances in the neuroendocrine hypothalamus may be coreleased. These other neurotransmitters could work synergistically and function at longer intervals, perhaps with longer lasting effects. Our data support a general hypothesis that glutamate is the major fast excitatory neurotransmitter that controls not only the neuroendocrine system but also other hypothalamic regions as well.

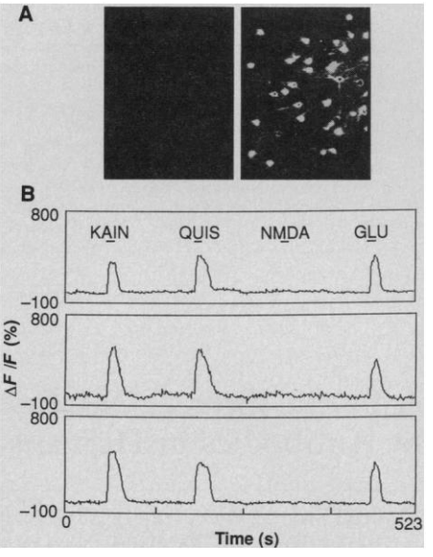

Fig. 2. Intracellular Ca^{2+} responses in cells from the neuroendocrine region of the mediobasal hypothalamus. The fluorescent Ca^{2+} indicator dye fluo-3 was used to study Ca^{2+} responses to the perfusion of glutamate and agonists in Hepes buffer (*12*). (**A**) Baseline fluorescence was subtracted from the video frame (*12*) before glutamate addition (left) and at the time of glutamate response (right). (**B**) Three cells responded to 10-s applications (short horizontal bars) of 10 μM kainate (KAIN), quisqualate (QUIS), and glutamate (GLU), but not to NMDA. All neurons (12 of 12) studied on this cover slip responded to glutamate, kainate, and quisqualate ($\Delta F/F$ range: 180 to 725%). Data are expressed as the percentage change in fluorescence over the baseline control fluorescence, with the baseline subsequently standardized as 0 for each neuron. At 100 μM, some cells responded to NMDA; this may in part be due to NMDA receptors or to binding of NMDA to a non-NMDA receptor. At 100 μM NMDA, hippocampal control neurons routinely responded to NMDA. Tetrodotoxin (1 μM) was used to block release of endogenous transmitter to ensure that only direct responses to glutamate agonists were detected. The data are presented as one averaged video frame per second. To avoid phototoxicity, a computer-controlled shutter blocked the light after each 350-ms recording. Sixty seconds after agonist application (vertical lines at bottom), cells were allowed to rest for 120 s in the dark with no recordings. Subsequent recording showed that the cells responded to 1 and 3 μM glutamate, and to an increase in extracellular K^+ (56 mM).

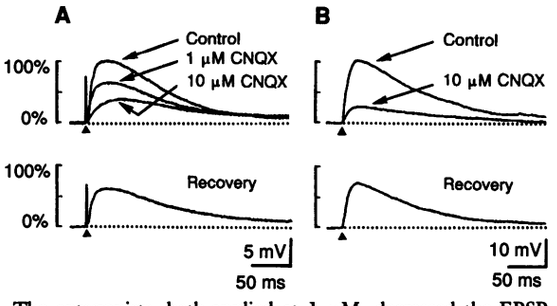

Fig. 3. Effect of the non-NMDA receptor antagonist CNQX on EPSPs in the (**A**) paraventricular nucleus (PVN) and (**B**) arcuate nucleus (ARC) in hypothalamic slices. Slices were cut with a Vibroslicer and transferred to a recording chamber where they were kept at 32°C, oxygenated with a mixture of 95% O_2 and 5% CO_2, and perfused with a standard artificial cerebrospinal fluid. Signals were amplified with an Axoclamp 2A and stored on videotape for subsequent analysis. The antagonists, bath-applied at 1 μM, decreased the EPSP amplitude by about 40% in PVN. At 10 μM, the EPSP amplitude was decreased by approximately 60% in PVN and 75% in ARC. Both cells were hyperpolarized to prevent action potentials. After more than 1 hour, recovery was obtained with 1 μM CNQX. Although nearly complete recovery could be obtained with low doses of CNQX (1 to 3 μM), total recovery from higher doses (10 to 30 μM) was never observed, even after washout periods of more than 90 min. The long-lasting effects of CNQX were probably due to its high binding affinity (*23*). Nonetheless, little or no change in resting potential or input resistance occurred during application of CNQX, even at the highest doses. To avoid the contamination of EPSP by a potential reversed inhibitory postsynaptic potential, the experiments were done in 50 μM picrotoxin. Each trace is an average of 20 responses. Stimulus intensity was 500 μA for PVN and 100 μA for ARC. Data from the two cells shown here are representative of data for 20 neurons from PVN and 6 from ARC examined in this study (▲, electrical stimulation).

