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P19 CELLS DIFFERENTIATE INTO GLUTAMATERGIC AND GLUTAMATE-RESPONSIVE NEURONS *IN VITRO*

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Abstract—The neurotransmitter L-glutamate has been associated with a number of developmental events within the central nervous system including synaptogenesis and the refinement of topographically ordered neural maps. As a model for studying such events at the molecular level, we have examined the expression of glutamate and glutamate receptors in neurons that develop from P19 cells in response to retinoids. We report here that many P19-derived neurons do contain glutamate in secretory vesicles and that this glutamate appears to function as a neurotransmitter. The neurotransmitter GABA is also present in these cultures and both glutamate and GABA appeared to co-localize in some neuronal processes. Both neurotransmitters were released from the neurons in response to membrane depolarization. These neurons also express various glutamate receptor subunits including GluR1, GluR4 and NMDAR1 as detected by immunological methods. Using whole-cell patch-clamping, we have recorded spontaneous postsynaptic potentials which increase in both amplitude and frequency with time in culture and which are sensitive to the glutamate antagonist kynurenic acid

Thus, P19-derived neurons mature in culture and form electrically active neural networks involving glutamate and glutamate receptors. © 1997 IBRO. Published by Elsevier Science Ltd.

Key words: neurotransmitter, embryonal carcinoma, retinoic acid, receptor.

L-Glutamate, the predominant excitatory neurotransmitter of the vertebrate CNS, is associated with a number of developmental events. Chief among these include the formation of active synapses and productive neural networks. For example, L-glutamate released from entorhinal axonal termini promotes the formation of synapses with target hippocampal pyramidal neurons[28] while it inhibits dendritic outgrowth from these same target cells.[26,27] *In vivo* studies have also shown that pharmacological agents which block the *N*-methyl-D-aspartate (NMDA)-type glutamate receptors disrupt the normal segregation of synapses and hence the formation of appropriate neural networks in the tectum of both frogs and goldfish[5,40,42,48] as well as the striatal cortex in cats.[15]

Glutamate has also been shown to play an important role in the establishment of long-term potentiation, a model for the development of memory.[6,12,20,33,35] Finally, glutamate and the NMDA receptor have been linked to excitotoxicity and neuronal death during ischemia and other traumas to the CNS.[4]

P19 embryonal carcinoma cells are a pluripotent stem cell line which can be induced to differentiate with retinoic acid into cell types similar to those derived from neuroectoderm (reviewed in Ref. 21) The neurons obtained in these cultures are irreversibly post-mitotic, show a typical neuronal morphology and exhibit a number of markers characteristic of CNS neurons such as neuron-specific enolase, the neurofilament proteins and the neuron-specific nuclear antigen NeuN. In addition, P19 neurons have been shown to undergo an electrophysiological maturation of their membranes where depolarizing current steps can elicit action potentials.[22] Whole-cell recordings and mRNA analysis have demonstrated the presence of both the NMDA and amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor types following retinoic acid induced P19 cell differentiation.[9,39,51] Transcripts encoding the NMDAR2A and the NMDAR2B receptor subunits appear to be

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Abbreviations: AMPA, amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; EGTA, ethyleneglycolbis (aminoethylether)tetra-acetate; FITC, fluorescein isothiocyanate; GluR, glutamate receptor; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; MK-801, dizocilpine maleate, NeuN, neuron-specific nucleoprotein; NMDA, *N*-methyl-D-aspartate; NMDAR, NMDA receptor; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; TTX, tetrodotoxin.

expressed in both undifferentiated and differentiated cells while the NMDAR1 subunit, critical to receptor function, is only expressed following the induction of the neuron phenotype.[39] These observations are consistent with those of Turetsky *et al.*[51] who demonstrated the activation of inward currents in P19 neurons in response to the extracellular application of NMDA. Stimulation of these NMDA receptors in retinoic acid-treated P19 cells has been shown to trigger an influx of Ca^{2+} from the extracellular medium.[2,32] This influx is dependent on the presence of glycine and can be blocked by both Mg^{2+} and the noncompetitive NMDA antagonist, dizocilpine maleate (MK-801).

The presence of AMPA/kainate-type glutamate receptors has also been demonstrated on P19-derived neurons. Addition of kainate to superfusates has been shown to induce whole-cell currents and after prolonged exposure result in widespread excitotoxicity in retinoic acid-treated P19 cultures.[51] Ray and Gottlieb[39] have also detected mRNA encoding the non-NMDA receptors in P19 neurons using non-specific polymerase chain reaction (PCR) primers for the glutamate receptor subunits GluR1 through GluR4. The exact identity of these non-NMDA receptors, however, has not yet been determined.

We set out to examine the role of glutamate in the development of functional synapses in P19-derived neurons. L-glutamate is the predominant excitatory neurotransmitter of the mammalian CNS while GABA is the most common inhibitory transmitter. Since P19-derived neurons show many of the characteristics of CNS neurons (reviewed in Ref. 21) including the synthesis and storage of GABA,[46] they were tested for the presence of L-glutamate.

EXPERIMENTAL PROCEDURES

Cell culture and differentiation

Undifferentiated P19 cells were grown in α-MEM (Gibco-BRL) supplemented with 7.5% calf serum and 2.5% fetal bovine serum. Cultures were maintained as monolayers in exponential growth between densities of 1.3×10^4 and 1.3×10^5 cells/cm^2. Differentiation by retinol and serum-free medium was carried out as described[21] by plating cells into serum-free medium comprised of Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 1 × Basal Medium Eagle's Vitamin Solution (100 × stock, Gibco), 1 × Trace Element Mix (100 × stock, Gibco), 1 μg/ml Human Transferrin, 20 nM hydrocortisone-21-phosphate, 10 nM L-carnitine, 100 μM putrescine, 15 nM selenious acid (Aldrich), 50 nM cadmium sulphate, 5 μg/ml retinol, 10 μg/ml DL-α-tocopherol (Gibco), 0.2 μg/ml DL-6,8-thioctic acid, 0.1 μg/ml linoleic acid, 5 μg/ml bovine insulin, and 5 mg/ml bovine serum albumin. Cultures used to detect glutamate receptors by immunofluorescence were prepared by exposing cells to 3×10^{-7} M retinoic acid; (Eastman Kodak) in serum-supplemented medium for 72 h. After the first 48 h on tissue culture dishes, cells were passaged and plated on bacterial grade dishes for the remaining 24 h. Cells were then plated on gelatin-coated (0.15%) coverslips set in 100 mm bacterial grade dishes. Twenty-four hours later the serum-supplemented media was replaced with serum-free media, and cells were incubated undisturbed until 14 days after initial retinoic acid treatment. Cells differentiated by the serum-free method were analysed 14 days following induction with retinol. Long-term cultures were generated by treating the cells with retinoic acid in the presence of serum and then transferring the cultures to serum-free medium at day 12.

Depolarization of P19-derived neurons was achieved by treating the cultures with KCl–Locke's Buffer: 50.4 mM NaCl, 53 mM KCl, 2.15 mM K_2HPO_4, 0.85 mM KH_2PO_4, 1.2 mM $MgCl_2$, 2.2 mM $CaCl_2$, 10 mM HEPES pH 7.2 and 5.6 mM dextrose.[50] Two-week-old neuron-enriched cultures were first rinsed in phosphate-buffered saline (PBS) to remove the growth medium and then depolarized for 2 min with KCl–Locke's buffer. Following this incubation, the buffer was removed and 50 ml of fresh KCl–Locke's were suffused over the culture. In most cases the cells were fixed immediately following the depolarization.

