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Local synaptic release of glutamate from neurons in the rat hypothalamic arcuate nucleus

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1. The hypothalamic arcuate nucleus (ARC) contains neuroendocrine neurons that regulate endocrine secretions by releasing substances which control anterior pituitary hormonal release into the portal blood stream. Many neuroactive substances have been identified in the ARC, but the existence of excitatory neurons in the ARC and the identity of an excitatory transmitter have not been investigated physiologically.
2. In the present experiments using whole-cell current- and voltage-clamp recording of neurons from cultures and slices of the ARC, we demonstrate for the first time that some of the neurons in the ARC secrete glutamate as their transmitter.
3. Using microdrop stimulation of presynaptic neurons in ARC slices, we found that local axons from these glutamatergic neurons make local synaptic contact with other neurons in the ARC and that all evoked excitatory postsynaptic potentials could be blocked by the selective ionotropic glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM) and D,L-2-amino-5-phosphonovalerate (AP5; 100 μM). To determine the identity of ARC neurons postsynaptic to local glutamatergic neurons, we used antidromic stimulation to reveal that many of these cells were neuroendocrine neurons by virtue of their maintaining axon terminals in the median eminence.
4. In ARC cultures, postsynaptic potentials, both excitatory and inhibitory, were virtually eliminated by the glutamate receptor antagonists AP5 and CNQX, underlining the functional importance of glutamate within this part of the neuroendocrine brain.
5. GABA was secreted by a subset of ARC neurons from local axons. The $GABA_A$ receptor antagonist bicuculline released glutamatergic neurons from chronic inhibition mediated by synaptically released GABA, resulting in further depolarization and an increase in the amplitude and frequency of glutamate-mediated excitatory postsynaptic potentials.

The hypothalamic arcuate nucleus (ARC) contains neurons that project to the median eminence and their axon terminals release substances which act to regulate secretions of the anterior pituitary. A large number of neuroactive substances have been identified in the ARC, many of these first isolated from this region of the brain. These include several neuroactive peptides including β-endorphin, somatostatin, galanin, α-melanocyte-stimulating hormone, neuropeptide Y, neurotensin, substance P, as well as GABA and dopamine (Swanson & Sawchenko, 1983; Everitt, Hokfelt, Wu & Goldstein, 1984; Chronwall, 1985). The transmitters that regulate the activity of ARC neurons therefore act as the final common neuronal regulators of endocrine secretions from the pituitary, ovaries, testes, thyroid and adrenal cortex.

Glutamate receptor agonists and antagonists administered to the neuroendocrine regions of the hypothalamus exert a large number of effects on endocrine secretions, affecting the release of most, if not all, hormones that have been examined (Wilson & Knobil, 1982; Mason, Bissette & Nemeroff, 1983; Gay & Plant, 1987; Lopez, Donoso & Negro-Vilar, 1992; Wayman & Wilson, 1992; Parker & Crowley, 1993; Bach & Yaksh, 1995; Brann, 1995). Cells in the ARC are electrically responsive to glutamate application (van den Pol, Wuarin & Dudek, 1990) and express a number of ionotropic (van den Pol, Hermans-Borgmeyer, Hofer, Ghosh & Heinemann, 1994; Meeker, Greenwood & Hayward, 1994; van den Pol, Obrietan, Cao & Trombley, 1995*a*) and metabotropic (van den Pol, Romano & Ghosh, 1995*b*) glutamate receptors. Furthermore, in one of the first examples of neuronal

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excitotoxicity, glutamate was shown to be toxic to neurons in the ARC (Olney & Sharpe, 1969; Olney, Ho & Rhee, 1971). Data demonstrating that cells respond to glutamate and that glutamate agonists alter hormone secretion support the argument that glutamate receptors are expressed by neurons involved in endocrine regulation, but as neurotransmitter receptors may exist on neurons where there is no evidence for the homologous presynaptic transmitter (Herkenham, 1987), physiological evidence for glutamate secretion is critical. Glutamate is released by axons of unidentified origin in the ARC (van den Pol *et al.* 1990). In parallel a glutamatergic innervation of the neurosecretory cells of the supraoptic (Gribkoff & Dudek, 1990) and paraventricular nucleus (van den Pol *et al.* 1990; Wuarin & Dudek, 1991) has been reported; the source of these excitatory inputs has not been determined. Although glutamate immunoreactivity is found in ARC neurons both in rodents (van den Pol *et al.* 1990) and primates (Goldsmith, Thind, Perera & Plant, 1994), suggesting that glutamate may be a transmitter of ARC neurons, the presence of glutamate by itself is not sufficient evidence for its release as glutamate may be found in most cells of the brain (van den Pol *et al.* 1990). Physiological approaches to this question are therefore a necessity.

With cultures of the mediobasal hypothalamus, digital calcium imaging and single- and dual-cell recording techniques established that glutamatergic neurons were present in the hypothalamus (van den Pol & Trombley, 1993), but their location in the hypothalamus was not determined. One study (Strecker & Dudek, 1994) attempted to detect glutamate release from local interneurons using slice recordings in the suprachiasmatic nucleus (SCN), but none were found despite strong evidence of substantial glutamatergic innervation of the SCN (Cahill & Menaker, 1989; Kim & Dudek, 1991). These SCN data indicated the absence of local glutamatergic neurons in this part of the medial hypothalamus and suggested that the excitatory input to the SCN originated elsewhere. Similar results were found in the hypothalamic paraventricular nucleus where microdrop stimulation around the nucleus failed to reveal any glutamatergic neurons, although many local GABAergic neurons were found (Tasker and Dudek, 1993). In other regions of the brain, such as the hippocampus for instance, glutamate-secreting neurons have been localized and well characterized in the pyramidal cells of CA1–3 and the granule cells of the dentate gyrus (for review see Crunelli, Forda & Kelly, 1985). In striking contrast, a glutamatergic identity has not been established for any neurons in specific regions of the hypothalamus despite the importance of glutamate in hypothalamic function (van den Pol *et al.* 1990).

The present experiments were designed to test the hypothesis that the ARC itself could be the origin of glutamatergic axons in the nucleus and that the axons from local glutamatergic neurons make synaptic contact with other neurons of the ARC and are the primary mediators of local excitatory actions within the nucleus. Our experiments utilized whole-cell patch-clamp recordings of ARC neurons both in culture and in hypothalamic slices which included the ARC. One way of establishing the identity of a putative transmitter released by neurons is to block the actions of the native transmitter released from its axons. Using this strategy, we tested the actions of glutamate receptor antagonists AP5 and CNQX on excitatory activity arising from ARC neurons.

METHODS

Tissue culture

After I.P. injection of sodium pentobarbitone (100 mg kg^{-1}) to induce deep anaesthesia, embryonic day (E) 20 and E21 Sprague–Dawley rats were removed and the mother was killed with an overdose (130 mg kg^{-1}) of the same anaesthetic. The fetal rats were further anaesthetized by hypothermia (4 °C) and decapitated. Postnatal day (P) 1 rats were anaesthetized with an I.P. injection of 100 mg kg^{-1} sodium pentobarbitone and/or hypothermia (4 °C) and decapitated. Brains were removed and the ARC was dissected from 350–400 μm thick slices of the developing hypothalamus. The tissue was then enzymatically treated (10 units ml^{-1} papain, 500 μM EDTA, 1·5 mM $CaCl_2$, 0·2 mg ml^{-1} L-cysteine in Earle's balanced salt solution) for 30 min, resuspended in standard tissue culture medium and triturated to form a single-cell suspension. The suspension was plated onto 22 mm square glass coverslips pre-coated with polylysine (540 000 Da; Collaborative Research). To maintain a high local neuronal density, the cells were plated within a 7 mm glass ring placed on top of the coverslip. The glass ring was removed 24 h after plating.

After 4 days *in vitro*, the proliferation of non-neuronal cells was inhibited by the application of cytosine arabinofuranoside (1 μM) to the tissue culture medium. At the same time, the glutamate receptor antagonists CNQX (10 μM) and AP5 (100 μM) were also added to the medium of some cultures to inhibit glutamate-dependent cytotoxicity. Cultures were maintained in a standard (glutamate and glutamine free) minimal essential medium (Gibco) with 10% fetal bovine serum, 100 units ml^{-1} penicillin–streptomycin and 6 g l^{-1} glucose at 37 °C and 5% CO_2 in a Napco 5410 incubator for 20–30 days *in vitro* before use. Tissue culture medium was changed twice a week.

Arcuate nucleus verification

After cutting out the arcuate nucleus from slices of the medial hypothalamus to use for tissue culture, the remaining section was routinely fixed in 4% (w/v) paraformaldehyde, later stained with propidium iodide (2 μg ml^{-1}; Molecular Probes) for 3 min and then washed in four changes of buffer. The stained slice was examined in a Biorad 500 confocal scanning microscope to verify that the tissue removed from the slice for culturing was restricted to the arcuate nucleus. An example of a such a section showing a typical slice after cutting the region of the E21 arcuate nucleus is shown in Fig. 1*A*. To facilitate identification in this section, the ARC was left in position next to the adjacent hypothalamus after the cut had been made.