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2. L. P. Renaud *et al.*, in *The Electrophysiology of the Secretory Cell*, A. M. Poisner and J. Trifaro, Eds. (Elsevier, Amsterdam, 1985), pp. 165–194.
3. Rabbit antisera against glutamate conjugated by glutaraldehyde to keyhole limpet hemocyanin were tested for specificity by enzyme-linked immunosorbent assay (ELISA) and immunodot blot. The antibody did not recognize any of 20 amino acids tested for cross-reactivity including aspartate, GABA, glycine, taurine, and glutamine, or the dipeptide *N*-acetylaspartylglutamate. On Western blots, the antibody did not bind to polyglutamate or to native

rat brain protein but did bind to glutamate cross-linked by glutaraldehyde to rat brain proteins. Solid-phase absorption of the antibody to glutamate conjugated to bovine serum albumin eliminated positive staining.

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6. The identity of 18 neurons from the arcuate and paraventricular nuclei as neuroendocrine was confirmed in another experiment in which cells projecting to the vascular system were labeled by retrograde axonal transport of intravenous injections of horseradish peroxidase [R. D. Broadwell and M. W. Brightman *J. Comp. Neurol.* **166**, 257 (1976)]. Presynaptic glutamate-immunoreactive boutons were found on each cell (C. Decavel and A. N. van den Pol, in preparation).
7. The number of gold particles per bouton varied with the concentrations of antisera. The ratio of labeling of highly immunoreactive to less reactive structures was similar over several antisera concentrations. The presence of high levels of glutamate in presynaptic boutons supports, but does not prove, the possibility that it is released. All neurons probably contain some glutamate. Those cells that use glutamate as a neurotransmitter appear to maintain higher concentrations of glutamate in their axon terminals than other cells do (*8, 9, 19*), as shown ultrastructurally by ratios of immunogold particles over different cells.
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11. Hypothalamic neurons from late embryonic or early postnatal rats were plated on glass cover slips treated with polylysine and collagen [A. N. van den Pol, U. di Porzio, U. Rutishauser, *J. Cell Biol.* **102**, 2281 (1986)]. After 5 to 60 days in vitro, cells were incubated in fluo-3 acetoxymethyl ester (Molecular Probes) for 45 min in Hepes buffer (10 mM Hepes, 25 mM glucose, 137 mM NaCl, 5.3 mM KCl, 3 mM $CaCl_2$, 1 mM $MgCl_2$, 1 μM tetrodotoxin, pH 7.4), and placed in a microscope chamber where glutamate or its agonists could be serially perfused. For experiments involving NMDA, the same buffer without $MgCl_2$ was utilized. To reduce phototoxicity, we used neutral density filters which allowed only 1% of the normal fluorescent light emitted by the mercury arc lamp to reach the cells. Fluo-3 fluorescent cells could barely be detected without digital intensification of the video image.
12. A Zeiss IM35 inverted microscope with a ×20 Olympus ultraviolet objective (numerical aperture = 0.70) was fitted with a computer-controlled shutter to block fluorescent light when a video frame was not being recorded. A Hamamatsu SIT video camera was interfaced with an ITI 151 video processor driven by an IBM AT computer. Processed images were stored on a 2023F Panasonic laser disk recorder. Additional details are found in A. H. Cornell-Bell, S. M. Finkbeiner, M. S. Cooper, S. J. Smith, *Science* **247**, 470 (1990) A Bio-Rad confocal scanning laser microscope was also used to confirm our results..
13. Although our results indicate an important role of kainate and quisqualate receptors in excitatory transmission of hypothalamic neurons, these experiments do not rule out a contribution from the NMDA receptor. Under the appropriate conditions, such as depolarization to about −30 mV, glutamate could induce inward current flow through NMDA receptor–activated channels, and an NMDA component to the decay phase of the EPSP in some paraventricular nucleus neurons could be detected. Nonetheless, our data suggest that NMDA receptors contribute to synaptic transmission less in the hypothalamus than in other brain areas such as the cerebral cortex or hippocampus.
14. Several studies in the supraoptic and paraventricular nuclei have combined intracellular recording and staining with vasopressin and oxytocin-neurophysin immunocytochemistry, antidromic stimulation, Nissl counterstaining, or visual determination of location in slices or sections; the purpose of these multidisciplinary studies has been to identify the electrophysiological properties of the major groups of neurons and neuroendocrine cells in these nuclei [R. D. Andrew, B. A. MacVicar, F. E. Dudek, G. I. Hatton, *Science* **211**, 1187 (1981); P. Cobbet, K. G. Smithson, G. I. Hatton, *Brain Res.* **362**, 7 (1986); J. G. Tasker and F. E. Dudek, *J. Physiol. (London)*, in press; N. W. Hoffman, J. G. Tasker, F. E. Dudek, *Soc. Neurosci. Abstr.* **15**, 1088 (1989) (*2*). The 13 paraventricular cells had high input resistance (200 to 500 megohms), a regular or slowly adapting discharge of action potentials with no low-threshold potential to a depolarizing current pulse, and linear current-voltage relation to about −100 mV. Identical neurons in the paraventricular nucleus have been injected with Lucifer Yellow or biocytin and shown to be magnocellular (Tasker and Dudek) and neurophysin-positive (Hoffman *et al.*); therefore, this cell type has been identified as a neurosecretory cell. These electrophysiological properties are identical to those recorded in the supraoptic nucleus (*2*), which contains primarily neurosecretory cells. The other seven cells displayed low-threshold calcium spikes and strong time-dependent inward rectification [P. Poulain and B. Carette *Brain Res. Bull.* **19**, 453 (1987); Tasker and Dudek; Hoffman *et al.*] This latter cell type is neurophysin-negative and is probably not neurosecretory. EPSPs in all of these cells were blocked in a dose-dependent manner by CNQX.
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5 June 1990; accepted 31 October 1990