Immunofluorescence

Cultures intended for immunofluorescence were grown on laminin-coated (GABA and glutamate staining) or gelatin-coated coverslips as previously described.[21] After rinsing the cells in PBS, cultures to be stained for glutamate or GABA were fixed in 4% paraformaldehyde plus 0.3% glutaraldehyde for 30–40 min. All other cultures were fixed in 4% paraformaldehyde plus 0.2% picric acid in 160 mM phosphate buffer (pH 6.9). Staining procedures were carried out as described.[21] Briefly, primary antibodies were incubated with the sample at 4°C overnight, except for NMDAR1, which was incubated for 72 h. After washing in PBS, secondaries were added for 30 min at 37°C and, where applicable, cells were washed again in PBS and tertiary was added for 30 min at 37°C. Rabbit anti-glutamate antibodies were diluted in PBS containing 1% bovine serum albumin and 0.1% sodium azide (1:2000, Arnel Products). Detection was by using a biotinylated donkey anti-rabbit antibody (1:50, Amersham) followed by streptavidin–Texas Red (1:100, Amersham) diluted in PBS only. For NMDAR1 staining, the primary, secondary and tertiary antibodies were all diluted in PBS as follows: mouse anti-NMDAR1 antibodies, 1:50 (Pharmingen), biotin-conjugated sheep anti-mouse secondary, 1:50 (Amersham) and CY3-conjugated streptavidin tertiary, 1:100 (Sigma). All other antibodies were diluted in PBS plus 0.3% Triton X-100. For double labelling, mouse anti-glutamate monoclonal antibodies (1:150, Pel-Freez Biologicals) and rabbit anti-GABA antibodies (1:200, Chemicon) were incubated together, as were either rabbit anti-glutamate receptor subunit 1 or 4 (GluR1, GluR4) (both at 1:100, Chemicon) with mouse anti-neuron-specific nucleoprotein (NeuN) (1:50, a gift from Dr R. Mullen). Secondaries were prepared 24 h prior to use as follows: biotinylated sheep anti-mouse antibodies (1:50, Amersham) and fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit antibodies (1:20, Dako) for glutamate/GABA staining, or biotin-conjugated donkey anti-rabbit and FITC-conjugated sheep anti-mouse (1:20, Amersham) for glutamate receptor/NeuN staining. The tertiary was either Texas Red-conjugated (glutamate) or CY3-conjugated (glutamate receptor) streptavidin. Slides were coverslipped with a mounting medium of PBS containing phenylaminediamine (0.1 mM) and 90% glycerol.

Northern analysis

Total cellular RNA was isolated by means of the lithium chloride-urea procedure[1] and separated in 1% agarose containing 1.1 M formaldehyde.[24] The RNA was transfered to Hybond N (Amersham) and hybridized according to standard protocols.[24] The probe for the NMDAR1 transcript was prepared by multiprime labelling and consisted of the 1.2 kilobase EcoRI fragment of pN60, a kind gift from Shigetada Nakanishi.[31] As a loading control, the blot was

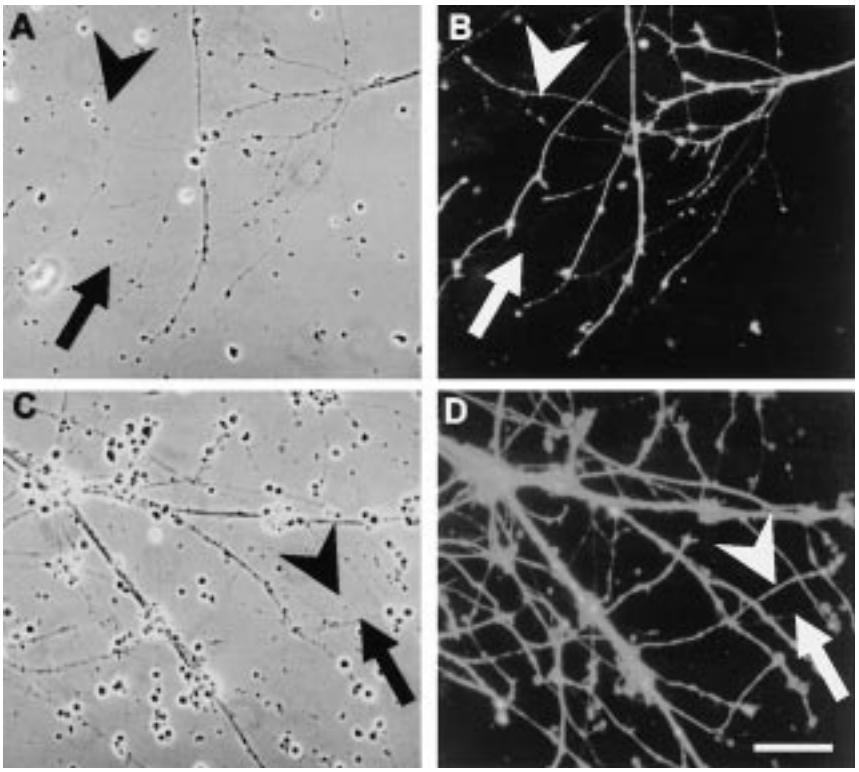

Fig. 1. P19-derived neurons contain glutamate and GABA. P19 cells were differentiated in serum-free medium containing 17 μM retinol, plated on laminin-coated coverslips and allowed to mature for 14 days. (A and C) Phase-contrast of regions of the cultures corresponding to the immunofluorescent photographs in B and D. (B) Immunofluorescent staining for glutamate. The primary was a rabbit anti-glutamate antibody (1:20) followed by a biotinylated donkey anti-rabbit antibody (1:50) and streptavidin–Texas Red (1:100). (D) Immunofluorescent staining for GABA. The primary was a rabbit anti-GABA antibody (1:200) followed by an FITC-conjugated swine anti-rabbit antibody (1:20). In each panel the arrow indicates a process that does not stain with the antibody used while the arrowhead indicates a process that does stain. Note that there is extensive fasciculation of the processes. Scale bar=50 μm.

stripped and reprobed with the PstI to XbaI fragment of the glyceraldehyde-3-phosphate dehydrogenase cDNA.

Electrophysiology

The whole-cell patch-clamp technique[11] was performed on P19-derived neuronal cultures ranging in age from four to 63 days post-retinoid treatment. The results presented are a subset from 93 successful whole-cell recordings. P19 Ringer, used as the external solution, consisted of 140 mM NaCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.3 with 1 N NaOH (280 mosm). The internal patch pipette solution contained 140 mM KCl, 12 mM NaOH, 5 mM EGTA, 10 mM HEPES, 5 mM KATP, and 0.3 mM NaGTP, pH 7.1 (the final solution was diluted by 10% with distilled water). Experiments were performed on the stage of a Zeiss inverted microscope using standard whole-cell attached patch-clamp methods.[11] Cultures were allowed to equilibrate for 1 h in the experimental set-up before any recordings were made. At all times, the cells were continuously superfused with fresh, oxygenated P19 Ringer at room temperature (bath size: 1.5 ml with 0.5 ml/min perfusion rate). Cells that were phase-bright and had neuron-like processes were chosen for study. Patch pipettes of Corning 7052 borosilicate glass with tested d.c. resistances (in recording solution) between 5–10 megohms pulled on a Flaming-Brown P-87 puller were used. The overall experimental protocol was as follows: i) measured seal resistance (10 gigohms), ii) adjusted membrane input capacitance to zero, iii) monitored the cell's increase in input capacitance in order to verify the whole-cell recording, iv) measured whole-cell recording capacitance, v) switched to current-clamp mode to measure resting membrane potential, and vi) recorded spontaneous synaptic activity and verified that cell attained a stable resting potential. Current recordings were made with an Axon Instruments Axoclamp 2A amplifier used in single electrode voltage-clamp mode. Data acquisition and analysis were performed using pCLAMP (Axon Instruments).

RESULTS

P19-derived neurons contain the neurotransmitters glutamate and GABA

Antibodies directed against L-glutamate have been used successfully to detect this neurotransmitter in the vertebrate retina,[8,13,25] cerebellum[45] and hippocampus.[47] P19 cells treated with retinol in glutamate-free, serum-free medium were established in culture for two weeks. Cells differentiated under these conditions gave cultures highly enriched in neurons with almost no non-neuronal cells (Fig. 1A, C). Figure 1B shows the immunofluorescence obtained using polyclonal antisera directed against glutamate in these