Whole-cell recording from cultured neurons

Whole-cell patch-clamp recordings were made with an Axoclamp-2B amplifier (Axon Instruments). Glass pipettes pulled from borosilicate glass capillaries of 2 mm o.d. and 0·2 mm wall thickness were filled

with an internal solution containing (mM): 145 potassium methylsulphate, 10 Hepes, 5 $MgCl_2$, 1·1 EGTA, 4 Na-ATP, 0·5 Na-GTP at pH 7·2. The osmolarity of the medium was 310 mosmol l^{-1}, measured with a Precision Systems Micro Osmometer. After filling, the electrodes had a resistance of 2–5 MΩ. The seal resistances were 8–10 GΩ. The external solution (buffer) contained: 10 μM CNQX, 100 μM AP5, 158·5 mM NaCl, 2·5 mM KCl, 2 mM $CaCl_2$, 10 mM Hepes, 1 μM glycine and 10 mM glucose, at pH 7·3 and osmolarity, 325 mosmol l^{-1}. Experiments were done at room temperature (20–22 °C). A flow-pipe perfusion system was used to perfuse cells in the experimental chamber. It consisted of several inputs into a final single short output terminated by a 0·5 mm i.d. glass pipette. This perfusion pipette was aimed at the recorded cells (100 μm away) which were continuously perfused at a flow rate of 2 ml min^{-1} from the source containing the AP5 and CNQX medium. To change from one solution to another, the flow of the first solution was stopped and flow of the second was started. The newly applied solution flooded the test cell in less than 0·5 s. In the present experiments we used tetrodotoxin (TTX, 1 μM) to block voltage-dependent Na^+ currents, $CoCl_2$ (2 mM) to block Ca^{2+} currents (Hille, 1992) and bicuculline (5–50 μM) to block the activity of $GABA_A$ receptors (all from Sigma).

Both current and voltage clamp were used for electrical recordings. Membrane resistance (R_{input}) was measured by voltage clamp using the application of negative square-wave voltage steps of 10 mV amplitude (in the range of 10–50 mV) from a holding potential (V_h) of −60 mV.

Slice preparation and recording

Sprague–Dawley rats (6 weeks old, weighing 100–150 g) were used to prepare ARC slices. After decapitation under full anaesthesia with halothane, the brain was rapidly removed and placed in ice-cold, aerated buffer (described below); the hypothalamus was dissected out and 350–400 μm thick frontal slices were cut with a vibroslicer (Technical Products International, Inc.).

After preparation, the slices were kept in artificial cerebrospinal fluid (ACSF) at room temperature for at least 1 hour before use. ACSF contained (mM): 124 NaCl, 3·0 KCl, 2·0 $CaCl_2$, 2·0 $MgCl_2$, 1·23 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose and was continuously aerated with 95% O_2–5% CO_2 at pH 7·4. A slice was then adhered to a piece of lens paper and transferred to the experimental interface chamber with constant flow of the oxygenated medium (4 ml min^{-1}). The patch electrodes were prepared from the same glass capillaries used for culture recordings. The standard filling solution contained (mM): 145 potassium methylsulphate, 2 $MgCl_2$, 0·1 $CaCl_2$, 1·1 EGTA, 10 Hepes, 2 Na-ATP and 0·3 Na-GTP at pH 7·2 and 290 mosmol l^{-1}. After filling, the patch electrodes had resistances of 5–7 MΩ and were inserted into the ARC during observation through a stereomicroscope (Microscopes West, Inc.). With positive pressure applied to the recording pipette to eject a small fluid stream, the recording pipette was advanced into the brain slices until a partial seal was obtained. Whole-cell access was then obtained by applying negative pressure to the pipette. The recordings were made at room temperature. The following agents were applied in the bath: AP5 (100 μM), CNQX (10 μM) and TTX (1 μM).

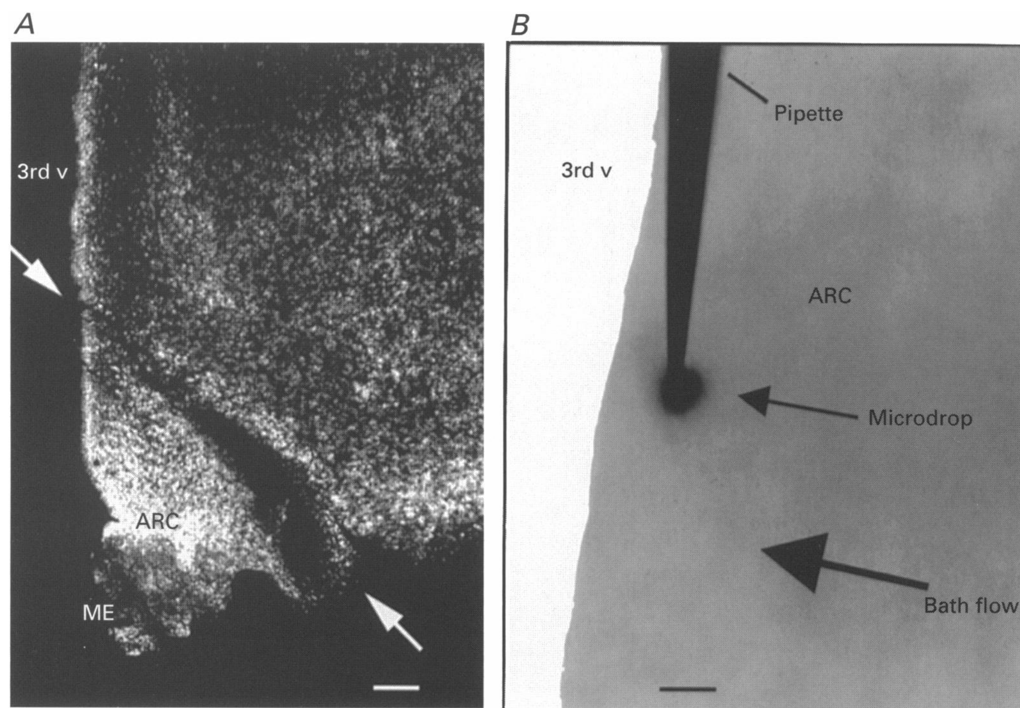

Figure 1. Arcuate nucleus histological verification

A, this confocal laser micrograph shows a thick section of the mediobasal hypothalamus from an E21 brain. The ARC has been surgically severed from the rest of the hypothalamus, but left in place to facilitate identification. This section is typical of ARC isolation to obtain cultured ARC neurons. Scale bar represents 100 μm. *B*, a slice of adult arcuate nucleus showing the size and diffusion of a microdrop of glutamate and black ink. The direction of bath perfusion is shown by the large arrow. ME, median eminence; 3rd v, third ventricle. Scale bar represents 45 μm.

To stimulate neurons in the ARC we used the microdrop technique (Christian & Dudek, 1988; Tasker & Dudek, 1993). Stimulating pipettes pulled from borosilicate glass (2 mm o.d.) were filled with 10 mM glutamate in ACSF. Pipette tips were broken to a diameter of 7–10 μm; a small drop from this pipette was ejected by pressure (Fig. 1*B*). A schematic representation of the location of recording and stimulation pipettes in ARC slices is shown in Fig. 8*A*. To obtain a direct response from a recorded neuron, microdrops were applied at a distance of 20–100 μm from a recorded neuron. To evoke the synaptic release of glutamate in the absence of a direct response to applied glutamate, microdrops were applied 200–500 μm away from the recorded cell. The flow of buffer in the bath was directed so that the perfusion was across the tissue, past the arcuate nucleus and then towards the third ventricle (Fig. 1*B*). This flow would then wash any microdrops of glutamate away from any non-arcuate hypothalamic tissue.

In some neurons a bipolar electrode was also placed on the median eminence to stimulate (50–75 μA) antidromic responses of neurons that had axons terminating here, identifying them as neuro-endocrine cells. Electrical impulses (0·2 ms, 0·5 Hz) were delivered through an isolation unit from a Grass 44 stimulator. To stimulate neurons and axons within the ARC, a second bipolar electrode was placed in the dorsal part of ARC. During some experiments, after the stimulation of the dorsal ARC, the electrodes were moved and placed in the ventrolateral part of the ARC. The stimulating current was 40–275 μA.

Data acquisition and analysis

In all electrophysiological experiments data were monitored and stored on a Macintosh Quadra 800 computer using AxoData 1.0 with the subsequent off-line analysis by AxoGraph 2.0 (Axon Instruments) or Igor Pro software (WaveMetrics, Lake Oswego, OR, USA). Most recordings were made at 400 or 2000 Hz frequency acquisition and some long-term recordings were made at an acquisition rate of 16 Hz. Data in the Results section are presented as means ± S.E.M. Student's *t* tests were used for the statistical comparison of two groups.

