Primary Culture of Identified Neurons from the Visual Cortex of Postnatal Rats

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We have examined the properties of neurons from the visual cortex of postnatal Long Evans rats in dissociated cell culture. Visual cortex from rat pups 1-15 d old was subjected to enzymatic and mechanical dissociation to yield a suspension of single cells. Neurons plated onto collagen or a feeder layer of astrocytes rapidly extended processes and survived for 4-10 weeks. Antisera to glutamic acid decarboxylase, choline acetyltransferase, and vasoactive intestinal polypeptide stained 22 ± 2, 2.3 ± 0.3, and 2.4 ± 0.2% of all neurons, respectively, suggesting that different neuronal classes survived roughly in proportion to their number in vivo.

In order to study a particular identified class of cortical neurons, we prelabeled cells in vivo by retrograde transport of a fluorescent tracer. Neurons in layer V of visual cortex that project to the superior colliculus were labeled after injecting fluorescent microspheres into the colliculus. Retrogradely labeled neurons were readily identified immediately after dissociation and throughout the period in vitro. After 2 weeks in culture, labeled cells exhibited many ultrastructural features characteristic of pyramidal neurons in vivo.

Intracellular recording techniques were used to evaluate the response properties of labeled layer V neurons, as well as other, unlabeled neurons, to excitatory amino acid agonists and antagonists. Glutamate and aspartate—as well as the synthetic agonists N-methyl-D-aspartate (NMDA), kainate, and quisqualate—excited every cortical neuron tested. The antagonist 2-amino-5-phosphonovaleric acid had no effect on responses to quisqualate and kainate but completely blocked depolarizations due to NMDA and aspartate and reduced depolarizations elicited by low concentrations of glutamate. Kynurenic acid, piperidine dicarboxylic acid, and y-aminobutyric acid antagonized responses to all 5 of the agonists. These results provide evidence that corticocollicular neurons in culture express both NMDA-type and non-NMDA receptors for excitatory amino acids.

The cerebral cortex is a laminated structure composed of many diverse neuronal cell types. In the visual cortex, each layer contains classes of neurons with characteristic morphology, visual response properties, and anatomical projection patterns (Gilbert, 1983; Peters, 1985). The projection from visual cortex to the superior colliculus, for example, arises from a population of pyramidal neurons in layer V, while 2 separate groups of neurons in layer VI innervate the claustrum and the lateral geniculate nucleus (Gilbert and Kelly, 1975; LeVay and Sherk, 1981; Selzner et al., 1981). Neurons in each of these populations also possess axon collaterals that ramify within the visual cortex itself (Gilbert and Wiesel, 1979; Katz et al., 1984; Parnavelas et al., 1983). Although much is known about the morphology and receptive field properties of cells in visual cortex, it has been difficult to study the electrophysiological and biochemical characteristics of particular neuronal classes because of the inaccessibility of individual neurons within the intact brain.

Information concerning the neurotransmitter(s) employed by specific populations of cortical neurons has come chiefly from immunocytochemical studies (Eckenstein and Thoenen, 1983; McDonald et al., 1982; Ribak, 1978; Schmechel et al., 1984; Somogyi et al., 1984) and from autoradiographic localization of neurons that accumulate radioactive transmitter candidates (Baughman and Gilbert, 1981; Chronwall and Wolff, 1980; Hamos et al., 1983). Several lines of evidence suggest that glutamate and aspartate may function as excitatory neurotransmitters in the cortex (Fonnum, 1984, Stutei, 1984), while GABA is a strong candidate for the major inhibitory transmitter (Houser et al., 1984). Evidence for at least 3 different receptor sites for glutamate has been presented (Foster and Fagg, 1984; Watkins and Evans, 1984), but the role of each receptor type in excitatory synaptic transmission has not been established.
Figure 2. Dissociated neurons from the visual cortex of animals aged 3 d (D), 7 d (B), 8 d (A), and 15 d (C). A, Photomicrograph taken with Nomarski optics immediately after dissociation. B–D, Phase-contrast photomicrographs of neurons in culture for 3 hr (B), 16 d (D), and 19 d (C). Bar, 30 μm.
Tissue culture of dissociated neurons has proven to be a useful technique for examining the function and development of cells from both the central and peripheral nervous systems (Fischbach and Nelson, 1977). Cells in culture are readily available for physiological recordings, and their fluid environment can be evaluated and found to be clearly inferior to papain for dissociation of centrifugal preparation of both hemispheres, placed in Earle's balanced salt solution (BSS) at 37°C, and cut into 500 μm slices with a scalpel.