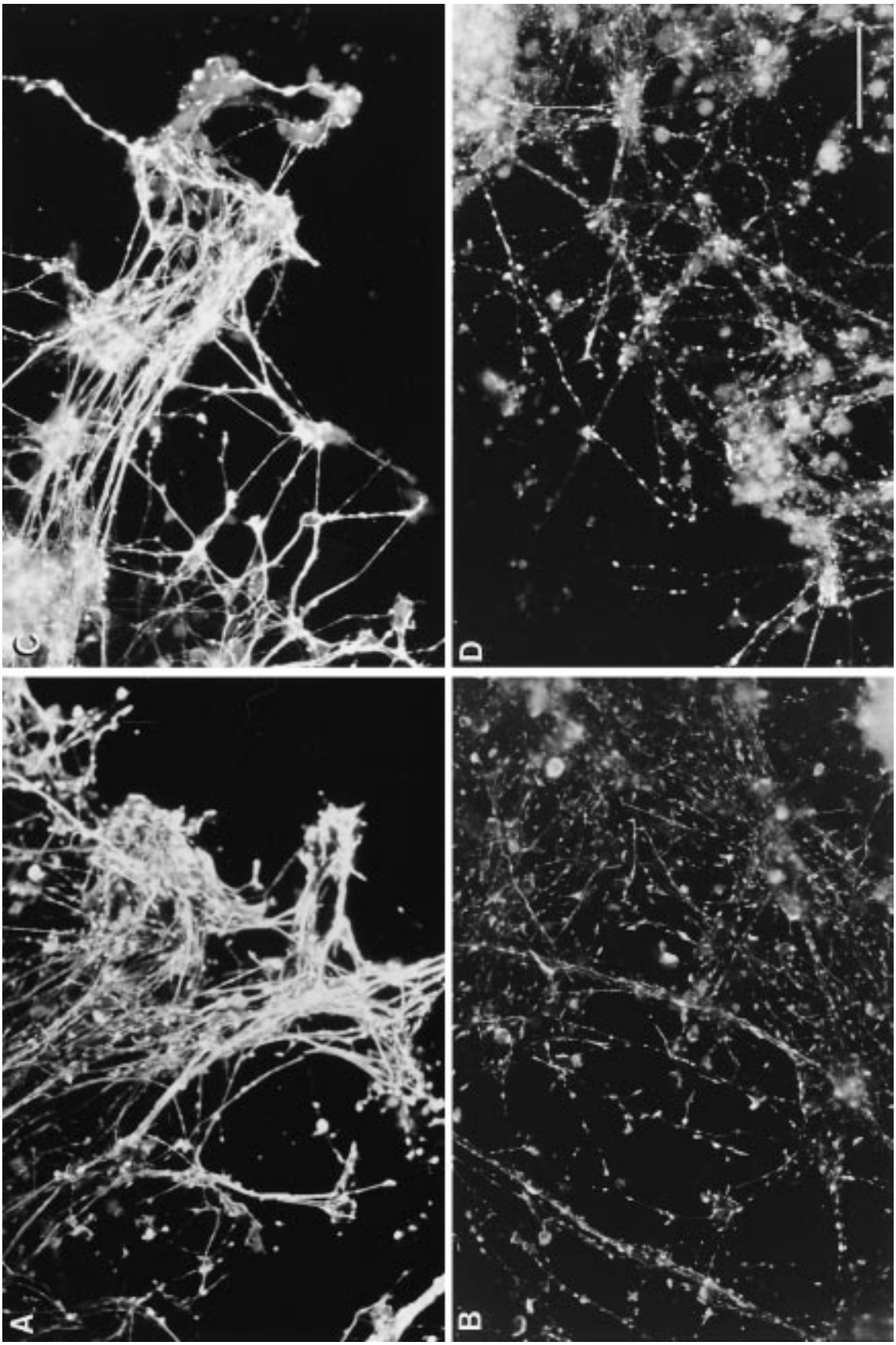

Fig. 2.

cultures. More than 80% of the neuronal processes were labelled with particularly high intensity evident in the varicosities and axonal termini, sites consistent with the storage of a neurotransmitter.[10] This localization of glutamate was detected in P19-derived neurons as early as four days following treatment with retinoids, coincident with the beginnings of neurite extension in culture. GABA was also localized to the varicosities and growth cones of cultured P19 neurons (Fig. 1D).

To ensure that the immunoreactive glutamate and GABA represented neurotransmitters and specifically that the glutamate staining obtained was not simply that of precursor to GABA synthesis, P19 neurons were depolarized using KCl–Locke's buffer. Treatment of neurons with this buffer high in potassium (56 mM) causes a depolarization of neuronal membranes and release of transmitters contained in vesicles. Figure 2A shows the immunofluorescence for P19-derived neurons using polyclonal antisera directed against glutamate. Figure 2B shows a sibling culture stained for glutamate immediately following treatment with KCl–Locke's buffer. Depolarization of P19 neurons caused a significant loss of staining suggesting that most of the glutamate detected in control cells was contained within transmitter vesicles. This loss of staining was not due to cell damage as cultures returned to medium following depolarization and maintained overnight at 37°C remained viable, and the cells again stained intensely with antibodies to glutamate indicating that the glutamate pools were replenished. Furthermore, cultures treated with KCl–Locke's buffer minus calcium stained intensely for glutamate (data not shown). Calcium influx upon depolarization is known to be essential for vesicle fusion with the neuronal cell membrane and release of neurotransmitters.[14] These same experiments were repeated for GABA staining with identical results (Fig. 2C, D).

To determine whether individual cells contained one or both neurotransmitters, we co-stained cultures using a monoclonal antibody against glutamate and a polyclonal serum against GABA. Processes containing glutamate but not GABA (glutamate+/GABA−) were detected (Fig. 3, double headed arrow) as were processes with the reciprocal phenotype, GABA+/glutamate− (Fig. 3, arrowhead). However, in the majority of processes, both glutamate and GABA staining appeared to co-localize (Fig. 3, single arrow). Neuronal processes fasciculate extensively in these cultures suggesting that these GABA+/glutamate+ processes could be derived from distinct neurons whose processes are closely juxtaposed. However, in certain areas, immunostaining for both transmitters

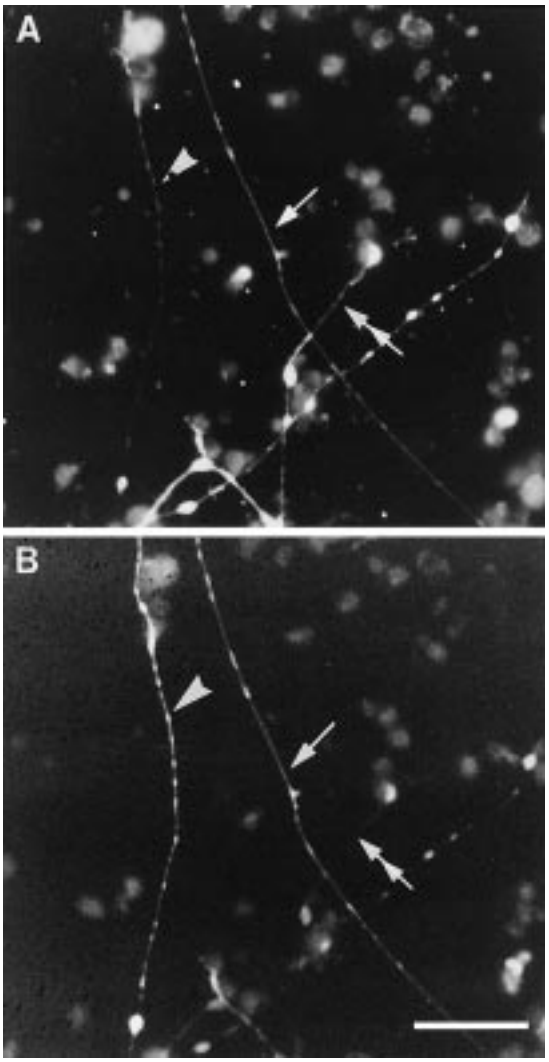

Fig. 3. Double labelling for glutamate and GABA in P19-derived neurons. Cells were differentiated and maintained in serum-free medium for 14 days. (A) Immunofluorescent staining for glutamate. The primary was a mouse anti-glutamate monoclonal antibody (1:150) followed by a biotinylated sheep anti-mouse antibody (1:50) and streptavidin–Texas Red (1:100). (B) Immunofluorescent staining for GABA. The primary was a rabbit anti-GABA antibody (1:200) followed by an FITC-conjugated swine anti-rabbit antibody (1:20). Double-headed arrow: glutamate+/GABA−; arrowhead: glutamate−/GABA+; single arrow: glutamate+/GABA+. Scale bar=100 μm.