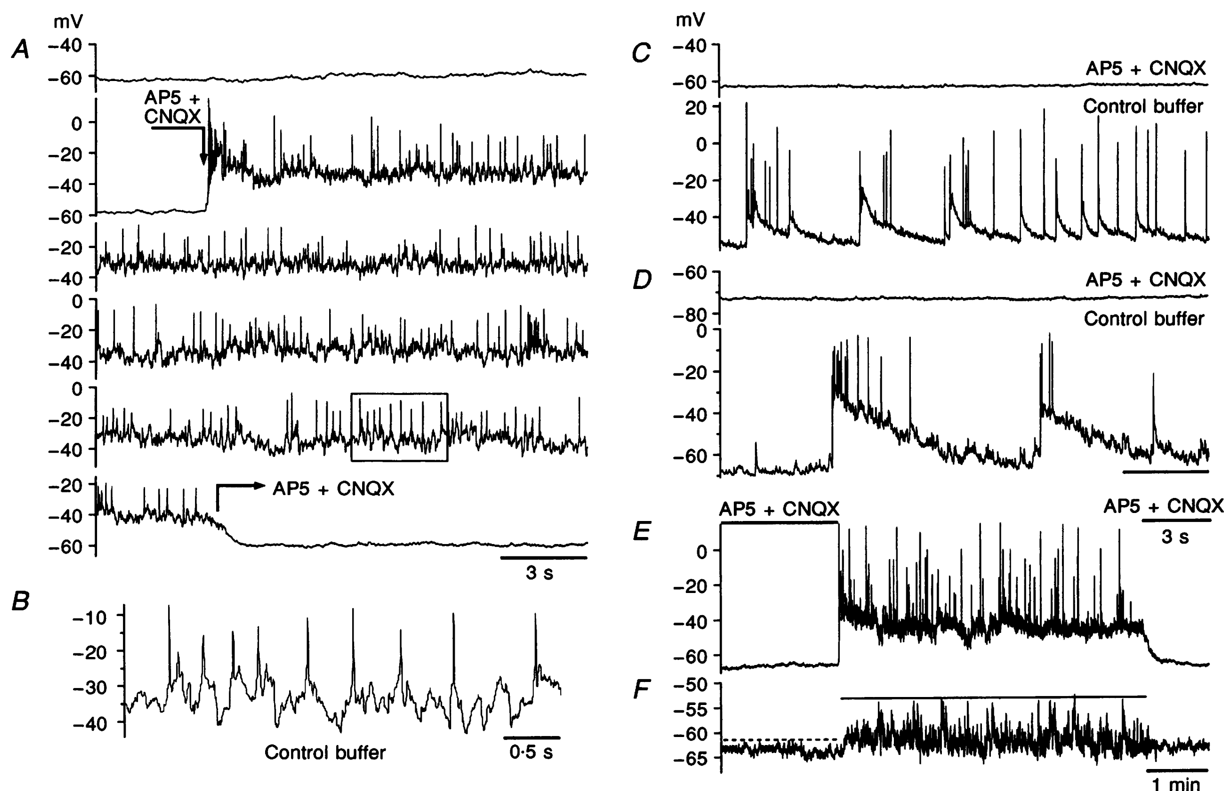

Figure 2. Glutamate-dependent activity in arcuate nucleus neuronal cultures

A, enhanced glutamate-dependent electrical activity was obtained in current-clamped ARC neurons after removal of a chronic (20–30 days) blockade of glutamate neurotransmission (10 μM CNQX + 100 μM AP5). Reintroduction of AP5 and CNQX-containing medium completely blocked this activity. Each trace in *A* is an immediate continuation of the previous one, located above. *B*, expanded time base of the indicated portion (box) of *A* shows glutamate-mediated EPSPs with greater resolution. *C* and *D*, samples of glutamate-mediated EPSPs recorded in ARC neurons after the removal of AP5 and CNQX are shown. *E*, sustained depolarization and heightened glutamate activity were observed in this ARC cell with relief from a chronic glutamatergic blockade. *F*, glutamate-dependent activity was revealed in control ARC cultures, raised in the absence of glutamatergic antagonists, after removal of AP5 and CNQX. Two lines are used in *F* to emphasize the difference in cell activity before (dashed line) and after (continuous line) removal of AP5 and CNQX.

RESULTS

Arcuate nucleus cultures

Glutamatergic activity in cultured neurons

A total of sixty-three ARC neurons from fifty-five coverslips were recorded in the culture experiments. In the presence of glutamate receptor antagonists AP5 (100 μM) and CNQX (10 μM), little excitatory electrical activity was found. Relief from AP5 and CNQX resulted in an immediate depolarization, an increase in action potential (AP) frequency and in the frequency of excitatory postsynaptic potentials (EPSPs) (Fig. 2).

Nine ARC neurons (8 coverslips) cultured for 25 days in the absence of AP5 and CNQX and incubated in AP5 (100 μM) and CNQX (100 μM) for 30 min before the experiment were recorded. When AP5 and CNQX were removed from these cells, a significant increase in electrical activity was detected, suggesting the release of glutamate from neurons in these cultures (Fig. 2*F*). After AP5 and CNQX washout the frequency of APs in these cells increased to $0{\cdot}9 \pm 0{\cdot}1$ spikes s^{-1} ($n = 9$) from $0{\cdot}0 \pm 0{\cdot}0$ in AP5 and CNQX buffer, and cells showed a $2{\cdot}1 \pm 0{\cdot}1$ mV depolarization ($n = 9$) from a membrane potential (V_m) of $-59{\cdot}9 \pm 0{\cdot}9$ mV measured before AP5 and CNQX withdrawal. The reintroduction of AP5 and CNQX led to recovery of the initial V_m baseline. Relatively fast (1–3 Hz) EPSPs (3–10 mV) were only detected in the absence of AP5 and CNQX. In the presence of AP5 and CNQX little evidence of EPSPs was found.

A dramatic increase in electrical activity was found upon removal of AP5 and CNQX in cells that had been subjected to a chronic (20–30 days) treatment with AP5 and CNQX. In twenty-five of these neurons tested, the V_m was depolarized from $-60{\cdot}3 \pm 1{\cdot}1$ mV in the presence of AP5 and CNQX to $-46{\cdot}3 \pm 1{\cdot}7$ mV ($n = 25$; $P < 0{\cdot}0001$) in the first 30 s after removal of the blockade (Fig. 2*A* and *E*). The

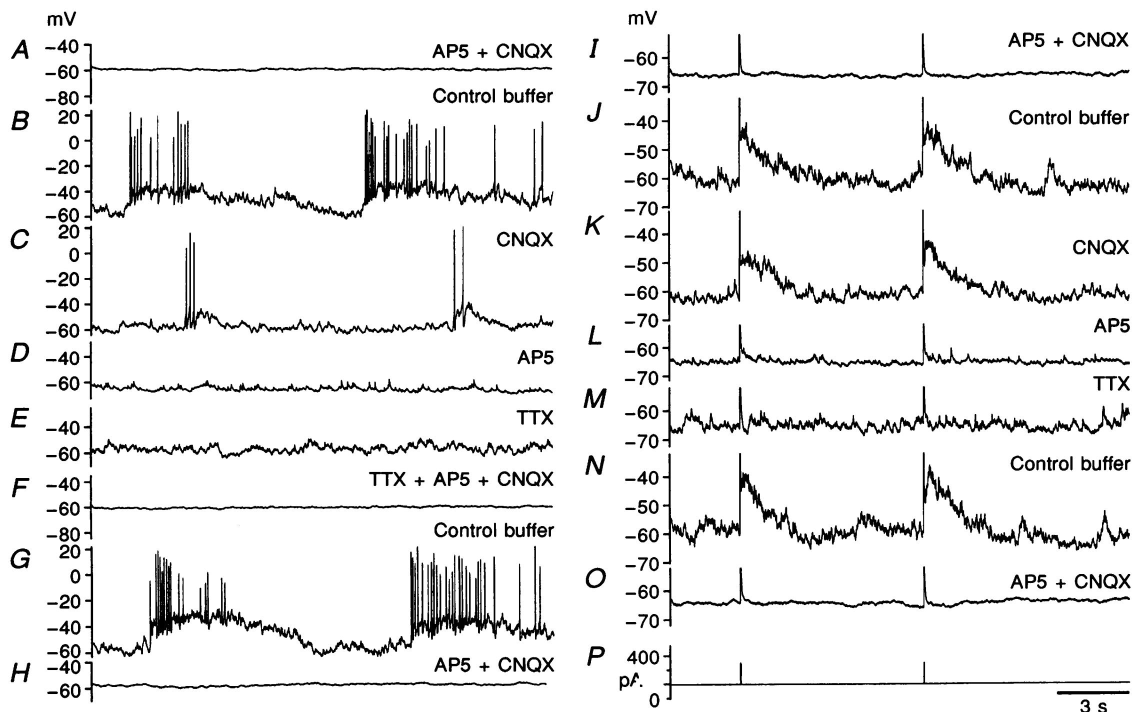

Figure 3. Effects of AP5, CNQX and TTX on glutamate-dependent activity

Current-clamp recordings of two neurons with spontaneous (left column) and evoked (right column) EPSPs are presented in this figure. *A*, no activity was found in the cell in the presence of AP5 and CNQX. *B*, large spontaneous EPSPs were recorded after removal of both AP5 and CNQX (this trace was taken 2 min after relief from a block). *C*, a decrease in the amplitude of EPSPs was found when the non-NMDA glutamate receptor antagonist CNQX was applied. *D*, EPSPs were blocked by the NMDA glutamate receptor antagonist AP5. *E*, they were also suppressed by TTX (1 μM), a blocker of voltage-dependent Na^+ currents. *F*, small amplitude excitatory activity remaining in the presence of TTX was depressed by AP5 and CNQX. Glutamate-dependent EPSPs recovered after reintroduction of the control buffer (*G*) and were blocked by the final application of AP5 and CNQX (*H*). *I–P*, all of the described effects were also observed in the second cell with evoked EPSPs. *P*, evoked EPSPs were obtained by the application of square-wave pulses of current (0·2 ms, 300 pA) in this neuron through the recording electrode (intracellular currents injections appear as two upward deflections).

frequency of APs was $0{\cdot}0 \pm 0{\cdot}0$ and $3{\cdot}2 \pm 0{\cdot}4$ spikes s^{-1} ($n = 25$; $P < 0{\cdot}0001$) during and after removal of the glutamate receptor antagonists, respectively (Fig. 2*A–D*). Relief from glutamate receptor blockade led to the generation of the fast (1–4 Hz) small (3–10 mV) EPSPs in all twenty-five ARC neurons studied and to the development of slow large transients in membrane potential (> 10 mV amplitude) in sixteen of these neurons. The large, slow depolarizations were never seen in the presence of AP5 and CNQX, indicating a dependence on glutamate release. The mean frequency and amplitude of the large, slow transients were $0{\cdot}28 \pm 0{\cdot}06$ Hz and $16{\cdot}7 \pm 2{\cdot}2$ mV, respectively (Fig. 2*C* and *D*). The reintroduction of AP5 and CNQX in 1–5 min caused an immediate return to the electrically quiet baseline in all of the tested neurons (Fig. 2*A*, *E* and *F*).