Slices of visual cortex from a single pup were incubated at 37°C with gentle stirring in 10 ml of enzyme solution (Bader et al., 1978; Lam, 1972; Townes-Anderson et al., 1985) equilibrated with 95% O₂/5% CO₂. The enzyme solution contained NaCl, 116 mM; KC₄, 5.4 mM; Na₂CO₃, 26 mM; NaH₂PO₄, 1 mM; CaCl₂, 1.5 mM; MgSO₄, 1 mM; EDTA, 0.5 mM; glucose, 25 mM; cysteine, 1 mM; and papain (Cooper Biomedical, Malvern, PA), 200 units. After 1.5 hr, the slices were rinsed briefly with Earle's BSS containing BSA, 1 mg/ml, and ovomucoid, 1 mg/ml. The slices were gently triturated with a fire-polished Pasteur pipet in 2-3 ml of this solution. Freshly dissociated cells that excluded the dye Erythrosin B (Phillips, 1973) were considered to be viable. Dissociated cells were harvested by low-speed centrifugation (10 min, 70 × g) through 5 ml of Earle's BSS containing BSA (10 mg/ml) and ovomucoid (10 mg/ml). Cells were resuspended in growth medium and plated in modified petri dishes (Brau, 1970). Typically, cells from a single rat pup were used to prepare 40–50 culture dishes. Optimal viability and morphological preservation of the dissociated cells were obtained using the enzyme papain. In preliminary experiments, other enzymes, including trypsin, collagenase, dispase, hyaluronidase, and neuraminidase were evaluated and found to be clearly inferior to papain for dissociation of postnatal tissue.

The growth medium consisted of Eagle's MEM (Gibco, Grand Island, NY) containing 0.5 mM glutamine, 20 mM glucose, and either gentamicin (50 μg/ml) or penicillin (100 Units/ml) and streptomycin (0.1 mg/ml, Gibco). The medium was supplemented with 3% adult rat serum, which was prepared as described by Mains and Patterson (1973) and stored at -80°C. Cells were plated onto glass coverslips coated with a layer of dried rat tail collagen (Borman, 1958). The cell density at plating was approximately 100–200 cells/mm². Proliferation of non-neuronal cells was inhibited several days after plating by the addition of 10 μM 1-β-D-arabinofuranosyl cytosine (ARA-C) to the growth medium for 24–48 hr. In some experiments, freshly dissociated cells were plated onto a confluent layer of astrocytes. The astrocyte layers were obtained from cultures of newborn rat occipital cortex, prepared as described above. Neurons in these cultures were killed by a single feeding with icv-cold growth medium. Freshly dissociated neurons plated onto a preexisting layer of astrocytes developed neurites much more rapidly than did cells plated onto collagen. In addition, the survival of neurons obtained from rat pups 9–15 d of age was enhanced by plating onto astrocytes. Cultures were maintained in a humid atmosphere of 95% air and 5% CO₂ at 37°C. The cultures were fed every 4–5 d with 1 ml of warm medium. We attempted to quantitate retinal ganglion cells (Armson and Bennett, 1983; Leifer et al., 1984; Sarthy et al., 1983) in vitro.

Work from several laboratories (Dichter, 1978; Romijn et al., 1982; Swaiman et al., 1982; Thomas, 1985; Yavin and Yavin, 1977) has shown that neurons dissociated from the whole cerebral cortex of embryonic rodents survive in culture and express a variety of differentiated properties, including the formation of excitatory and inhibitory synaptic connections (Dichter, 1978). In the present study, we have developed dissociation and culture procedures that allow for the long-term survival of neurons from the visual cortex of postnatal Long Evans rats. Retrograde transport of fluorescent latex microspheres (Katz et al., 1984) has been used to prelabel neurons in layer V that project to the superior colliculus. As an initial step toward characterizing synaptic transmission between cortical neurons, we have used intracellular recording techniques to evaluate the response properties of labeled layer V neurons to excitatory amino acid agonists and antagonists. Preliminary reports of this work have been published (Huettner and Baughman, 1984, 1985).

Materials and Methods

Tissue culture

Long Evans rat pups (Charles River Breeding Labs), aged 1–15 d, were killed by cervical dislocation. Rectangular blocks of tissue, 2 × 3 mm, comprising area 17 (Krieg, 1946; Miller and Vogt, 1984) were removed from the occipital cortex of both hemispheres, placed in Earle's balanced salt solution (BSS) at 37°C, and cut into 500 μm slices with a scalpel.
Figure 4. Immunohistochemical localization of glutamic acid decarboxylase-like immunoreactivity in normal (A) and colchicine-treated (B) cultures. Cultures were prepared from a 7-d-old animal and fixed after 20 d in vitro. Some GAD-positive varicosities (arrowheads) are closely associated with the somas of unstained neurons. Bar, 30 μm.

cell survival by counting neurons at weekly intervals; however, cultures that were counted deteriorated much more rapidly than sister cultures that were not counted. To provide an estimate of neuronal survival, cell counts of cultures fixed after 3–4 weeks in vitro were compared to the initial plating density of 100–200 cells/mm². Based on counts of 35 cultures, we estimate that 50–70% of the neurons initially plated remained viable after 3–4 weeks in vitro. Using the methods described above, we have prepared viable dissociated neurons from adult rats, 3–5 months old. However, neurons from animals older than 15 d of age have not survived in long-term cultures.