Fig. 2. Depolarization of P19 neurons causes glutamate and GABA neurotransmitter release. P19 cells were differentiated and maintained in serum-free medium for 14 days as described in Fig. 1. (A and B) Immunofluorescent staining for glutamate. The primary was a rabbit anti-glutamate antibody (1:20) followed by a biotinylated donkey anti-rabbit antibody (1:50) and streptavidin–Texas Red (1:100). Exposure time for each panel was 4 s. (C and D) Immunofluorescent staining for GABA. The primary was a rabbit anti-GABA antibody (1:200) followed by an FITC-conjugated swine anti-rabbit antibody (1:20). Exposure time for each panel was 8 s. (B and D) Cultures were depolarized with KCl–Locke's Buffer (56 mM KCl) immediately prior to fixation for immunofluorescence. Scale bar=100 μm.

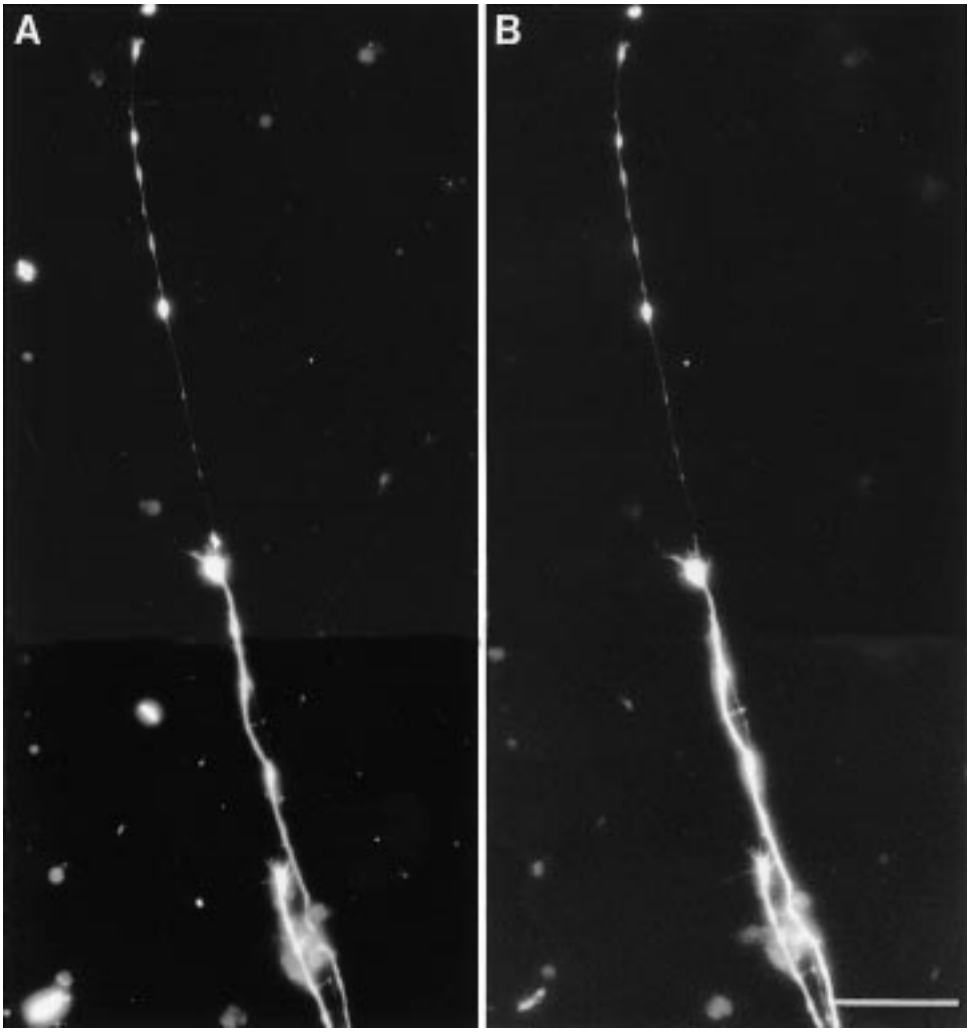

Fig. 4. Glutamate and GABA appear to co-localize to the same neuron. Cells were grown, fixed and stained exactly as for Fig. 3. (A) Immunofluorescent staining for glutamate. (B) Immunofluorescent staining for GABA. Scale bar=50 μm.

was strong in the varicosities and growth cones of neurons (Fig. 4) and the distribution of the two transmitters was identical in the filopodia of each growth cone. Thus single neurons might carry vesicles containing both glutamate and GABA.

Because of the widespread fasciculation of processes, it was difficult to determine the proportion of neurons that reacted with antibodies to glutamate and to GABA. Our best estimate is that about 60% of process-bearing cells contain immunoreactive glutamate and an equal proportion contain GABA.

P19-derived neurons express the glutamate receptor subunits GluR1, GluR4 and NMDAR1

Transcripts encoding the NMDAR1 glutamate receptor subunit were detected in P19 cells that had differentiated into neurons but not in undifferentiated P19 cells (Fig. 5). Total RNA was isolated from various P19 cultures and examined by Northern analysis using the NMDAR1 cDNA as probe. Cultures enriched in neurons by the serum-free method were harvested at 14 days and the NMDAR1 transcripts were evident. Long-term cultures differentiated with retinoic acid in the presence of serum were harvested at 34 days post-induction but the NMDAR1 transcript not detected. These serum-containing cultures are rich in non-neuronal cells such as astrocytes which proliferate and vastly outnumber the neurons, so the proportion of neuronal RNA in these cultures is very low. Astrocytes do not express the NMDAR1 transcript.[7] The NMDAR1 probe detected two transcripts in P19 neurons as well as the mouse and rat brain RNA controls. Alternative splicing of the NMDAR1 transcript yields at least seven isoforms of the NMDAR1 receptor (denoted R1A through R1G) varying in size from 3789 to 4011 nucleotides.[49]

The Northern analysis demonstrating the existence of the NMDAR1 receptor subunit was confirmed

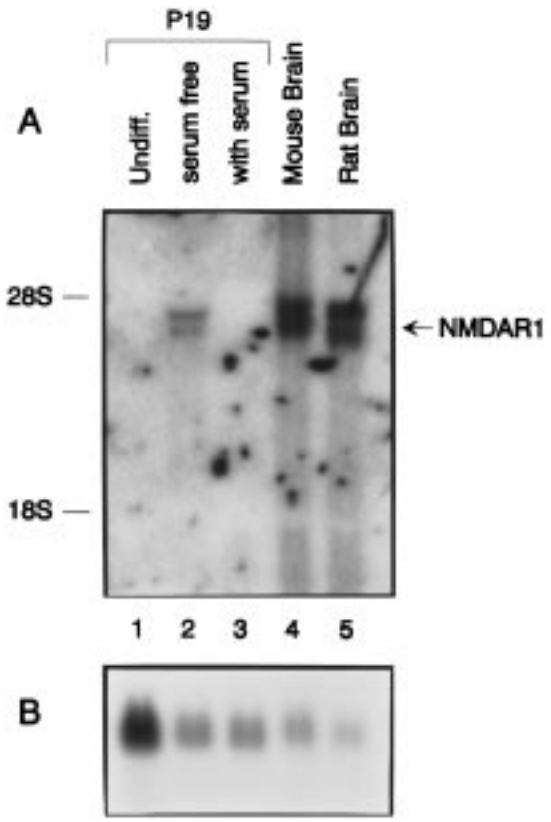

Fig. 5. Northern blot showing expression of NMDAR1 subunit transcripts. Lane 1, undifferentiated P19 cells. Lane 2, P19 cells differentiated and maintained in serum-free medium for 14 days. Lane 3, P19 cells differentiated with retinoic acid in the presence of serum and transferred to serum-free medium at day 12. Cultures were harvested at day 34. Lane 4, total brain from adult mice. Lane 5, total brain from adult rats. Each lane contains 20 μg of total RNA. (A) Northern blot probed with the EcoRI fragment of pN60 prepared by multiprime labelling. Following hybridization, the blot was washed in 0.2 × standard saline citrate plus 0.1% sodium dodecyl sulphate at 42°C. The blot was exposed to X-ray film overnight at −70°C. The NMDAR1A transcript is 3948 nt. (B) As a loading control, the blot was reprobed with a multiprime-labelled PstI to XbaI fragment of the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase cDNA.

by immunohistochemistry. The NMDAR1 subunit protein was not detected in undifferentiated P19 cells (Fig. 6A) but appeared as early as day 5 post-differentiation. By day 7 post-differentiation, NMDAR1 protein was detected in more than 85% of P19 cells with neuronal morphology (Fig. 6B). NMDAR1 expression was maintained until to at least day 28. These results have been confirmed by Western blot examining protein extracts from retinoic acid-treated P19 cultures at day 7 and day 12 (data not shown).