Relative contribution of NMDA and non-NMDA receptors

To test whether the glutamatergic excitatory activity in cultured ARC cells treated with AP5 and CNQX during culture was specifically dependent on either NMDA or non-NMDA ionotropic receptors, we selectively blocked either the NMDA glutamate receptors with AP5 (100 μM), or non-NMDA ionotropic receptors with CNQX (10 μM). When glutamate-dependent EPSPs and APs stabilized 2 min after the removal of AP5/CNQX (Fig. 3*B*), a 2 min treatment with either CNQX (Fig. 3*C*) or AP5 (Fig. 3*D*) revealed a general decrease in spontaneous electrical activity ($n = 13$). The depressing effect of AP5 on the frequency of APs was more pronounced compared with that of CNQX, indicating a greater contribution of NMDA receptors in this activity. In the presence of CNQX the frequency of APs decreased to $1{\cdot}2 \pm 0{\cdot}2$ from $3{\cdot}5 \pm 0{\cdot}5$ spikes s^{-1} in the control buffer ($n = 13$; $P < 0{\cdot}001$). All APs disappeared during AP5 administration ($n = 13$). The effect of AP5 on V_m was also more dramatic than the effect of CNQX: V_m was $-53{\cdot}2 \pm 1{\cdot}1$ mV in control buffer, $-57{\cdot}6 \pm 0{\cdot}9$ mV in the presence of CNQX, $-59{\cdot}8 \pm 1{\cdot}0$ mV after AP5 administration, and $-60{\cdot}3 \pm 1{\cdot}1$ mV when both glutamate receptor antagonists were applied. The frequency of large EPSPs did not significantly change when CNQX was added to the control medium: $0{\cdot}22 \pm 0{\cdot}05$ and $0{\cdot}23 \pm 0{\cdot}05$ Hz ($n = 13$) in control and CNQX, respectively; but AP5 completely and reversibly blocked large EPSPs. The effect of either AP5 or CNQX did not depend on the order of their administration and was repeatable.

The effects of CNQX and AP5 were also observed in the recorded neuron when EPSPs were evoked by square-wave pulses of current (0·2 ms, 300 pA) through the recording electrode of this neuron (Fig. 3*I–L*). The evoked EPSPs were slightly decreased by CNQX (Fig. 3*K*) and significantly depressed by AP5 (Fig. 3*L*). These EPSPs may have been a result of excitatory glutamate-dependent axonal feedback to the recorded cell.

Relative contribution of action potential-dependent and independent glutamate release

To test whether the generation of glutamatergic excitatory activity in ARC cultures was specifically determined by

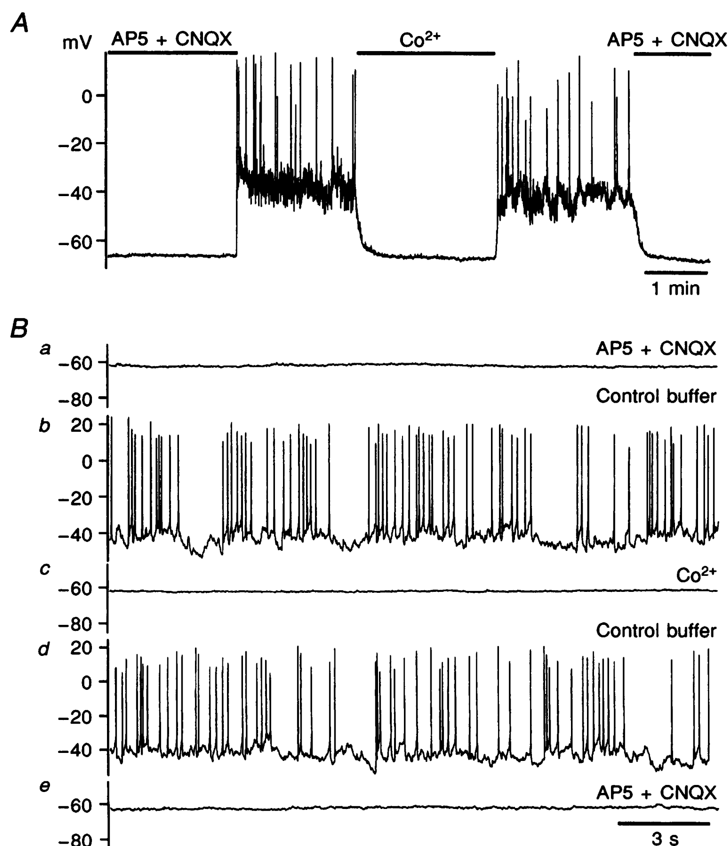

Figure 4. Effect of Ca^{2+} channel blockade on glutamate-dependent activity

Current-clamp recordings of two cells (*A* and *B*) are shown. Sustained depolarization and spontaneous action potentials recorded in the cells after the removal of AP5 and CNQX were completely and reversibly depressed by $CoCl_2$ (2 mM), a Ca^{2+} channel antagonist. Activity of the ARC neuron in the presence of AP5 and CNQX (*Ba*), in control buffer (*b*), during application of $CoCl_2$ (*c*), and during the following reintroduction of the control (*d*) and AP5- and CNQX-containing (*e*) buffers are presented in *B*.

either AP-dependent or -independent glutamate release, we blocked voltage-dependent Na^+ channels with TTX (1 μM). When TTX was applied either 2 min after the removal of glutamatergic block or immediately after the withdrawal of AP5 (as shown in Fig. 3*E* and *M*), AP-mediated electrical activity in neurons ($n = 13$) was blocked for the entire period of TTX application (2–4 min). These data showed the substantial contribution of AP-dependent release of glutamate to the generation of glutamate-mediated activity in ARC neurons. A large number of low amplitude (3–10 mV) potentials recorded in the presence of TTX (Fig. 3*F*) were completely blocked by AP5 and CNQX in all five neurons tested (Fig. 3*F*), indicating the release of vesicle quanta in the absence of APs. Spontaneous EPSPs and APs (Fig. 3*G*; $n = 13$), as well as evoked EPSPs (Fig. 3*N*; $n = 1$), completely recovered after the reintroduction of the control buffer and were blocked during the final administration of AP5 and CNQX (Fig. 3*H* and *O*).