Immunohistochemistry

Cultures used for immunocytochemistry were washed 3 times with warm MEM containing 20 mM glucose and immersed in fixative. To examine neurofilament-like (NF-IR) and glial fibrillary acidic protein-like (GFAP-IR) immunoreactivities, cultures were fixed for 20 min in 0.1 M sodium phosphate, pH 7.4, containing 4% paraformaldehyde. After 3 washes with Tris-buffered saline (TBS), pH 7.4, cultures were incubated for 5 min in TBS containing 0.2% Triton X-100 (TX-100). Cultures were then washed 3 times with TBS and incubated for 15 min in TBS with 3% normal goat serum. Cultures were incubated for 1 hr in rabbit antiserum to α-melanocyte-stimulating hormone (Immunonuclear, Stillwater, MN) in TBS containing 3% normal goat serum, 2% BSA, and 0.1% TX-100. This antiserum has been shown to cross-react with the 150 kDa subunit of neurofilaments (Drüger et al., 1983). The cultures were rinsed 3 times with TBS and incubated for 1 hr with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin (Cappel, Cochranville, PA) diluted 1:40 in TBS containing 3% normal goat serum and 2% BSA. After 3 washes with TBS, cultures were incubated for 1 hr with secondary antibodies to GFAP (Bignami et al., 1972) diluted 1:10 in TBS containing 2% BSA, 3% normal rabbit serum, and 0.1% TX-100. Cultures were washed with TBS and incubated for 1 hr with rhodamine isothiocyanate-conjugated rabbit anti-rat immunoglobulin (Cappel), diluted 1:40 in TBS containing 2% BSA and 3% normal rabbit serum. Cultures were washed again and examined with a Zeiss microscope equipped for phase microscopy and epifluorescent illumination.

To examine glutamic acid decarboxylase-like (GAD-IR) and vasoactive intestinal polyptide-like (VIP-IR) immunoreactivities, cultures were fixed for 10 min in 0.1 M sodium phosphate, pH 7.4, containing 4% paraformaldehyde and 0.1% glutaraldehyde. This was followed by a second fixation for 10 min in 0.1 M sodium borate, pH 9.75, containing 4% paraformaldehyde. To stain for choline acetyltransferase-like (ChAT-IR) immunoreactivity, cultures were fixed for 20 min with 0.1 M sodium phosphate containing 4% paraformaldehyde and 15% picric acid. Cultures were washed 3 times with TBS and incubated 18–24 hr with primary antiserum. Sheep anti-GAD (Oertel et al., 1981) and mouse anti-ChAT (Eckensstein and Thoenen, 1982) were diluted 1:1000 with
Figure 5. Immunohistochemical localization of vasoactive intestinal polypeptide-like immunoreactivity. Cultures were prepared from an 8-d-old animal and fixed after 24 d in vitro. A, Cell body and processes of a VIP-positive neuron. B, Higher magnification photomicrograph of a VIP-positive fiber with varicosities encircling an unstained cell soma (asterisk). Bar: A, 30 μm; B, 20 μm.

Figure 6. Immunohistochemical localization of choline acetyltransferase-like immunoreactivity. Cultures were prepared from a 7-d-old animal and fixed after 20 d in vitro. A and B, Two ChAT-positive neurons and many unstained neurons are shown. Bar, 30 μm.

TBS containing 2% BSA, 0.1% TX-100, and 20% normal rabbit serum. Rabbit anti-VIP (Eckenstein and Baughman, 1984) was used at a dilution of 1:1000 in TBS containing 2% BSA, 0.1% TX-100, and 20% normal goat serum. Following the primary incubation, cultures were rinsed 3 times and incubated for 1 hr with secondary antisera diluted 1:40 in TBS containing 2% BSA and 3% normal rat serum. Goat anti-rabbit immunoglobulin, rabbit anti-mouse immunoglobulin, and a rabbit anti-goat immunoglobulin that cross-reacts with sheep immunoglobulin were used as the secondary antisera (Sternberger-Meyer, Jarretsville, MD). Cultures were then rinsed 3 times with TBS and incubated for 1 hr with goat, mouse, or rabbit peroxidase-anti-peroxidase (PAP, Sternberger-Meyer) diluted 1:40 in TBS containing 2% BSA and 3% normal rat serum. The cultures were rinsed again with TBS and incubated for another hour with secondary antisera, then rinsed and incubated with PAP for an hour. After a final 3 rinses with PBS, the cultures were incubated with 0.1 M sodium phosphate containing 0.03% H₂O₂ and 0.1% 3,3'-diaminobenzidine tetrahydrochloride (DAB). The reaction was terminated by washing with PBS. Cultures were then treated briefly with PBS containing 0.1% osmium tetroxide, dehydrated with ethanol, and mounted on slides.