The presence of mRNAs encoding various AMPA receptor subunits was previously demonstrated by Ray and Gottlieb[39] using reverse transcription-PCR and a pair of non-specific oligonucleotides for GluR subunit types 1 through 4. Using immunological detection procedures, we found that both the GluR1 and GluR4 subunits were present on P19 neurons. The proportion of P19 neurons expressing GluR1 was quantified by labelling neurons with antibody to the neuron specific nucleoprotein, NeuN.[34] Over 75% of NeuN-positive neurons also expressed GluR1 (Fig. 6C, D). As observed for the NMDAR1 subunit, expression of GluR1 appeared by day 5 post-differentiation, and the level of expression reached by day 7 was maintained until at least day 21 (also confirmed by Western blot, data not shown).

Definitive positive staining for the GluR4 subunit was not observed in NeuN-positive P19 neurons by immunohistochemistry. However, Western blot analysis using antibodies directed against GluR4 did show a positive band at 110000 mol. wt in P19 neurons 12 days post- differentiation (data not shown).

P19-derived neurons form glutamatergic synapses in vitro

P19-derived neurons contain the neurotransmitter glutamate and express glutamate receptors suggesting that they could form functional glutamatergic synapses *in vitro*. It has been previously demonstrated that these neurons form synapses *in vitro* and are immunoreactive for synaptophysin, an integral membrane protein present on presynaptic neurons.[29a] The expression of this protein correlates with synapse formation.[41] To further investigate the nature of these synapses, whole-cell patch-clamp recordings were made. In 24-day-old cultures, spontaneous synaptic activity was recorded from the soma of these neurons (Fig. 7A). When 1 mM L-glutamate was briefly injected into the superfusate, the neurons exhibited dramatic increases in synaptic activity, leading to generation of action potentials (Fig. 7B). The brief hyperpolarization following the action potentials (Fig. 7B2) indicates the presence of delayed rectifier-type K^+ channels. Thus P19 neurons undergo electrophysiological maturation in culture much the same as they do when grafted into the striatum of adult rats.[23] That these action potentials fire in response to added glutamate is further evidence for the presence of functional glutamate receptors on these neurons.

Spontaneous synaptic activity could be observed from cells as young as six days in culture (data not shown). The amplitude, frequency and complexity of these spontaneous potentials increased steadily over the three-week period of our experiments. Figure 8 illustrates that some of the synaptic inputs are glutamatergic in origin. In this example, the same cell had its synaptic activity recorded first in P19 Ringer (Fig. 8A), then in the presence of the non-specific glutamate antagonist kynurenic acid (1 mM; Fig. 8B), and then again following a wash in normal P19 Ringer solution (Fig. 8C). Kynurenic acid caused a clear reduction in the synaptic activity of P19-derived

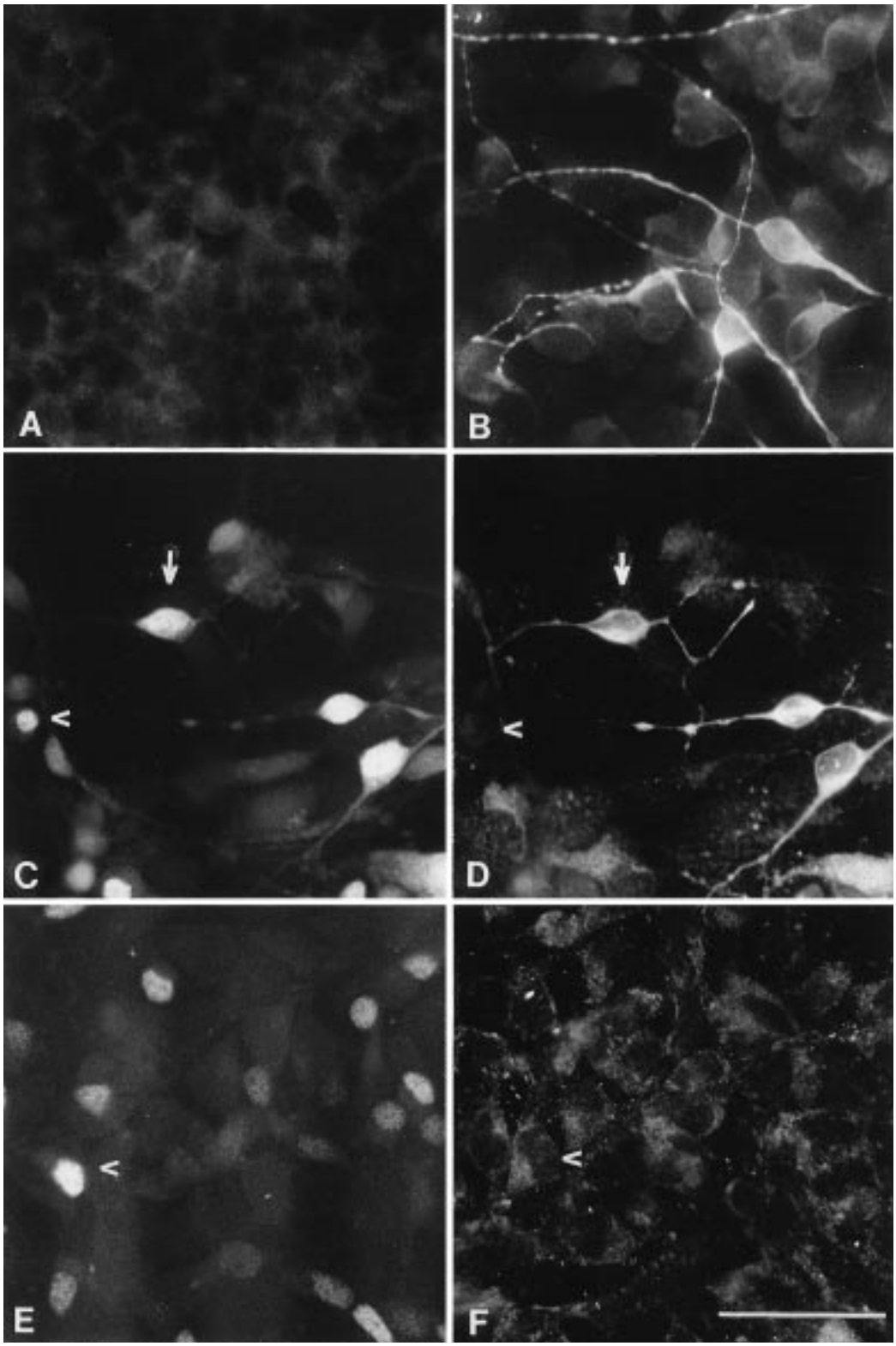

Fig. 6. Immunofluorescent staining for glutamate receptor subunits and neuronal markers in undifferentiated P19 cells and P19-derived neurons. (A) Undifferentiated P19 cells show no expression of the NMDAR1 subunit, whereas NMDAR1 is present in a high proportion of differentiated P19 cells with neuronal morphology (B). GluR1 (D) is expressed in a high percentage of P19 neurons which also stain positive for the neuron-specific nucleoprotein marker, NeuN (C). GluR4 (F) staining was not apparent in any of the NeuN-positive P19 neurons (E). Arrows indicate double-positive neurons for NeuN and respective receptor. Arrow heads indicate neurons which are NeuN positive only. Scale bar=50 μm.