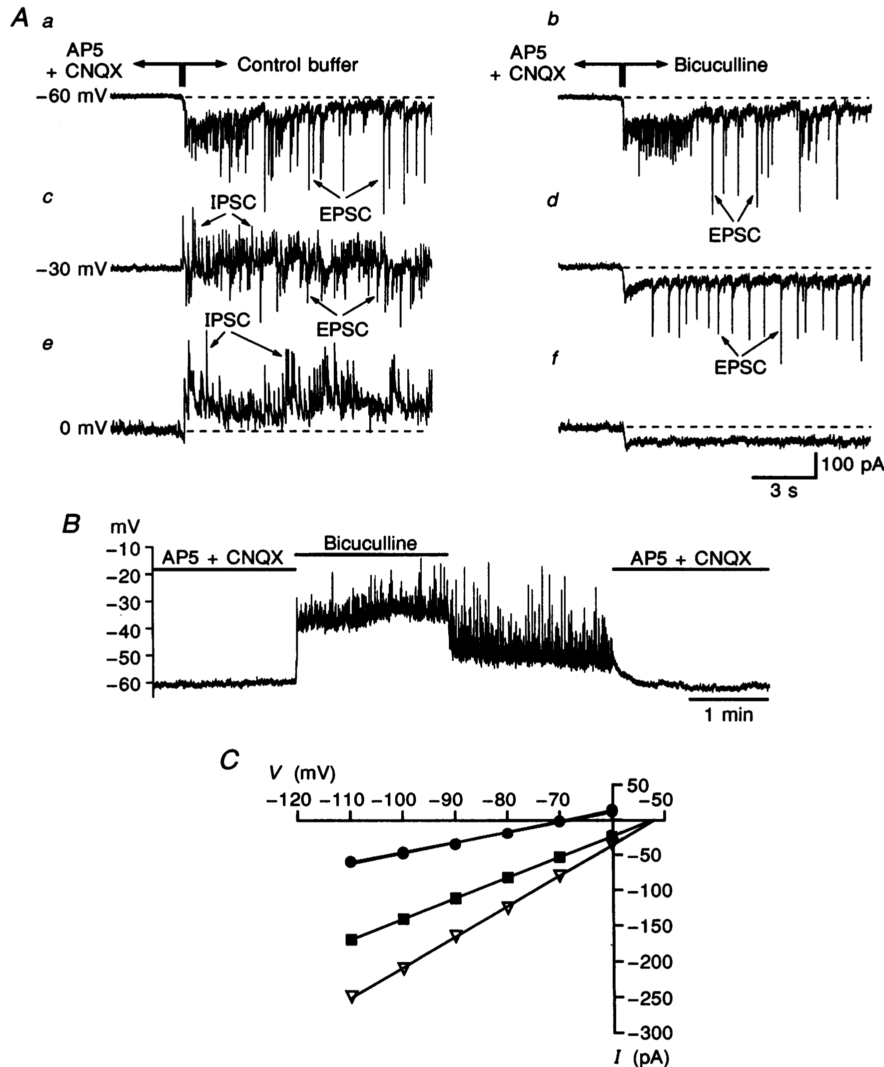

Figure 5. Synaptic release of glutamate increases GABA secretion in cultured neurons

A shows the EPSCs (*a*) that occurred after washout of AP5 and CNQX with control buffer and which were not significantly affected by the $GABA_A$ antagonist bicuculline (50 μM) (*b*) at a V_h of −60 mV. Both EPSCs and IPSCs were recorded with the same experimental protocol at −30 mV (*c*), but only EPSCs remained after bicuculline application (*d*). At a V_h of 0 mV only IPSCs were recorded in the control buffer (*e*) that were blocked by bicuculline (*f*). Removal of AP5 and CNQX revealed an activation of sustained inward current in this cell at a V_h of −60 mV. At a V_h of 0 mV this sustained inward current was masked by a sustained outward current, but could be detected again after the blockade of $GABA_A$ receptors with bicuculline. *B*, in the absence of AP5 and CNQX, the amplitude of the sustained depolarization was larger in the presence of bicuculline compared with the amplitude of depolarization recorded in the control buffer. *C*, washout of AP5 and CNQX solution with the control buffer (▽) significantly decreased the input resistance of the recorded neuron. Bicuculline (50 μM, ■) added to the control medium significantly increased input resistance. Initial input resistance (○) recovered after reintroduction of AP5 and CNQX (●) (points superimposed). Voltage-clamp recordings (*A*), current-clamp recordings (*B*) and *I–V* curves obtained using voltage clamp (*C*) are shown on this figure.

Dependence of glutamate-mediated activity on Ca^{2+} channels

To determine the contribution of Ca^{2+} channels to glutamate-mediated activity in ARC neurons, we blocked these channels with Co^{2+}. The blockade of Ca^{2+} channels completely and reversibly depressed both spontaneous EPSPs, APs and sustained depolarizations revealed in neurons ($n = 6$) after removal of a chronic glutamatergic blockade (Fig. 4*A*, *Bb* and *c*). Upon Co^{2+} washout glutamate-mediated electrical activity recovered (Fig. 4*A* and *Bd*). The reintroduction of AP5 and CNQX depressed all activity and the V_m returned to the initial quiet baseline (Fig. 4*A* and *Be*).

Activation of both excitatory and inhibitory postsynaptic currents after removal of glutamate receptor antagonists

The addition of the $GABA_A$ receptor blocker bicuculline (50 μM) increased excitatory glutamatergic activity indicating the presence of inhibitory GABA-secreting neurons. Using voltage clamp we found that at a V_h of −60 mV, close to the reversal potential for Cl^- and K^+ currents, the relief from AP5 and CNQX led to an immediate activation of fast excitatory postsynaptic inward currents (EPSC) in all eleven cells tested (Fig. 5*A a*). At this V_h bicuculline did not usually affect the EPSC pattern (9 of 11 cells; 82%) (Fig. 5*A b*), but in some cases (2 of 11; 18%) resulted in an enhancement of the amplitude of EPSCs (not shown). Both EPSCs and fast inhibitory postsynaptic outward currents (IPSCs) were recorded with the same experimental protocol at a V_h of −30 mV (Fig. 5*A c*), but only EPSCs remained after bicuculline application (Fig. 5*A d*). IPSCs were more detectable when the V_h was shifted in a positive direction to 0 mV (Fig. 5*A e*). At this V_h, close to the reversal potential for EPSPs (Johnston & Brown, 1981), bicuculline completely and reversibly blocked all recorded fast currents (Fig. 5*A f*), showing that GABA acting at $GABA_A$ receptors was responsible for inhibitory postsynaptic activity.

At a V_h of −60 mV, removal of AP5 and CNQX resulted in immediate sustained inward currents (Fig. 5*A a*) in a majority of the recorded neurons (10 of 11; 91%). The mean amplitude of the sustained inward current measured in the first 30 s after the removal of AP5 and CNQX was $-78{\cdot}4 \pm 5{\cdot}8$ pA ($n = 10$). In five of ten cells (50%) this current persisted for 5 min of the test until the AP5 and CNQX block was restored. Another five cells (50%) showed an initial inward current followed by a slow gradual return to the background baseline. However, at a V_h of 0 mV the sustained inward current was often masked by a sustained outward current (Fig. 5*A e*). The sustained outward current was probably a GABA-mediated Cl^- current because it could be blocked by bicuculline (Fig. 5*A f*). On the other hand, the sustained inward current was probably due to Na^+ and Ca^{2+} with a reversal potential positive to 0 mV and close to the reversal potential for glutamate-activated cation currents (data not shown). This current was blocked with AP5 and CNQX reintroduction.

When bicuculline was applied in the absence of AP5 and CNQX in current-clamp mode, the amplitude of spontaneous glutamate-dependent EPSPs and the amount of sustained depolarization were usually significantly higher than those recorded in control buffer (Fig. 5*B*). In the first 30 s after the switch from AP5 and CNQX to either control or bicuculline-containing buffer, the mean amplitudes of sustained depolarization were $14{\cdot}0 \pm 1{\cdot}2$ mV ($n = 25$) and

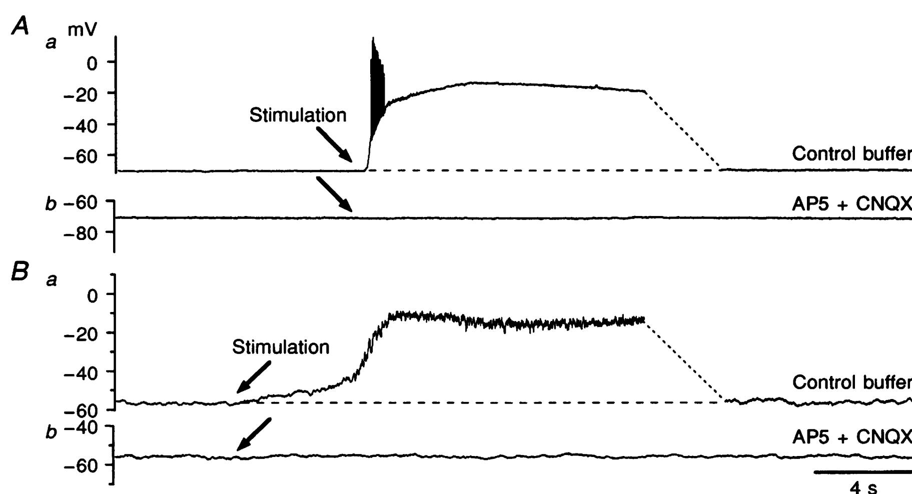

Figure 6. Direct effect of glutamate on neurons in arcuate nucleus slices

A, glutamate (10 mM) applied by microdrop to neurons in ARC slice led to a rapid depolarization and a burst of action potentials in a cell directly stimulated by the amino acid application (*a*). This response was blocked by AP5 (100 μM) and CNQX (10 μM) (*b*). *B*, some cells showed a more gradual depolarization, but no increase in spike frequency (*a*). These responses were totally blocked by AP5 and CNQX (*b*).

20·3 ± 1·5 mV ($n = 10$), respectively. The amplitude of slow, large EPSPs also was slightly greater in the presence of bicuculline (22·7 ± 2·0 mV; $n = 7$) compared with that in control medium (16·7 ± 2·2 mV; $n = 16$). The mean amplitude of the fast small EPSPs also increased during application of bicuculline.

Analysis of R_{input} measured in ARC neurons using voltage-clamp recording showed that the removal of glutamate receptor antagonists significantly decreased R_{input} from 637 ± 138 MΩ in the presence of AP5 and CNQX to 331 ± 73 MΩ ($P < 0·002$; $n = 7$) (Fig. 5C). This effect revealed an increase in conductance of some ionic currents through the cell's membrane most likely due to synaptic release of glutamate from cultured neurons. However, when the $GABA_A$ receptor antagonist bicuculline (50 μM) was added to the control buffer it significantly increased R_{input} from 331 ± 73 to 509 ± 89 MΩ ($P < 0·05$; $n = 7$) (Fig. 5B), showing that the total increase in membrane conductance found after the removal of AP5 and CNQX was partly due to the activation of anion channels. These data suggest that the activity of both excitatory glutamate-secreting and inhibitory GABA-secreting neurons was increased after the removal of the glutamate receptor blockade.