Retrograde labeling

Neurons in layer V of the visual cortex that project to the superior colliculus were labeled in vivo by retrograde transport of fluorescent latex microspheres (Katz et al., 1984; microspheres can be obtained from Tracer Technology, P.O.B. 7, Bardinia, NY 10924). Rat pups were anesthetized with halothane and their heads were secured with plasticine. A small hole was drilled in the skull directly behind the lambdoid suture, and microspheres suspended in saline were deposited bilaterally into the superior colliculi by pressure ejection from a blunt micropipet mounted on a micromanipulator. The mean diameter of the microspheres in the injected suspension was approximately 20 μm (Katz et al., 1984, Andreas Burkhalter, personal communication). After 36-48 hr survival time, animals were used to prepare cultures or were perfused with 0.1 M sodium phosphate, pH 7.4, containing 4% paraformaldehyde for histologic examination. Fixed brains were equilibrated with 30% sucrose and sectioned on a cryostat. Mounted sections were counterstained with 4',6-diamidino-2-phenylindole (Sigma) and air-dried. The slides were immersed for 30 sec in 100% alcohol and 30 sec in xylene before coverslapping with krystalon (Harleco, Gibbstown, NJ).
Figure 7. Fluorescence photomicrographs of labeled cells in layer V of the visual cortex after an injection of fluorescent latex microspheres into the superior colliculus. A, Roman numerals indicate the layers of the cortex. B, Higher magnification photomicrograph of labeled neurons within layer V. Fluorescent particles are present in the cell bodies and apical dendrites of 3 pyramidal neurons. Also shown are Nomarski (C) and fluorescence (D) photomicrographs of a freshly dissociated neuron obtained from an 8-d-old animal that received an injection of fluorescent microspheres in the superior colliculus on postnatal day 5. The cell contains numerous fluorescent particles and is thus identified as a corticocollicular neuron. Bar: A, 75 μm; B–D, 30 μm.
Figure 8. Fine structure of corticocollicular neurons in culture for 15 d. A and D, Phase-contrast photomicrographs of cortical neurons in cultures prepared from an 8-d-old animal. B and E, Fluorescence photomicrographs of the same fields as in A and D, respectively. One neuron in each field is brightly labeled. C, Low-power electron micrograph of the labeled neuron in A and B. The nucleus (N) has a folded envelope that forms a complete partition in this plane of section. Well-ordered stacks of granular endoplasmic reticulum (ER) lie adjacent to the plasmalemma. The
Fluorescent microspheres in cultured neurons and in tissue sections were visualized with a rhodamine filter set (exciter BP 510–560 nm, beam splitter FT 580 nm, barrier LP 590 nm) on a Zeiss microscope equipped for epifluorescent illumination. For most experiments, animals were injected on postnatal day 5 or 6 and sacrificed on day 7 or 8.

Electron microscopy
Cultures grown on aclar (Allied Chemical Co., Morristown, NJ) coverslips were fixed for 1–2 hr with 0.12 M sodium phosphate, pH 7.4, containing 3% glutaraldehyde. Neurons containing fluorescent latex microspheres were photographed, and their locations on the coverslips were marked. The cells were then postfixed with 2% osmium tetroxide for 30 min, stained en bloc with 1% uranyl acetate for 2–3 hr, dehydrated with ethanol, and embedded in Epon (Landis, 1976). Retrogradely labeled neurons were relocated and mounted for thin sectioning. Sections were stained with lead citrate and examined under the EM.

Electrophysiology
Recording procedures were similar to those described by O’Lague et al. (1978). The culture dish was mounted on the stage of an inverted microscope with phase-contrast optics. A heated brass collar held the dish rigidly in place and maintained the temperature at 30°C. A stainless-steel annulus was inserted into the culture dish to restrict the fluid volume to approximately 0.2 ml. The culture was continuously perfused at a rate of 1 ml/min with medium containing 10% MEM (vol/vol), 1% heat-inactivated horse serum (Gibco), and in final concentrations (mM): NaCl, 135; KCl, 5.3; NaHCO₃, 4.2; Na,H.PO₄, 0.8; MgSO₄, 1; CaCl₂, 3; glucose, 30; HEPES, 5 (pH 7.4). A polyethylene tube filled with 2.5% agar in saline served as a salt bridge from the culture well to the reference electrode (Ag:AgCl). Various media containing drugs could be selected for perfusion by means of a multichannel distribution valve.

Microelectrodes were pulled from thin-walled “omega dot” tubing (Glass Company of America, Bargaintown, NJ) and filled with 0.2 M potassium acetate containing 10 mM HEPES, pH 7.2, or occasionally 0.2 M potassium methylsulfate, 10 mM HEPES, pH 7.2 (resistance in perfusion medium 70–100 MΩ). In some experiments, electrodes were filled with 0.2 M lithium acetate containing 10 mM HEPES, pH 7.2, and 0.4% Lucifer Yellow (Stewart, 1981). A bridge circuit was used to pass current while recording.

