Neuroscience 200 (2012) 248–260

ULTRASTRUCTURAL CHARACTERIZATION OF RAT NEURONS IN PRIMARY CULTURE

F. ROBERT,[1] J-F. CLOIX AND T. HEVOR*

Laboratoire de Neurobiologie, Université d'Orléans, BP 6759, Rue de Chartres, F-45067 Orléans CEDEX 2, France

Abstract—Few studies have addressed the ultrastructure and morphology of neurons in primary pure culture. We therefore use immunohistochemistry and electron microscopy to investigate the ultrastructure of cultured neurons during extended incubation *in vitro*. Rat cerebral cortex neurons were cultured in Neurobasal™ medium. Adherent cells developed as networks of single neurons or clusters depending on the plating density. Almost all surviving cells were neurons as demonstrated by neurofilament immunolabeling. The number of cultured neurons increased substantially to 14–21 days *in vitro* (DIV) and then plateaued and subsequently declined. From DIV 1–10 neurons extended large neurites, followed by the development of fine and dense neurites, and neurones survived until DIV 30–50. Notably, numerous mitochondria were observed along fibrous elements within neurites, suggestive of active intracellular trafficking. Electron microscopy also revealed that multiple types of synapses were formed between neurons. These ultrastructural results confirm previous reports of electrophysiological activity in cultured neurons. However many neurons contained distorted mitochondria and abnormal organelles including multilamellar vesicles and multivesicular myeloid bodies. The proportion of neurons containing abnormal organelles increased significantly in culture medium supplemented with antibiotics. On long-term culture neuronal death and apoptotic nuclei were observed. Despite the presence of abnormal organelles, the ultrastructure of cultured neurons was very similar to that of *in vivo* neurons; *in vitro* culture therefore provides a useful tool for studies on neuronal development, aging, and neurotransmission.

Key words: electron microscopy, neuronal growth, neurites, synapses, multilamellar vesicles, apoptosis.

The high diversity of cell types in the brain, often with long processes that traverse different brain regions, presents a significant obstacle to neurochemical and physiological investigations into the roles that distinct cell types play in the CNS. To circumvent this issue Booher and Sensenbrenner (1972) described methods for obtaining pure cultures of neurons and astrocytes. Nevertheless, the neuronal cultures obtained were often contaminated by astroglial cells, and moreover, the life span of the neurons was short (Pettmann et al., 1979; Louis et al., 1981). A culture medium without serum was developed by Brewer and colleagues (Brewer et al., 1993) that allows the preparation of pure cultures of long-living neurons, and this method has been used to investigate many aspects of neuronal function including growth and development, the role of reactive oxygen species, and the regulation of neuronal firing. Despite numerous biochemical, physiological, and pathological studies on cultured mammalian neurons, a thorough ultrastructural investigation of these neurons has not yet been performed. In the present investigation, we sought to study the growth, morphology, and ultrastructure of such cultured rat neurons. We report that the ultrastructure of cultured neurons is very similar to that of *in vivo* neurons, and we observed robust formation of multiple types of synapse in culture. However, abnormal mitochondria, multilamellar vesicles, and multivesicular myeloid bodies were frequent in these neurons. Because antibiotics are often added to culture media, we addressed their potential influence on neuronal structure. Finally, we studied the survival and cell death of neurons maintained in culture.

EXPERIMENTAL PROCEDURES

Reagents

Neurobasal™ medium and B27 supplement were purchased from Gibco (catalog numbers 21103 and 17504 respectively, Gibco-Invitrogen, Carlsbad, CA, USA). Anti-rat neurofilament antibodies raised in rabbit (catalog number N4142) and biotin-conjugate anti-rabbit IgG antibodies raised in goat (catalog number B7389) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rat glial fibrillary acidic protein (GFAP) was detected using a kit containing all compounds necessary for the GFAP immunochemistry, especially anti-GFAP antibodies raised in rabbit, extravidin-peroxidase conjugate (Sigma, catalog number IMMH-6). Hoechst 33258 fluorochrome (catalog number B2883), and kit for the measurement of lactate dehydrogenase activity (catalog number TOX7) were from Sigma-Aldrich. Antibiotics and glutamine were purchased from Sigma (catalog numbers: P4333 and G2150 respectively).

Neuronal cell culture

Cerebral cortices of 16-day rat embryos were dissected out, freed from meninges, and dissociated into single cells by trituration in Hanks' balanced salt solution without Ca^{2+}. Hanks' balanced salt solution medium containing Ca^{+2} was then added to a final concentration of 0.65-mM Ca^{2+}. The cell suspension was layered over fetal calf serum and then centrifuged at $3000\times g$ for 5 min. Cells pelleted through the fetal calf serum were suspended in Neurobasal™/B27 medium; viable cells were plated at 8×10^5 cells per poly-L-lysine-coated 60-mm Petri dish. Cells were incubated in a CO_2 incubator (Forma Scientific, Marietta, OH, USA) at

[1] Present address: Unité d'Immunologie des Parasites, Institut Pasteur, 28 Rue du Dr.Roux, F-75724 Paris CEDEX 15, France.
*Corresponding author. Tel: +33 2-38-41-70-96; fax: +33 2-38-41-72-44.
E-mail address: tobias.hevor@univ-orleans.fr (T. Hevor).
Abbreviations: DIV, days *in vitro*; EM, electron microscopy; LDH, lactate dehydrogenase; TUNEL, terminal deoxynucleotityl transferase-mediated dUTP nick end labeling.