Arcuate nucleus slices

Direct response to glutamate application in arcuate nucleus slices

A total of forty-nine cells from thirty ARC slices were recorded in these experiments. Twenty-one of these cells exhibited voltage-dependent Na^+ and K^+ currents when positive voltage steps of 10 mV amplitude (in the range of 10–50 mV) from a V_h of −60 mV were applied during voltage clamp. The remaining cells did not have voltage-dependent Na^+ or K^+ channels and may have been astrocytes or their processes. The direct application of glutamate by the microdrop method evoked a substantial depolarization of 39·7 ± 5·1 mV from a mean V_m of −62·1 ± 1·1 mV in all cells tested (Fig. 6A and B). In sixteen of twenty-one neurons that showed voltage-dependent Na^+ and K^+ currents, the depolarization was accompanied by a large burst of action potentials (Fig. 6A). In the other five neurons no increase in action potentials accompanied the depolarization (Fig. 6B). No significant change in the amplitude of the direct glutamate-mediated depolarization was found during TTX (1 μM) application, although PSPs were eliminated. The glutamate microdrop evoked a steady depolarization of V_m with no action

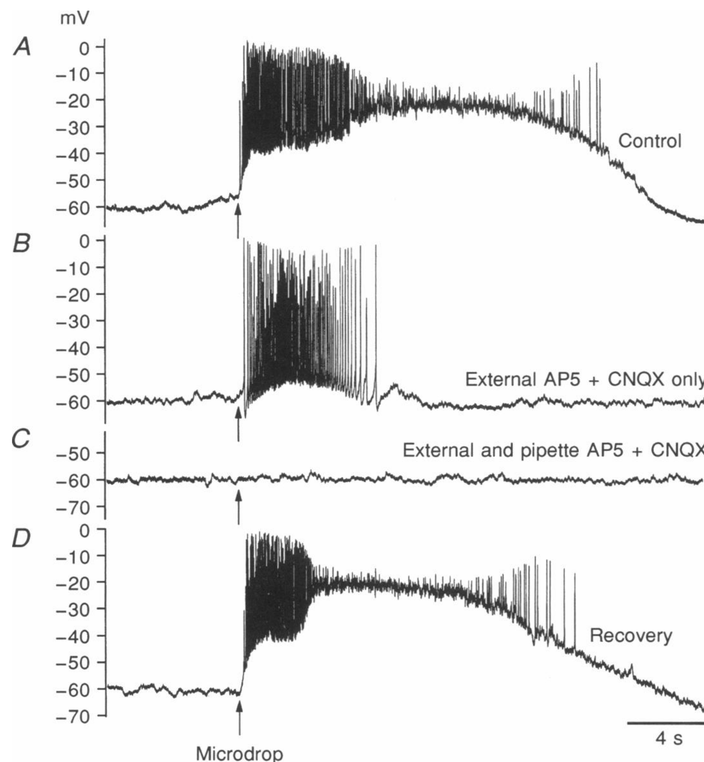

Figure 7. AP5 and CNQX block direct effects of glutamate

A, a microdrop of 10 mM glutamate evoked a large depolarization and burst of action potentials in the neuron shown. *B*, when AP5 (100 μM) and CNQX (10 μM) were introduced into the bath, the amplitude of the depolarization was decreased, but an effect of glutamate was still obvious. *C*, however, when AP5 and CNQX was applied both in the bath and together with glutamate in the stimulating pipette, all cellular actions of glutamate were blocked. *D*, after antagonist washout, the glutamate responses fully recovered.

potentials or transients of the membrane potential, recorded in control medium in eighteen of twenty-five cells that were presumably glial cells.

All direct effects of glutamate either in neurons or in putative glia were completely blocked by AP5 (100 μM) and CNQX (10 μM) applied both in the external medium and in the micropipette medium together with glutamate (total of 11 neurons and 5 glial cells tested) (Figs 6*A b*, *B b* and 7). When AP5 and CNQX were applied only in the bath, cell responses to the direct application of glutamate were not blocked, but were partially reduced (Fig. 7*B*). When AP5 and CNQX were applied both in the bath solution and together with glutamate in the stimulating pipette, then all responses to glutamate were blocked (Fig. 7*C*). Thus the incomplete blockade when AP5 and CNQX were only introduced into the bath solution was probably due to the microbolus of AP5- and CNQX-free glutamate briefly washing off the glutamate receptor antagonists, which then rapidly diffused back. Similar results were found in cultured neurons, where glutamate receptor antagonists were required both in the bath and in the stimulating glutamate solution if complete block were to be achieved. Direct depolarizing responses to glutamate were found only when the stimulating pipette was less than 100 μm away from the recorded cell. This distance is roughly similar to the spread of the glutamate microdrop in tissue, as assessed by adding dye to the drop (Fig. 1*B*).

Synaptic release of glutamate from neurons in arcuate nucleus slices

Twenty-one of forty-nine cells exhibiting voltage-dependent Na^+ and K^+ currents were used to test glutamatergic postsynaptic activity in ARC slices. For this purpose a stimulating pipette containing 10 mM glutamate was moved further away (200–500 μm) from a recorded cell. To find neurons that sent axons to the median eminence, and therefore would probably be involved in neuroendocrine regulation, we used a bipolar stimulating electrode on the median eminence in some experiments, and searched for ARC neurons that showed an antidromic response to an action potential generated in the median eminence. Eleven

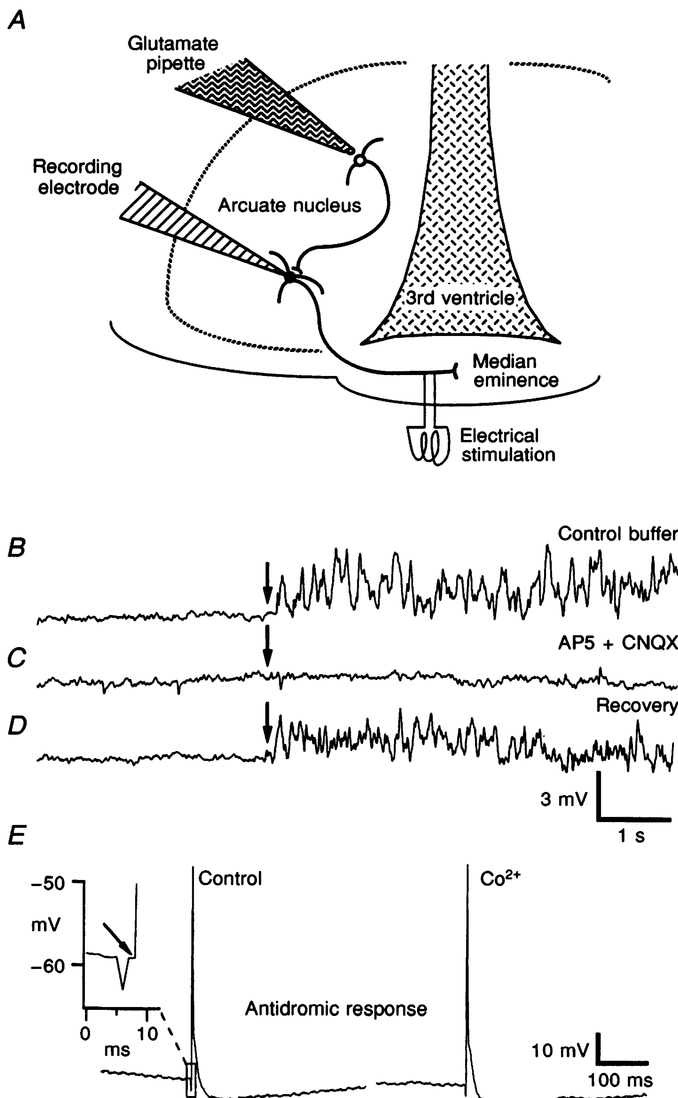

Figure 8. Glutamate receptor antagonists block effect of synaptically released excitatory transmitter in slice

Whole-cell patch-clamp recording from ARC slice: glutamate microdrop stimulation. *A*, the relative placement of the recording and glutamate (10 mM)-containing stimulating pipette in ARC and stimulating electrodes in the median eminence of a rat brain slice are shown. *B*, glutamate microdrop, applied at arrow, evokes an increase in EPSPs that could not be accounted for simply by a direct action of the glutamate microdrop on the recorded cell. *C*, AP5 (100 μM) and CNQX (10 μM) block this effect. *D*, recovery is found after washout of AP5 and CNQX. *E*, antidromic response recorded in an ARC neuron that showed activation of EPSPs during application of a microdrop of glutamate (10 mM). The antidromic response was obtained in the control medium during electrical stimulation of the median eminence (stimulus intensity, 50 μA) and it was not affected by the complete blockade of synaptic activity (Co^{2+}; 2 mM). A 2–5 ms latency between the stimulus artifact and the antidromic spike (arrow) was routinely found, as shown in the small box in *E*. This latency is typical for these small unmyelinated fibres.

neurons were found that showed an antidromic response to electrical stimulation of the median eminence. The relative location of the recording electrode, glutamate-containing pipette and median eminence-stimulating electrode is shown in Fig. 8*A*.