The sensitivity of neurons to excitatory amino acid agonists was tested with a pressure-ejection system (Choi and Fischbach, 1981). A blunt micropipet (10–30 μm tip diameter) containing an agonist dissolved in recording medium was positioned 100–200 μm from the soma. Drug solution was ejected from the pipet with 1–5 sec pulses of pressure (2–5 psi). The inert dye Fast green was added to the drug solution (0.1%) to allow for visualization of the ejected material. The area affected by each puff of drug solution was approximately 200–300 μm in diameter. In some experiments, excitatory amino acid antagonists were added to the bath and to the agonist pipet.

Results
Mild enzymatic and mechanical dissociation of cortical tissue from early postnatal Long Evans rats yielded a high proportion of viable cells (Fig. 1A). Approximately 90% of the cells obtained from rats 1–15 d old excluded the dye Erythrosin B. The total number of dissociated cells obtained from the 2 visual cortices...
Immunohistochemistry

GAD-IR was observed in the processes and cell bodies of a subpopulation of cortical neurons in culture. Figure 4A shows a GAD-positive cell body with multipolar morphology; in this, as in most cells, the DAB reaction product was excluded from the nucleus. GAD-positive processes possessed many varicosities and were frequently observed in close contact with neuronal somata (Fig. 4, arrows). In some experiments, cultures were incubated with colchicine (100–500 nM) for 24–36 hr prior to fixation. This colchicine treatment enhanced the staining of cell bodies and proximal dendrites and reduced, but did not eliminate, staining of processes and varicosities (Fig. 4B). Cell counts of colchicine-treated cultures revealed that 22 ± 2% (n = 11) of the neurons exhibited GAD-IR.

Antiserum to the neuropeptide VIP stained the cell bodies and processes of a small percentage of cortical neurons (Fig. 5). In cultures treated with colchicine, approximately 2.4 ± 0.2% (n = 8) of all neurons possessed VIP-IR. The processes of individual VIP-positive neurons could be followed for 100–300 μm. Frequent swellings or varicosities were observed along the length of most stained processes (Fig. 5B). Preadsorption of the antiserum with synthetic VIP completely eliminated the staining. Antiserum to the enzyme choline acetyltransferase stained a subpopulation of cortical neurons comprising 2.3 ± 0.3% (n = 8) of all cells (Fig. 6). Optimal staining for ChAT-IR was obtained using 4% paraformaldehyde and 15% picric acid as the fixative; however, this fixation yielded relatively poor morphological preservation of neuronal processes. Substitution of preimmune sheep, rabbit, or mouse serum for each of the primary antisera above resulted in no detectable staining. In some experiments, cultures stained for GAD-IR, VIP-IR, or ChAT-IR also contained neurons that had been labeled, prior to the dissociation, by retrograde transport of fluorescent microspheres from the superior colliculus (see below). Corticocollicular neurons in vivo do not exhibit immunoreactivity for GAD, ChAT, or VIP (Eckenstein and Baughman, 1984; Ribak, 1978). In culture, the retrogradely labeled neurons were never observed to stain with these 3 antisera.

Retrograde labeling

Bilateral injections of fluorescent latex microspheres into the superior colliculus produced bright labeling of neurons in layer V of the visual cortex (Fig. 7A), as well as in the retina and other parts of the CNS that are known to innervate the superior col-
Figure 10. Effect of kynurenate, 2-amino-5-phosphonovalerate, and glutamic acid diethyl ester on the responses of a corticocollicular neuron to N-methyl-D-aspartate, kainate, and quisqualate. The cell was first exposed to brief applications of 50 μM NMDA (1 sec), 10 μM kainate (1 sec), and 0.5 μM quisqualate (3 sec) in normal recording medium. The agonist applications were repeated during superfusion of the culture with 250 μM kynurenate, 50 μM APV, and 500 μM GDEE, and during a final wash with normal medium. The culture was prepared from a 7-d-old animal and the recording was obtained after 20 d in vitro.

Electron microscopy

Retrogradely labeled neurons maintained in culture for 15 d exhibited many ultrastructural features characteristic of cortical pyramidal neurons in vivo. Figure 8 shows 2 cells that were identified in vitro as labeled corticocollicular neurons and were then processed for electron-microscopic examination. Each cell had a large nucleus containing homogeneous karyoplasm and a single nucleolus. In general, the outline of the nucleus formed a gentle curve with occasional moderate folds. In some cells, however, a sharp crease or cleft was observed on one side of the nucleus (Peters, 1985; Peters et al., 1976). Prominent Golgi bodies and plentiful mitochondria and polyribosomes were observed in the cytoplasm as well as abundant rough endoplasmic reticulum that was frequently arranged into well-organized stacks adjacent to the nucleus or the plasmalemma. Numerous multivesicular bodies were found throughout the cytoplasm, while granular lysosomes were less common. Usually, the neurons exhibited 3–6 large processes that arose as gradually tapering extensions of the perikaryal cytoplasm and rescaled dendrites. Thinner neurites with a more abrupt origin from the perikaryon were observed occasionally; it was not possible, however, to determine whether these processes were axons or dendrites since they quickly became lost in the tangle of neurites surrounding each cell.