A Borna Virus cDNA Encoding a Protein Recognized by Antibodies in Humans with Behavioral Diseases

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Borna disease virus (BDV) causes a rare neurological disease in horses and sheep. The virus has not been classified because neither an infectious particle nor a specific nucleic acid had been identified. To identify the genome of BDV, a subtractive complementary DNA expression library was constructed with polyadenylate-selected RNA from a BDV-infected MDCK cell line. A clone (B8) was isolated that specifically hybridized to RNA isolated from BDV-infected brain tissue and BDV-infected cell lines. This clone hybridized to four BDV-specific positive strand RNAs (10.5, 3.6, 2.1, and 0.85 kilobases) and one negative strand RNA (10.5 kilobases) in BDV-infected rat brain. Nucleotide sequence analysis of the clone suggested that it represented a full-length messenger RNA which contained several open reading frames. In vitro transcription and translation of the clone resulted in the synthesis of the 14- and 24-kilodalton BDV-specific proteins. The 24-kilodalton protein, when translated in vitro from the clone, was recognized by antibodies in the sera of patients (three of seven) with behavioral disorders. This BDV-specific clone will provide the means to isolate the other BDV-specific nucleic acids and to identify the virus responsible for Borna disease. In addition, the significance of BDV or a BDV-related virus as a human pathogen can now be more directly examined.

BORNA DISEASE IS AN INFECTIOUS neurological disease that occurs sporadically in horses and sheep in Central Europe (*1*). Brain homogenates from infected animals can be used to infect a large number of animal species, from rodents to nonhuman primates (*2–4*). Studies in rats have shown that the agent is highly neurovirulent and invades the brain from peripheral sites by axonal transport (*2*). It replicates in specific groups of neurons in the cerebral cortex and causes biphasic behavioral disease; the short-term effects of Borna disease include aggression and hyperactivity, and the long-term effects include apathy and eating disorders (*3*). In tree shrews (*Tupaia*