Glutamate microapplication in one region of the ARC stimulated cells directly only in the immediate area of microdrop stimulation, and importantly, evoked EPSPs in four of eight neurons recorded in another, more distant, ARC region (Fig. 8*B*). These cells showed no steady depolarization typical of responses to direct contact with the glutamate microdrop. IPSPs were inhibited by the addition of the $GABA_A$ receptor antagonist bicuculline (5–50 μM) in all buffers. EPSPs were completely blocked by AP5 (100 μM) and CNQX (10 μM) (Fig. 8*C*). The washout of AP5 and CNQX resulted in the recovery of the EPSPs in response to chemical stimulation (Fig. 8*D*). The antidromic activation of axons by electrical stimulation of the median eminence is shown in Fig. 8*E*. The mean V_m of these neurons was -60 ± 1 mV. Little change in baseline V_m was found in the cells during microapplications of glutamate.

The EPSPs (Fig. 9*A*) resulting from the actions of glutamate microdrops were dependent on the activation of presynaptic ARC neurons because they could be blocked by the voltage-dependent Na^+ channel antagonist TTX (1 μM) (Fig. 9*D*). Washout of either AP5 and CNQX or TTX led to the recovery of evoked EPSPs (Fig. 9*C* and *E*).

Electrical and chemical stimulation of neurons in synaptic contact with neuroendocrine neurons

Low intensity (50 μA) stimulation of the median eminence revealed an antidromic response in the neuron in Fig. 8. The response remained in the presence of bath application of 2 mM Co^{2+} that blocked synaptic activity, indicating that the focal stimulation of the medial eminence revealed antidromic stimulation of axons projecting there, rather than orthodromic responses. The chemical stimulation of cells within the ARC evoked EPSPs in this neuron that were blocked by the glutamate receptor antagonists AP5 and CNQX. Microdrops of glutamate usually stimulated cells in the area of the microdrop even in the presence of AP5 and CNQX, as described earlier, due to the brief washout of the antagonists at the site of microdrop application. However, it could be argued that a reduction in the direct response of a presynaptic cell to applied glutamate in the presence of AP5 and CNQX could result in the loss of EPSPs in a postsynaptic cell. For that reason we also used focal electrical stimulation within the arcuate nucleus to evoke EPSPs, as described below, and TTX to block action potential-mediated transmitter release after chemical excitation, presented above.

In some ARC neurons that showed an antidromic response to median eminence stimulation, we used electrical stimulation in various parts of the arcuate nucleus to detect evoked glutamate release.

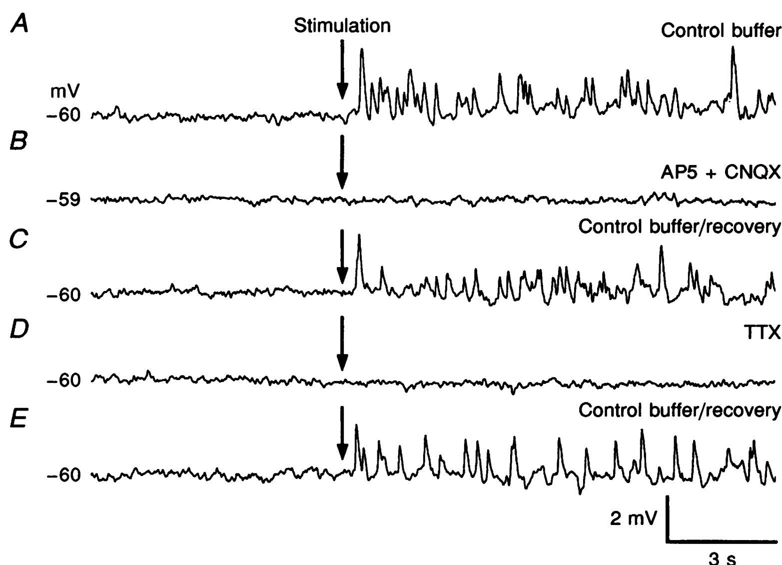

Figure 9. Action potential dependence of evoked EPSPs

Glutamate-evoked EPSPs blocked by TTX. *A*, stimulation of ARC nucleus perikarya by the application of a microdrop of glutamate (10 mM) revealed the activation of EPSPs. *B*, EPSPs were blocked by AP5 and CNQX applied in the bath. *C*, the evoked EPSPs recovered after washout of glutamatergic antagonists. *D*, TTX reversibly depressed glutamate-dependent EPSPs, indicating the EPSPs were due to action potential-mediated transmitter release. *E*, EPSPs recovered after TTX washout. Application of each antagonist was followed by at least 10–15 min of washout and recovery. To reduce GABA-mediated inhibitory postsynaptic potentials in this experiment, bicuculline (5 μM) was added to all solutions.

When a relatively low stimulating current (150–275 μA) was applied to the dorsal part of ARC, an evoked EPSP was detected in all four neurons tested in the control medium (Fig. 10*A*). Increasing the intensity of stimulation (up to 300–350 μA) led to the appearance of an evoked IPSP in the neuronal response (not shown). The evoked IPSP, which was masked at low intensities of stimulation by the evoked EPSP, could also be revealed during application of the glutamate receptor antagonists AP5 (100 μM) and CNQX (10 μM) (Fig. 10*A b*). This IPSP was blocked by the $GABA_A$ receptor antagonist bicuculline (50 μM) applied in the external medium together with AP5 and CNQX (Fig. 10*A c*). The postsynaptic responses recovered after washout of all antagonists. When the stimulating electrodes were moved and placed in the ventrolateral part of ARC, either evoked EPSPs or both EPSPs and IPSPs were recorded in a neuron during application of the lower range of stimulating currents (150–275 μA) (Fig. 10*A e*). Of note, regardless of where the stimulating electrode was placed within the arcuate nucleus, all EPSPs could consistently be fully blocked with the glutamate receptor antagonists AP5 and CNQX. All four neurons showed an antidromic response during stimulation of the median eminence (50–75 μA) that was not blocked by 2 mM Co^{2+} (Fig. 10*B*).

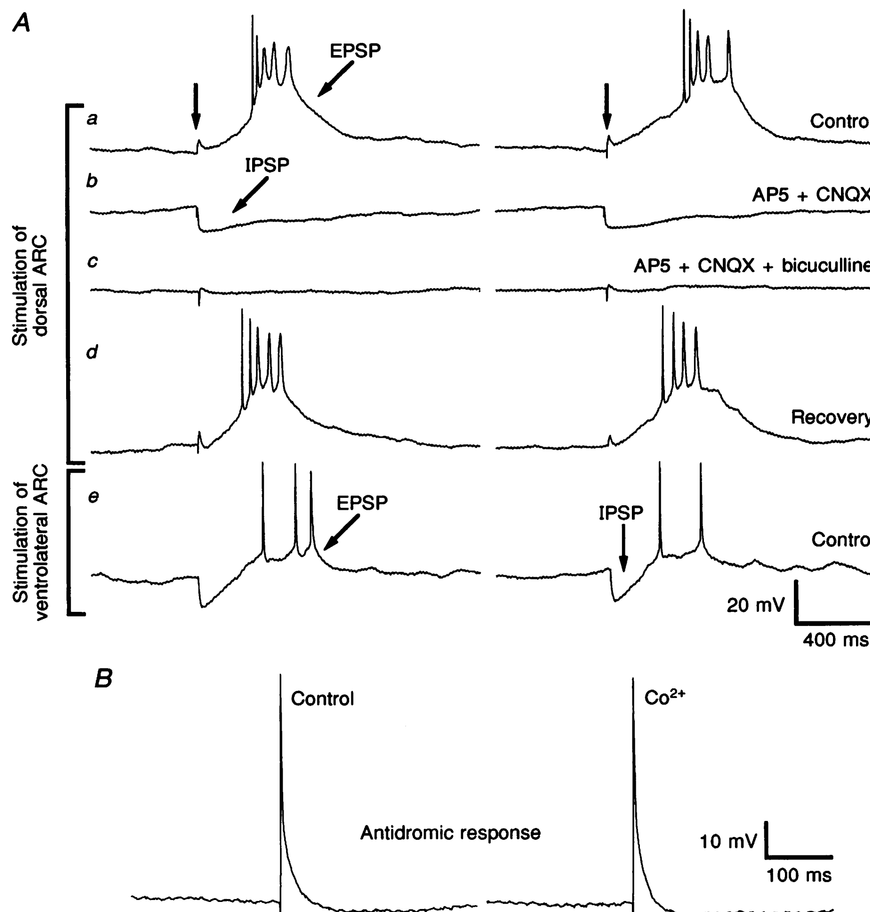

Figure 10. Median eminence projection of neuron postsynaptic to glutamate- and GABA-secreting axons

Whole-cell current-clamp recording from ARC slice: electrical stimulation. *A*, EPSP evoked in ARC slice in control medium during electrical stimulation of the dorsal ARC (*a*) is blocked by AP5 (100 μM) and CNQX (10 μM) (*b*). Application of glutamate receptor antagonists revealed evoked IPSP, which was masked by EPSP in the control medium. This IPSP is blocked by the application of $GABA_A$ receptor antagonist bicuculline (50 μM) (*c*). Evoked EPSP recovers after wash out of the antagonists applied (*d*). Both evoked EPSPs and evoked IPSPs were recorded in this neuron when the stimulating electrodes were placed in the ventrolateral part of ARC (*e*). The stimuli are marked by vertical arrows; IPSPs and EPSPs are marked by diagonal arrows. The right side of the figure shows a repetition of the experiment in the same cell shown on the left. Responses were very similar. *B*, antidromic response revealed during stimulation of the median eminence shows that this neuron has the median eminence projections. This response was not affected by the complete blockade of synaptic activity (Co^{2+}; 2 mM). Membrane potential of the neuron was −59 mV measured in the control medium. Stimulus intensity: 250 μA, dorsal and ventrolateral ARC stimulation; 45 μA, median eminence stimulation.