Synaptic contacts were observed on the cell bodies and proximal dendrites of all 11 neurons examined in the EM, and numerous synaptic profiles were found in the neuropil adjacent to each labeled cell. Figure 8G shows a synapse on the cell body of the corticocollicular neuron shown in panels A–C. Two examples of synaptic profiles in the neuropil surrounding this cell are shown in Figure 8, H and I. In the present study we did not evaluate the number or distribution of synapses, nor did we attempt to classify the synaptic morphology. We were not able to localize the fluorescent microspheres at the ultrastructural level. Individual microspheres are approximately 20 nm in diameter, and it is known that they dissolve in the organic solvents used for infiltration and embedding of the cells (Katz et al., 1984). Multivesicular bodies, which represent a possible storage site for retrogradely transported microspheres, were found in all of the neurons examined, including many unlabeled cells that were adjacent to retrogradely labeled neurons. We could not
Figure 11. Effect of 2-amino-5-phosphonovaleric acid on the responses of a cortical neuron to glutamate and N-methyl-D-aspartate. Traces on the left show response of the cell to 1 sec applications of 20 μM glutamate and 100 μM NMDA in normal recording medium. The culture was then superfused with 20 μM APV and 3 brief pulses (1 sec) of glutamate and NMDA were applied (at the triangles). Traces on the right show responses of the cell to glutamate and NMDA after washing out the APV with normal recording medium. The culture was prepared from a 7-d-old animal and the recording was obtained after 15 d in vitro.

detect any difference in the appearance of these structures within the labeled and unlabeled neurons.

Electrophysiology
Intracellular recordings were obtained from 71 retrogradely labeled layer V neurons and from many unlabeled neurons as well. Cells from different platings displayed similar properties, and no significant variation was observed for neurons after 2–10 weeks in culture. Resting potentials ranged from −45 to −68 mV, and stable impalements were maintained for 30 min to several hours. Brief depolarizing current pulses elicited one or more action potentials in all neurons studied. In addition, virtually every neuron exhibited frequent spontaneous EPSPs and IPSPs that were reversibly abolished by addition of CoCl₂ (2.5 mM) or CdCl₂ (50–200 μM) to the perfusion medium. In medium containing 3 mM calcium and 1 mM magnesium, many EPSPs exceeded threshold. Elevation of the magnesium concentration to 10 mM also reversibly blocked spontaneous synaptic potentials in most of the cells.

Excitatory amino acids dissolved in recording medium were applied to 53 retrogradely labeled neurons by pressure ejection from a micropipet. Glutamate (10–50 μM) and aspartate (20–200 μM), as well as the synthetic agonists N-methyl-D-aspartate (NMDA, 10–200 μM), quisqualate (0.5–5 μM), and kainate (5–20 μM), produced rapid depolarizations in every neuron tested (Figs. 9–13). Control application of recording medium alone
Figure 13. Effect of cis-2,3-piperidine dicarboxylic acid on the responses of a corticocollicular neuron to excitatory amino acids. Responses of the cell to 1 sec applications of 20 µM glutamate, 20 µM aspartate, 20 µM NMDA, 10 µM kainate, and 0.5 µM quisqualate are shown before (top row of traces), during (middle row), and after (bottom row) superfusion of the culture with 1 mM PDA. The culture was prepared from an 8-d-old animal and the recording was obtained after 21 d in vitro.

had no effect. Application of excitatory compounds also produced a transient increase in the frequency of EPSPs and IPSPs recorded in most cells. To determine whether the depolarizing responses to excitatory amino acids were due to direct excitation of the impaled neuron or solely to summation of EPSPs, we blocked synaptic transmission with MgCl₂ or CdCl₂. Figure 9A shows recordings from a cell that exhibited suprathreshold EPSPs. The addition of 10 mM MgCl₂ to the perfusion medium greatly reduced the frequency and amplitude of the spontaneous EPSPs in this cell. Nevertheless, application of each of the 5 agonists in the presence of high magnesium elicited strong depolarizations, indicating that the agonists were acting directly on this neuron. In spinal cord neurons, the current evoked by NMDA is reduced by Mg²⁺ and to a much lesser degree by Cd²⁺ (Mayer and Westbrook, 1985). Cortical cells superfused with 10 mM MgCl₂ were depolarized by high concentrations of NMDA (100–200 µM) but not by lower concentrations (10–20 µM) that were routinely effective in control medium (3 mM Ca²⁺/1 mM Mg²⁺). Sensitivity to glutamate and aspartate was also diminished in 10 mM MgCl₂ but responses to quisqualate and kainate were not affected. Cadmium chloride at 200 µM completely blocked synaptic potentials but did not antagonize responses to any of the excitatory compounds (data not shown).