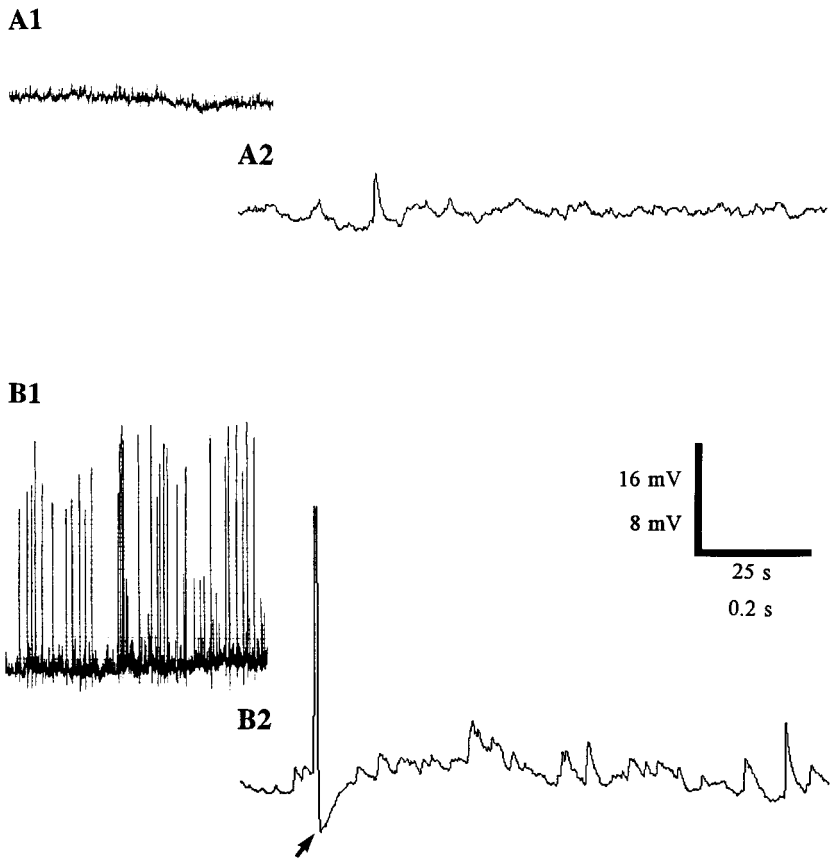

Fig. 7. Electrophysiological maturation of P19-derived neurons in culture. Shown are representative tracings from whole-cell patch-clamp experiments performed on a P19-derived neuron 24 days post-differentiation. (A) Spontaneous synaptic activity. A2 is a higher speed recording of a region of A1. (B) Recordings from the same region of the cell soma after it had been puffed with 1 mM L-glutamate (10 ms puffs, delivered at 2 Hz for 25 s). Note that the depolarizing response of the cell to the applied glutamate is not shown. There is a 2.5 min gap between the end of A1 and the beginning of B1. The large amplitude events in B1 are action potentials whose amplitude has been clipped by the chart recorder. B2 is a higher speed recording of a region of B1. The arrow in B2 shows a high gain example of one of these action potentials. Note that the increased activity in B1 lasts far longer than the period of glutamate application.

neurons, decreasing both the mean amplitude and the absolute number of synaptic events. Following the wash, synaptic activity returned to near pretreatment levels. Data for three cells treated in this manner are shown in Fig. 9. Thus, much of the synaptic transmission occurring between P19-derived neurons *in vitro* appears to be glutamatergic.

Tetrodotoxin (TTX) at 0.3–1.0 μM reduced spontaneous synaptic activity recorded in the cell soma but did not eliminate it (data not shown). The persistent synaptic signals in TTX suggests that at least some of the activity was due to spontaneous transmitter release and not that resulting from action potentials in presynaptic neurons.

DISCUSSION

Here we have demonstrated that P19-derived neurons contain L-glutamate as a neurotransmitter. Glutamate immunoreactivity was particularly intense at varicosities along the axons and growth cones, areas consistent with transmitter storage.[10] In addition, depolarization of these neurons with buffers high in potassium caused a calcium-dependent reduction in staining we attribute to release of glutamate from secretory vesicles. The depletion of glutamate stores did not occur in high potassium solutions without calcium suggesting that voltage-dependent calcium channels are present in the neurons and direct electrophysiological measurements indicate the presence of such calcium channels.[2] However, voltage-dependent calcium channels have been reported to be absent from P19-derived neurons[3,16,32] so the presence and nature of these channels remains controversial.

The presence of spontaneous depolarizing potentials which are sensitive to the non-specific glutamate receptor antagonist kynurenate is consistent with the idea that the glutamate contained in these neurons can fullfil the role of transmitter and is capable of

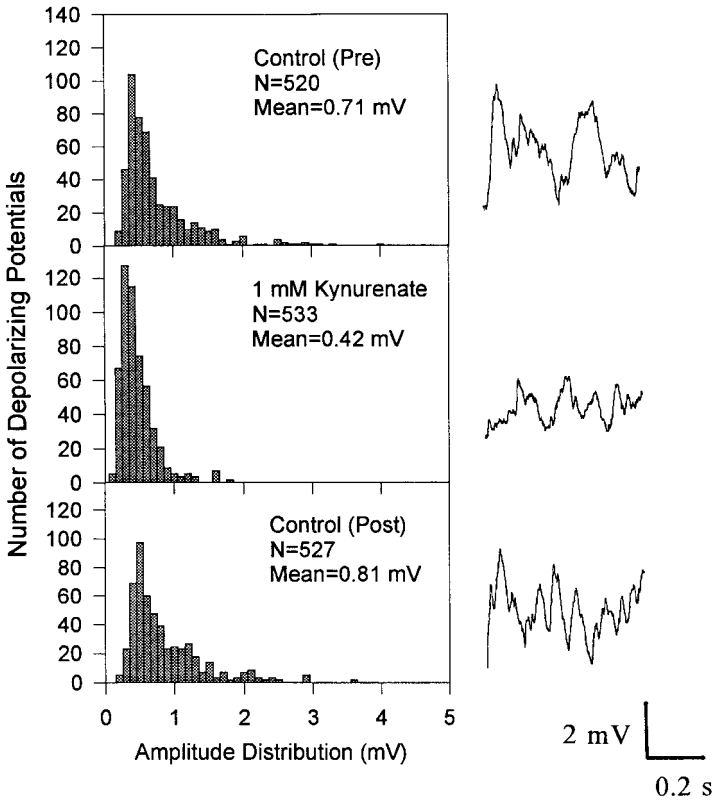

Fig. 8. Spontaneous synaptic activity of P19-derived neuron from a 17-day-old culture. Histograms represent counts of depolarizing potentials during stable, carefully monitored membrane potential and with low electrode noise during a 16 s period. All depolarizing potentials above baseline noise were measured on a digitizing bitpad from high-gain printouts of the digital records. Examples on the right are taken from the data block used to generate the histogram. Kynurenate (1 mM) results in a decreased mean amplitude, but comparable numbers of depolarizing events. This cell and the others in Fig. 9 were recorded using KCl-containing electrodes.

synaptic transmission. That this spontaneous activity was not eliminated by kynurenate indicates that other transmitters capable of generating depolarizing synaptic potentials must also be contained in P19-derived neurons, consistent with previous studies demonstrating the presence of various neurotransmitters in these neurons.[46] In particular, it is probable that some of these potentials are attributable to GABA or glycine-generated potentials which are depolarizing at early stages of neuronal development[19,22] and with the use of KCl-filled recording electrodes. The dampening effect of kynurenic acid on the synaptic potentials plus the strong depolarizations resulting from the addition of exogenous glutamate suggest the formation of glutamatergic neural networks in mature P19 neuronal cultures. By three weeks post-differentiation, the P19-derived neurons had matured electrophysiologically to the point of being able to generate action potentials and exhibited glutamate-mediated synaptic transmission. These observations are in agreement with similar results on P19-derived neurons implanted into rat striatum.[22,23]

A recent report demonstrates that pairs of cultured P19 neurons can be coupled by synapses.[9] Using electrophysiological recordings of pairs of P19 neurons, these workers showed that stimulation of one cell could evoke fast excitatory or inhibitory currents in neighbouring cells. The presence of both NMDA and non-NMDA receptor-mediated responses was elegantly demonstrated in this work. Thus, the anatomically-defined synapses described earlier[29a] have been shown to be functional and both excitatory and inhibitory in nature.