DISCUSSION

Excitatory neurons in the arcuate nucleus secrete glutamate

Our results based both on selective cultures of ARC neurons and on ARC slices strongly support our hypothesis that many neurons here are excitatory and secrete glutamate. In culture the actions of the excitatory transmitter released by other ARC neurons were completely blocked by the glutamate antagonists AP5 and CNQX, demonstrating glutamate release. That the glutamate response is dependent on action potentials and not on glutamate transporters or astrocyte release is demonstrated by the block of glutamate actions by TTX. Whereas the tissue culture experiments demonstrate that the postsynaptic cells are stimulated by synaptic glutamate release, neurons in culture may make synaptic contacts with cell partners others than those they normally contact. For this reason we used ARC slices to demonstrate that excitatory neurons release glutamate at synaptic endings which terminate on other ARC neurons in normal conditions. Anatomical studies based on Golgi silver chromate impregnations had previously demonstrated local axons arising from ARC neurons, but the transmitter used by these cells was not identified (van den Pol & Cassidy, 1982).

We used electrical and chemical stimulation to demonstrate synaptic glutamate release in the ARC slices. Whereas electrical stimulation can excite both neurons and axons that might originate from unknown sources, making it difficult to determine the origin of excitatory axons (van den Pol *et al.* 1990), chemical microdrop stimulation with glutamate, as used in the present study, excites perikarya, but does not stimulate axons of passage (Christian & Dudek, 1988). That the excitatory effect on the recorded cell was not due to direct diffusion from our stimulating pipette is demonstrated by the blockade of the effect with TTX, which blocks AP-mediated release of glutamate, but does not block a direct depolarization evoked by glutamate. Additionally, a direct response to glutamate is accompanied by a sustained depolarization, whereas synaptically released glutamate can generate rapid EPSPs, as shown in the Results. Additionally, transmitter release from axon terminals is dependent on Ca^{2+} entry; in our experiments, blocking Ca^{2+} channels with Co^{2+} blocked glutamate release, but would not block a direct response to a glutamate microdrop.

Importance of glutamate

A number of papers have demonstrated significant effects of glutamate on hormone secretions; hormones and peptides influenced by glutamate agonists include α-melanocyte-stimulating hormone, gonadotropin-releasing hormone, β-endorphin, luteinizing hormone-releasing hormone, prolactin, growth hormone, oxytocin, vasopressin and somatostatin (Arnauld, Cirino, Laayton & Renauld, 1983; Gay & Plant, 1987; Lopez *et al.* 1992; Wayman & Wilson, 1992; Costa, Yasin, Hucks, Forsling, Besser & Grossman, 1992; Bach & Yaksh, 1995; Brann, 1995). These studies establish that glutamate could have a widespread and complex functional role in endocrine regulation. A large number of neuroactive substances are found in the ARC; many of these are synthesized by neurons here and others are synthesized by cells outside the ARC that send an axonal projection to the nucleus. It is striking that virtually all excitatory electrical activity among ARC neurons is blocked with selective glutamate receptor antagonists. Additionally most inhibitory synaptic activity is also reduced or eliminated by glutamate receptor antagonists, presumably due to a loss of glutamate-mediated excitation of GABAergic neurons. Neurons postsynaptic to local glutamatergic ARC neurons sent axons to the median eminence, identified by antidromic stimulation, identifying them as neurons that were probably involved in regulating the endocrine system through the anterior pituitary gland. These data suggest that the glutamatergic innervation of the ARC may be critical for endocrine regulation by these cells.

Other neuroactive substances found in the ARC may require glutamatergic excitation to manifest their actions. This hypothesis is supported by studies showing that substances found in the ARC such as neuropeptide Y and dopamine or adenosine have relatively little effect on hypothalamic neuronal activity in the absence of glutamate excitation, but have a dramatic modulatory effect in reducing glutamate release at a site on presynaptic terminals or modulating the postsynaptic response to glutamate (Obrietan, Belousov, Heller & van den Pol, 1995; Belousov & van den Pol, 1995; van den Pol, Obrietan, Chen & Belousov, 1996*b*).

Some cultured cells in the present study were raised in the presence of glutamate receptor antagonists to reduce cell death arising from an excitotoxic response to glutamate released by other neurons (Furshpan & Potter, 1989; Obrietan & van den Pol, 1995; van den Pol, Obrietan & Belousov, 1996*a*). When relieved of this chronic block, neurons showed an exaggerated response to glutamate released from other ARC neurons, detected as an increase in the size and frequency of EPSPs and a sustained depolarization. Both NMDA and α-amino-3-hydroxy-5-methylisoxazole-4-propionate/kainate (AMPA/kainate) receptors on ARC neurons were involved in this response. The contribution of NMDA-type glutamate receptors in neuroendocrine regulation has received considerable attention (Wilson & Knobil, 1982; Gay & Plant, 1987). The role of AMPA/kainate and metabotropic glutamate receptors has received less attention (Brann, 1995). The striking increase in glutamate-mediated activity in all synaptically coupled cells tested suggests that there are a high number of glutamatergic neurons in the nucleus, or that the glutamate-releasing neurons can make a substantial number of synaptic contacts with other neurons.

GABA

In a previous paper based on ultrastructural immunocytochemistry we showed that single neuroendocrine cells of

the ARC with axons that terminate in the median eminence received synaptic contact from axons immunoreactive for glutamate and other axons immunoreactive for GABA (Decavel & van den Pol, 1992). In the present study we found that the $GABA_A$ antagonist bicuculline caused a large increase in the excitatory activity mediated by glutamate, consistent with the concept that many ARC neurons release glutamate, but other ARC axons release GABA from local synaptic endings. Removing glutamate receptor blockers from the bath resulted in an increase not only in glutamate-mediated EPSCs, but also in GABA-mediated IPSCs, indicating that inhibitory neurons received an excitatory input from glutamate-secreting neurons.

Many if not all cells in the ARC have been shown cytochemically to express one or more neuroactive peptides or dopamine, sometimes colocalized with GABA (Everitt *et al.* 1984). Although we have no direct evidence at this time, glutamate-secreting neurons in the ARC may release other colocalized neuroactive substances that could act to modulate the actions of glutamate.

Glutamatergic neurons have a restricted distribution in the hypothalamus

As described in the Introduction, other regions of the hypothalamus have been studied using the glutamate microdrop approach. Of significant interest is the apparent absence of excitatory neurons in the suprachiasmatic nucleus (Strecker & Dudek, 1994) and the regions dorsal, lateral, and ventral to the paraventricular nucleus (Tasker & Dudek, 1993). Although glutamate secreting cells were not found in these regions, inhibitory GABAergic neurons were common. In striking contrast, our study reveals that neurons of the arcuate nucleus release glutamate from local synaptic terminals in the nucleus. Together, these data suggest that glutamatergic neurons are not randomly distributed in the hypothalamus, but rather are localized in specific regions. Another possibility we cannot exclude is that, if glutamatergic neurons do exist in the SCN or paraventricular region, their axons may have a trajectory leading out of the thick tissue slice, or may simply not make local synaptic contact. Whether the local excitatory synaptic interactions we described here arise from collaterals of glutamatergic cells that ramify widely in many brain regions (Chronwall, 1985) or are local circuit neurons with axonal projections restricted to the ARC remains to be determined.

Functional implications

Application of glutamate to various parts of the hypothalamus (including ARC) or adjacent third ventricle stimulates the release of many hormones and neuropeptides. Excitatory interneurons have been postulated to exist in ARC or in the other hypothalamic nuclei involved in endocrine control (Wayman & Wilson, 1992; Brann, 1995; Bach & Yaksh, 1995). Ours is the first paper to demonstrate that glutamate is secreted by ARC neurons, that axons from these glutamatergic neurons innervate other ARC neurons, and that some of the neurons postsynaptic to local glutamatergic neurons send axons to the median eminence. Because local projections from interneurons typically account for the majority of presynaptic axons (Zaborszky & Makara, 1979), these ARC glutamatergic neurons may play a critical role in the integration of endocrine feedback signals. Pituitary tropins are secreted by action potential-dependent mechanisms from axon terminals in the median eminence (Urbanski & Ojeda, 1990; Brann & Mahesh, 1992; Lopez *et al.* 1992). That virtually all spontaneous excitatory synaptic potentials and most action potentials are lost in the presence of glutamate receptor antagonists suggests that other transmitters exert little independent excitatory synaptic activity in the absence of glutamate release, underlining the functional role of glutamate at the cellular level.

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