We tested 5 antagonists of excitatory amino acid receptors for their ability to block responses to each of the 5 agonists (see Discussion). The compound 2-amino-5-phosphonovaleric acid (APV, 20–50 µM; Davies and Watkins, 1982) had no effect on depolarizations produced by quisqualate or kainate, but it completely antagonized 10–200 µM NMDA (Fig. 10) and aspartate. Responses to low concentrations of glutamate were also diminished by APV (Fig. 11). Kynurenic acid (Genong et al., 1983; Jahr and Jessell, 1985), cis 2,3 piperidine dicarboxylic acid (PDA; Davies et al., 1981) and γ-aminobutyric acid (DGG; Davies and Watkins, 1981) antagonized the depolarizing responses to all 5 of the agonists. Partial blockade was obtained using 250–300 µM kynurenic acid, PDA, or DGG (Figs. 10, 12) and complete antagonism occurred at 1 mM (Figs. 9, 13). Addition of each of these 3 compounds at 1 mM to the perfusion medium also reversibly eliminated spontaneous synaptic activity. Glutamate diethyl ester (GDEE; Davies and Watkins, 1979; McLennan and Lodge, 1979) at concentrations up to 1 mM had no effect on responses to any of the agonists (Fig. 10; other data not shown) and did not alter spontaneous synaptic potentials. Finally, in several experiments we recorded from retrogradely labeled neurons with electrodes containing the fluorescent dye Lucifer Yellow, which was injected iontophoretically to visualize the cell and its neurites. Each of the dye-filled neurons possessed 3–6 branching processes that extended 100–500 µm from the cell soma (Fig. 14, A, C). At high magnification, processes that resembled axons and dendrites could be distinguished (Fig. 14E). Dendritic processes arose from a thick base and possessed numerous short (1–2 µm) spines along most of their length. Each cell had a single axon-like fiber that was thinner than the rest of the processes and that did not bear any spines. Although several of the dye-filled neurons clearly resembled pyramidal cells in their overall morphology, most of the cells were multipolar in appearance.

Discussion

Dissociated cell cultures of neocortical neurons have been prepared previously from embryonic animals (Diehler, 1978; Romijn et al., 1982; Swaiman et al., 1982; Thomas, 1985; Yavin and Yavin, 1977). The present study demonstrates that cortical neurons from young rats, 1–15 d of age, survive in culture and exhibit many differentiated properties. Several lines of evidence suggest that a number of distinct subpopulations of cortical neurons are present in these cultures. Approximately 20–25% of all neurons possessed immunoreactivity for GAD, the en-
Figure 14. Fluorescence photomicrographs of 2 corticocollricular neurons that were injected with Lucifer Yellow after 42 (A and B) and 51 (C and D) d in culture. B and D, Fluorescence photomicrographs taken with a rhodamine filter set to visualize the fluorescent latex microspheres.
neuron is prominent, the cell body appears multipolar. Bar: 75 μm; E, 15 μm.

Arrows indicate the labeled cells. A, C, and E, Fluorescence photomicrographs of the same field taken with a filter set for Lucifer Yellow. Arrowheads indicate the axon of each cell. E, High-magnification photomicrograph of processes from the cell shown in C and D. This photomicrograph was taken near the arrowhead in C. Numerous spines can be seen along the dendrite. A and B, Culture prepared from a 8-d-old animal. This cell has a distinctly pyramidal morphology. C and D, Culture prepared from a 7-d-old animal. Although one dendrite of this neuron is prominent, the cell body appears multipolar. Bar: A-D, 75 μm; E, 15 μm.

The morphology of retrogradely labeled layer V neurons was examined at both the light- and electron-microscopic level. The neurons in layer V of visual cortex that project to the superior colliculus are large pyramidal cells (Sefton et al., 1981). Pyramidal neurons in vivo are distinguished from other cell types by the shape of their somas and by their apical dendrite that extends radially through the cortex toward the pia (Feldman, 1984; Paravelas et al., 1977). Dendritic spines are found on both the apical dendrite and the smaller basal dendrites of all pyramidal cells. When cells are dissociated and grown in culture, it is more difficult to classify their morphology (Banker and Cowan, 1979). In a study of embryonic cortical cultures, Kriegstein and Dichter (1983) classified Lucifer Yellow-filled cells as either pyramidal, fusiform, or multipolar. Neurons were considered to be pyramidal if they had spiny dendrites, if their cell body had a triangular profile, and if one of their dendrites was more prominent than the rest. In the present investigation, 18 layer V neurons were injected with Lucifer Yellow. Each cell exhibited a single thin axonlike fiber and 3–6 thicker, spiny dendrites. Several neurons had triangular cell bodies, but only 5 cells appeared distinctly pyramidal. The morphology of the remaining 13 cells was multipolar. At the ultrastructural level, mature neurons in culture bore the full complement of organelles found in vivo. Dendrites arose from the cell body as gradual taperings of the perikaryal cytoplasm and contained numerous polyribosomes. In a study of embryonic hippocampal neurons in culture, Bartlett and Banker (1984a, b) observed polyribosomes in dendrites but not in presumptive axons. These authors also found that morphological differentiation of axonal and dendritic processes occurred in neurons that did not contact any other cell type, suggesting that the development of axons and dendrites is an intrinsic property of the neurons (Bartlett and Banker, 1984a).