Several reports have now documented the presence of both NMDA and non-NMDA type glutamate receptors in P19 neurons. Using the Ca^{2+} specific fluoroprobe Fura-2, we have previously measured the internal Ca^{2+} concentration of P19 neurons in the presence of glutamate and NMDA as well as NMDA receptor-specific antagonists.[32] Calcium influx from the external medium, which was dependent on the presence of glycine and could be blocked by pretreating the cells with either Mg^{2+} or MK-801, established the presence of the NMDA receptor in these cultured neurons. Similarly, Turetsky *et al.*[51] have shown that inward whole-cell currents can be activated by excitatory amino acids in P19 neurons, while undifferentiated cells show no such response. The immunofluorescence data indicate the presence of the

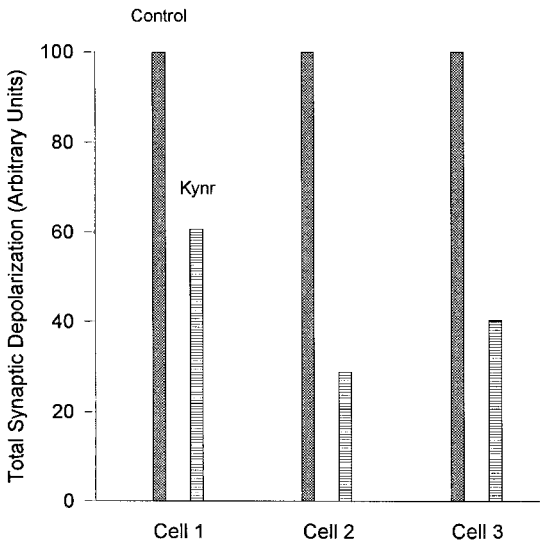

Fig. 9. Histograms showing effects of 1 mM kynurenate on spontaneous synaptic activity. The product of the number of events and their amplitudes was measured as in Fig. 8. Cell 1 is the same as depicted in Fig. 8. For each cell, control measures are indicated on the left and measures during kynurenate application are on the right. Ages of the cultures for these cells were 1, 17 days; 2, 17 days; 3, 14 days.

NMDAR1 and GluR1 receptors on both the cell soma and processes, a staining pattern similar to that seen in the monkey and rat hippocampus.[38,44] Moriyoshi *et al.*[31] have shown that NMDAR1 subunit mRNA can be detected throughout much of the rat brain so the presence of the NMDAR1 protein in most P19-derived neurons is consistent with these neurons resembling those of the CNS.

Ray and Gottlieb[39] demonstrated that undifferentiated P19 cells express the NMDAR2A and NMDAR2B transcripts but not those encoding NMDAR1. The NMDAR1 subunit mRNAs were only detected in P19 cells following induction with retinoic acid. Since functional NMDA receptors require the NMDAR1 subunit,[17,30] these observations explain the absence of NMDA-triggered whole-cell currents and Ca^{2+} influx in undifferentiated P19 cells despite the presence of NMDAR2A and 2B transcripts.[39] Consistent with the work of Turetsky *et al.*[51] and that of Ray and Gottlieb,[39] we have found that the NMDAR1 subunit is expressed only in P19 neurons and not in undifferentiated cells. NMDA receptor activity and function then are apparently regulated in these cells at the level of expression of the R1 subunit.

With respect to AMPA/kainate subtypes of glutamate receptor subunits, Turetsky *et al.*[51] have demonstrated that kainate and glutamate both elicit linear current–voltage relations in P19 neurons. This they argue suggests that the GluR2 receptor subunit is expressed on these cells. The specific identity of other non-NMDA type subunits has, however, remained unclear. Ray and Gottlieb[39] demonstrated the presence of AMPA/kainate receptor subunit transcripts using non-specific PCR primers selective for GluR1 through GluR4. Here we demonstrate that P19 neurons express the GluR1 protein. The detection of GluR4 by Western blot only may indicate that GluR4 is expressed in P19 neurons at relatively low levels, below the detection limit of immunohistochemistry. If the GluR1/GluR4 ratio is high as the immunological detection suggests and if GluR2 is also present in P19-derived neurons, this pattern of AMPA/kainate receptor subunit expression is similar to that seen in areas CA1 and CA3 of the hippocampus.[37] That P19 neurons bear a resemblance to both hippocampal and cortical neurons has been suggested previously by characterization of their neurotransmitter profile[46] and their electrophysiology.[22]

In the adult CNS glutamate and GABA are the predominant excitatory and inhibitory transmitters, respectively. The apparent co-localization of both neurotransmitters in single P19-derived neurons was unexpected although not unprecedented. Bipolar neurons in the nuclear layer of the primate[13] and salamander[52] retina as well as horizontal and amacrine cells in the retina of the lizard *Anolis*[43] have been shown to contain both glutamate and GABA as transmitters. Alternatively, these GABA+/glutamate+ cells may represent very early neurons with plastic transmitter character. In many instances the choice of transmitter synthesized is directly influenced by the interactions between the presynaptic cell and its postsynaptic partner. As a consequence, the phenotype of a neuron need not be firmly preprogrammed early in development and such a neuron may initially express a variety of transmitters. Most sympathetic neurons, for example, are catecholaminergic. However, a subset of sympathetic neurons-initially expressing norepinephrine switch their phenotype and synthesize acetylcholine upon innervating the sweat glands.[18] Glucocorticoids have been shown to regulate the phenotype of postmitotic differentiated sympathetic neurons in the developing superior cervical ganglion[29] as well as influence transmitter synthesis within the adult brain. Neurons of the paraventricular nucleus of the hypothalamus express at least eight different transmitters/peptides simultaneously and expression of these transmitters fluctuates in response to hormone levels.[36] P19 neurons may then exercise a similar plasticity of phenotype, expressing glutamate or GABA at the expense of the other only in response to specific hormones or neurotrophins not present in culture, or only following the firm establishment of functional synapses.

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REFERENCES

1. Auffray C. and Rougeon F. (1980) Purification of mouse immunoglobulin heavy-chain messenger RNA from total myeloma tumor RNA. *Eur. J. Biochem.* **107,** 303–314.
2. Canzoniero L. M. T., Sensi S. L., Turetsky D. M., Finley M. F. A., Choi D. W. and Huettner J. E. (1966) Glutamate receptor-mediated calcium entry in neurons derived from P19 embryonal carcinoma cells. *J. Neurosci. Res.* **45,** 226–236.
3. Cheun J. E. and Yeh H. H. (1991) Differentiation of a stem cell line toward a neuronal phenotype. *Int. J. dev. Neurosci.* **9,** 391–404.
4. Choi D. W. (1988) Glutamate neurotoxicity and diseases of the nervous system. *Neuron* **1,** 623–634.
5. Cline H. T., Debski E. A. and Constatine-Paton M. (1987) NMDA receptor antagonist desegregates eye-specific stripes. *Proc. natn. Acad. Sci. U.S.A.* **84,** 4342–4345.
6. Collingridge G. L. and Singer W. (1990) Excitatory amino acid receptors and synaptic plasticity. *Trends pharmac. Sci.* **11,** 290–296.
7. Conti F., Minelli A., Molnar M. and Brecha N. (1994) Cellular localization and laminar distribution of NMDAR1 mRNA in the rat cerebral cortex. *J. comp. Neurol.* **343,** 554–565.
8. Ehinger B., Ottersen O. P., Storm-Mathisen J. and Dowling J. E. (1988) Bipolar cells in the turtle retina are strongly immunoreactive for glutamate. *Proc. natn. Acad. Sci. U.S.A.* **85,** 8321–8325.
9. Finley M. F., Kulkarni N. and Huettne J. E. (1996) Synapse formation and establishment of neuronal polarity by P19 embryonic carcinoma cells and embryonic stem cells. *J. Neurosci.* **16,** 1056–1065.
10. Gordon-Weeks P. R., Lockerbie R. O. and Pearce B. R. (1984) Uptake and release of [^{3}h]GABA by growth cones isolated from neonatal cat brain. *Neurosci. Lett.* **52,** 205–212.
11. Hamill O. P., Marty A., Neher E., Sakmann B. and Sigworth F. J. (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.* **391,** 85–100.
12. Izquierdo I. (1991) Role of NMDA receptors in memory. *Trends pharmac. Sci.* **12,** 128–129.
13. Kalloniatis M. and Marc R. E. (1992) Glutamate, GABA and glycine immunoreactivity in the Macaque monkey retina. *Soc. Neurosci. Abstr.* **18,** 393.
14. Kelly R. B. (1993) Storage and release of neurotransmitters. *Cell* **72 (Suppl.),** 43–53.
15. Kleinschmidt A., Bear M. F. and Singer W. (1987) Blockade of NMDA receptors disrupts experience-dependent plasticity of kitten striate cortex. *Science* **238,** 355–358.
16. Kubo Y. (1989) Development of ion channels and neurofilaments during neuronal differentiation of mouse embryonal carcinoma cell lines. *J. Physiol.* **409,** 497–523.
17. Kutsuwada T., Kashiwabuchi N., Mori H., Sakimura K., Kushiya E., Araki K., Meguro H., Masaki H., Kumanishi T., Arakawa M. and Mishina M. (1992) Molecular diversity of the NMDA receptor channel. *Nature* **358,** 36–41.
18. Landis S. C. (1980) Developmental changes in the neurotransmitter properties of dissociated sympathetic neurons: A cytochemical study of the effects of medium. *Devl Biol.* **77,** 349–361.
19. Lin P., Kusano K., Zhang Q., Felder C. C., Geiger P. M. and Mahan L. C. (1996) $GABA_A$ receptors modulate early spontaneous excitatory activity in differentiating P19 neurons. *J. Neurochem.* **66,** 233–242.
20. Lynch G., Larson J., Kelso S., Barrionuevo G. and Schottler F. (1983) Intracellular injections of EGTA block induction of hippocampal long-term potentiation. *Nature* **305,** 719–721.
21. MacPherson P. A. and McBurney M. W. (1995) P19 embryonal carcinoma cells: A source of cultured neurons amenable to genetic manipulation. *Methods, A Companion to Methods in Enzymology* **7,** 238–252.
22. Magnuson D. S. K., Morassutti D. J., McBurney M. W. and Marshall K. C. (1995) Neurons derived from P19 embryonal carcinoma cells develop responses to excitatory and inhibitory neurotransmitters. *Devl Brain Res.* **90,** 141–150.
23. Magnuson D. S. K., Morassutti D. J., Staines W. A., McBurney M. W. and Marshall K. C. (1995) *In vivo* electrophysiological maturation of neurons derived from a multipotent precursor (embryonal carcinoma) cell line. *Devl Brain Res.* **64,** 130–141.
24. Maniatis T., Fritsch E. F. and Sambrook J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbour Laboratory. Cold Spring Harbour, New York.
25. Marc R. E., Liu W.-L. S., Kalloniatis M., Raiguel S. F. and Van Haesendonck E. (1990) Patterns of glutamate immunoreactivity in the goldfish retina. *J. Neurosci.* **10,** 4006–4034.
26. Mattson M. P., Murrain M., Gutherie P. B. and Kater S. B. (1989) Fibroblast growth factor and glutamate: Opposing roles in generation and degeneration of hippocampal neuroarchitecture. *J. Neurosci.* **9,** 3728–3740.
27. Mattson M. P., Dou P. and Kater S. B. (1988) Outgrowth-regulating actions of glutamate in isolated hippocampal pyramidal neurons. *J. Neurosci.* **8,** 2087–2100.
28. Mattson M. P., Lee R. E., Adams M. E., Gutherie P. B. and Kater S. B. (1988) Interactions between entorhinal axons and target hippocampal neurons: A role for glutamate in the development of hippocampal circuitry. *Neuron* **1,** 865–876.

29a McBurney M. W., Reuhl K. R., Ally A. I., Nasipuri S., Bell J. C. and Craig J. (1988) Differentiation and maturation of embryonal carcinoma-derived neurons in cell culture. *J. Neurosci.* **8,** 1063–1073.

29. McLennan I. S., Hill C. E. and Hendry I. A. (1980) Glucocorticoids modulate transmitter choice in developing superior cervical ganglion. *Nature* **283,** 206–207.
30. Monyer H., Sprengel R., Schoepfer R., Herb A., Higuchi M., Lomeli H., Burnashev N., Sakmann B. and Seeburg P. H. (1992) Heteromeric NMDA receptors: Molecular and functional distinction of subtypes. *Science* **256,** 1217–1221.
31. Moriyoshi K., Masu M., Ishii T., Shigemot R., Mizuno N. and Nakanishi S. (1991) Molecular cloning and characterization of the rat NMDA receptor. *Nature* **354,** 31–37.

32. Morley P., MacPherson P., Whitfield J. F., Harris E. W. and McBurney M. W. (1995) Glutamate receptor-mediated calcium surges in neurons derived from P19 cells. *J. Neurochem*. **65,** 1093–1099.
33. Morris R. G. M., Anderson E., Lynch G. S. and Baudry M. (1986) Selective impairment of learning and blockade of long-term potentiation by an NMDA receptor antagonist AP5. *Nature* **319,** 774–776.
34. Mullen R. J., Buck C. R. and Smith A. M. (1992) NeuN: A neuronal specific nuclear protein in vertebrates. *Development* **116,** 201–211.
35. Nicoll R. A., Kauer J. A. and Malenka R. C. (1988) The current excitement in long-term potentiation. *Neuron* **1,** 97–103.
36. Patterson P. H. and Nawa H. (1993) Neuronal differentiation factors/cytokines and synaptic plasticity. *Cell*. **72**. **10 (Suppl.),** 123–137.
37. Petralia R. S. and Wenthold R. J. (1992) Light and electron immunocytochemical localization of AMPA-selective receptors in the rat brain. *J. comp. Neurol.* **318,** 329–354.
38. Petralia R. S., Yokotani N. and Wenthold R. J. (1994) Light and electron microscope distribution of the NMDA receptor subunit NMDAR1 in the rat nervous system using a selective anti-peptide antibody. *J. Neurosci.* **14,** 667–696.
39. Ray W. J. and Gottlieb D. I. (1993) Expression of ionotropic glutamate receptor genes by P19 embryonal carcinoma cells. *Biochem. biophys. Res. Commun.* **197,** 1475–1482.
40. Reh T. A. and Constatine-Paton M. (1985) Eye-specific segregation requires neural activity in the three-eyed *Ranas pipens*. *J. Neurosci.* **5,** 1132–1143.
41. Schilling K., Barc E. B., Rhinehart D. and Pilgrim C. (1989) Expression of synaptophysin and neuron-specific enolase during neuronal differentiation *in vitro*: Effects of dimethyl sulfoxide. *J. Neurosci. Res.* **24,** 347–354.
42. Schmidt T. J. (1990) Long-term potentiation and activity dependent retinotropic sharpening in the regenerating retinotectal projection of goldfish: Common sensitive period and sensitivity to NMDA blockers. *J. Neurosci.* **10,** 233–246.
43. Sherry D. M. and Ulshafer R. J. (1992) Neurotransmitter-specific identification and characterization of neurons in the all-cone retina off *Anolis carolinensis*. II Glutamate and aspartate. *Vis. Neurosci.* **9,** 313–323.
44. Siegel S. J., Borse N., Janssen W. G., Gasic G. P., Jarn R., Heinemann S. F. and Morrison J. H. (1994) Regional, cellular and ultrastructural distribution of *N*-methyl-D-aspartate subunit 1 in monkey hippocampus. *Proc. natn. Acad. Sci. U.S.A.* **91,** 564–568.
45. Somogyi P., Halasy K., Somogyi J., Storm-Mathisen J. and Ottersen O. P. (1986) Quantification of immunogold labeling reveals enrichment of glutamate in mossy and parallel fiber terminals in cat cerebellum. *Neuroscience* **19,** 1045–1050.
46. Staines W. A., Morassutti D. J., Reuhl K. R., Ally A. I. and McBurney M. W. (1994) Neurons derived from P19 embryonal carcinoma cells have varied morphologies and neurotransmitters. *Neuroscience* **58,** 735–751.
47. Storm-Mathisen J., Leknes A. K., Bore A. T., Vaaland V. L., Edminson P., Huang F. M. S. and Ottersen O. P. (1983) First visualization of glutamate and GABA in neurons by immunocytochemistry. *Nature* **301,** 517–520.
48. Stretava D. W., Schatz C. J. and Stryker M. P. (1988) Modification of retinal ganglion cell axon morphology by prenatal infusion of tetrodotoxin. *Nature* **336,** 468–471.
49. Sugihara H., Moriyoshi K., Ishii T., Masu M. and Nakanishi S. (1992) Structures and properties of seven isoforms of the NMDA receptor generated by alternative splicing. *Biochem. biophys. Res. Commun.* **185,** 826–832.
50. Trifaró J. M. and Lee R. W. H. (1980) Morphological characteristics and stimulus-secretion coupling in bovine adrenal chromaffin cell cultures. *Neuroscience* **5,** 1533–1546.
51. Turetsky D. M., Huettner J. E., Gottlieb D. I., Goldberg M. P. and Choi D. W. (1993) Glutamate receptor-mediated currents and toxicity in embryonal carcinoma cells. *J. Neurobiol.* **24,** 1157–1169.
52. Yang C. Y. and Yazulla S. (1994) Glutamate-, GABA-, and GAD-immunoreactivities co-localize in bipolar cells of tiger salamander retina. *Vis. Neurosci.* **11,** 1193–1203.

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