Most cortical neurons in vivo are excited by glutamate and aspartate (Hayashi, 1954; Kajnevev and Phillips, 1963). In culture, all of the neurons that we tested, including 53 labeled layer V cells and many unlabeled neurons, were depolarized by glutamate and aspartate, and by the synthetic agonists NMDA, kainate, and quisqualate. Recent pharmacological studies have provided evidence that excitatory amino acids act on more than one receptor site (Davies et al., 1982; Fraser and Fagg, 1984; Watkins and Evans, 1981). In agreement with earlier work (Collingridge et al., 1983; Davies et al., 1981b; Rothman and Samaite, 1985), we found that APV selectively antagonized responses to NMDA and aspartate. APV also reduced responses to low concentrations of glutamate, but did not alter depolarizations due to kainate or quisqualate. Three other antagonists—kynurenate, PDA, and DGG—blocked responses to all 5 of the agonists. Kynurenate and PDA are known to antagonize responses to NMDA, kainate, and quisqualate in a number of systems (Collingridge et al., 1983; Davies et al., 1981a; Ganong et al., 1983; Jahr and Jesse, 1985; Perkins and Stone, 1982). DGG is often found to diminish responses to NMDA and kain-
ate selectively relative to responses elicited by quisqualate (Davies and Watkins, 1981; Rothman and Samaie, 1985). In some cases, however, DGG at higher concentrations can also block responses to quisqualate (Collingridge et al., 1983; Crucelli et al., 1983; O’Brien, 1985). In our hands, 1 mM DGG was necessary to achieve complete antagonism of NMDA and kainate depolarizations, and at this concentration, responses to quisqualate, glutamate, and aspartate were also abolished. A final antagonist, GDEE, is known to selectively block responses of spinal cord neurons in vivo to quisqualate (Davies and Watkins, 1979; McLennan and Lodge, 1979). In slices of cortex (Hori et al., 1982) and hippocampus (Collingridge et al., 1983), however, GDEE is much less effective. We could not discern any effect of GDEE at concentrations up to 1 mM on responses to the agonists that we tested.

The results of our pharmacological experiments provide strong evidence that corticocellular neurons in culture express both NMDA-type and non-NMDA receptors for excitatory amino acids. In the spinal cord, the differential sensitivity of kainate and quisqualate responses to antagonism by DGG (Davies and Watkins, 1981) and GDEE (Davies and Watkins, 1979; McLennan and Lodge, 1979) has been proposed as evidence for 2 subtypes of non-NMDA receptors (Watkins and Evans, 1981). We find that GDEE is without effect on cortical neurons in culture and that responses to both kainate and quisqualate are antagonized by DGG. These results are consistent with the presence of a single type of non-NMDA receptor that is activated by both kainate and quisqualate (O’Brien, 1985).

The primary motivation for using postnatal animals in this study was that retrograde labeling of specific cell populations would be difficult or impossible to achieve in the embryo. A second reason for using older animals was that the neocortex of embryonic rodents is relatively immature. While most cells destined to be neurons have undergone their final mitosis by embryonic day 21 (Berry and Rogers, 1965), many of the attributes of adult neurons are not fully expressed until 2–3 weeks after birth (Chronwall and Wolff, 1980; Ivy and Killackey, 1981; McDonald et al., 1982). At present, it is not known whether each class of cortical neurons is composed of cells whose fate is fully determined early in development, or whether the ultimate fate of any given neuron depends upon interactions with the local environment during migration and differentiation (Caviness, 1982; Jensen and Killackey, 1984; Jones et al., 1982; McConnell, 1985) or upon interactions with the targets that it innervates. Cortical neurons obtained from animals 1–2 weeks of age, such as those used in the present study, have been exposed to normal environmental influences for a much longer period of time than have embryonic cells. As a result, these older neurons may be more firmly established on a particular development course.

This study has shown that neurons from the visual cortex of postnatal rats, up to 15 d of age, survive in dissociated culture, regenerate processes, and form synaptic connections. Specific subpopulations of living neurons were identified in long-term culture by labeling the cells in vivo with retrogradely transported fluorescent latex microspheres. Identified corticocellular neurons exhibited many normal morphological and physiological properties, including both NMDA and non-NMDA receptors for excitatory amino acids. Antisera to GAD, ChAT, and VIP labeled subpopulations of neurons in culture that were roughly proportional to the percentage of cells that stain for these antigens in vivo. Corticocellular neurons were not stained by these 3 antisera. Further experiments on the electrophysiological characterization (Nerbonne et al., 1985) and synaptic pharmacology (Huettner and Baughman, 1985) of identified cortical neurons are in progress.

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