0306-4522/12 $36.00 © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2011.10.002

37 °C under 95% air/5% CO_2 in a water-saturated atmosphere. One half of the medium was replaced by fresh medium once per week or as otherwise specified. Four squares each of 1 cm^2 were delimitated in 60-mm-diameter Petri dishes containing cultures for cell counting. A reticle was stuck under the dishes to make easy cell counting using a photonic microscope. In a given dish, the number of cells after 3 days *in vitro* (DIV 3), when cells able to attach adhered to the bottom of the plates, was considered as 100% of cell number. Cells from the same squares were counted on the following DIVs, and the ratio between the obtained numbers and DIV 3 number was used to draw the growth curve.

Cell characterization

Adherent cells were washed with 0.15-M phosphate-buffered saline (PBS) pH 7.4 and fixed with 4% w/v paraformaldehyde in PBS pH 7.4. Cells were then washed in a PBS containing 3% v/v bovine serum albumin and 0.1% v/v Triton X100 for 1 h before incubation overnight with anti-neurofilament antibody. After thorough washing in PBS, cells were then incubated with biotinylated anti-rabbit IgG antibodies raised in goat. Development was with streptavidin–peroxidase and diaminobenzidine. To assess possible contamination by astrocytes, immunolabeling was performed using antibodies against rat GFAP; development was by the same protocol as for anti-neurofilament antibodies.

Ultrastructure studies

Cells were rinsed with 0.15-M PBS pH 7.4, fixed in the same medium containing 2.5% w/v glutaraldehyde, washed in PBS, and postfixed in a solution containing 1% w/v osmium tetroxide and 1.5% w/v potassium ferricyanide. After dehydration in graded ethanol, the cells were embedded in Epon, sectioned, and contrasted with 4% w/v uranyl acetate followed by 0.4% w/v lead citrate. Sections were coated with carbon powder and examined under a transmission electron microscope (Philips, model CM 10, provided by FEI, Eindhoven, the Netherlands; for detailed procedures see Hevor and Delorme (1991). The method for evaluating organelle numbers was as described elsewhere (Robert and Hevor, 2007). Briefly, in areas delimitated by electron microscope grids, the number of organelles were counted in slices from all neurons. In each neuron, the organelles were classified according to their shapes. The electron microscopy (EM) observations were made by two teams consisting of two persons each. In each team, one person loaded the section and the other examined it, blinded of which section was loaded. The observations were well matched between the teams (Robert and Hevor, 2007).

Evaluation of cell death

To assess neuronal integrity, cells were stained with the fluorochrome Hoechst 33258. After washing in 0.15-M PBS pH 7.4, cells were fixed for 10 min in acetic acid: methanol (1:3, v/v), washed with PBS, and stained with Hoechst 33258 (1 μg/ml in PBS) for 10 min. After final washing, cells were imaged using a Zeiss inverted epifluorescence microscope (Zeiss Axiovert 135, Göttingen, Germany), excitation wavelength 365 nm, emission wavelength 465 nm. Where appropriate, fluorescence images were superimposed over images recorded using conventional light microscopy.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) was performed according to the manufacturer's protocol as follows. Cells were permeabilized in 0.1% w/v sodium citrate containing 0.5% v/v Triton X100. TUNEL was conducted in medium comprising 560-μl H_2O, 140-μl TdT 5× buffer, 5-μl TdT, 4-μl biotin–dUTP. The reaction was halted by the addition of 30-mM sodium acetate and 300-mM sodium chloride. After washing, 3% w/v bovine serum albumin was added followed by 0.1 M PBS containing 0.05% v/v Tween and streptavidin–peroxidase. Development was with diaminobenzidine.

Lactate dehydrogenase activity both in cells and the culture medium was measured using a commercial kit (Sigma) according to the protocol provided by the manufacturer.

Statistical analysis

Except for the growth curve done on one typical culture batch out of five replicates, numerical data were expressed as mean±SD and were statistically analyzed by means of paired and small sample Student's *t*-test. The minimal level of significance accepted was $P<0.05$. Comparisons were made versus controls, which were either the means obtained in culture without antibiotics or the means obtained at DIV 3 or DIV 7.

RESULTS

The growth, ultrastructure, and integrity of primary cultured rat brain neurons were evaluated after different times in culture (DIV) that were selected according to the items studied. Two–three hours after seeding, cells were seen to adhere to the culture dish; by DIV 1 neurite outgrowth was observed, and a primary network could be seen at DIV 7 (Fig. 1A). Neurite complexity was particularly marked at around DIV 14, with a marked outgrowth of secondary neurites (Fig. 1C). At a four-fold higher seeding density, cells predominantly grew as clusters with few isolated cells (Fig. 1B).

Cultured neurons contained a substantial amount of neurofilaments as revealed by anti-neurofilament immunolabeling; this highlighted both cell bodies and neurites and improved the visualization of neuronal networks (Fig. 1E, F). Comparison of anti-neurofilament and anti-GFAP staining patterns revealed that astrocytes were undetectable for the first 2 weeks; the proportion of contaminating astrocytes was in the range 0–3% in DIV 21 cultures.

Cell counting at different times after plating allowed growth curves to be established. This analysis revealed a clear increase in the number of neurons in culture, with a three-fold rise between DIV 3 and DIV 11–18 (Fig. 1D). Between DIV 11 and DIV 18 the number of neurons plateaued and subsequently declined. Neuronal networks were clearly visible at DIV 30–50 depending on the status of the medium renewal. Replacement of 50% of the medium by fresh medium (once per week; Experimental procedures) prolonged survival, whereas no renewal gave a markedly shorter mean survival time (Fig. 1D). Conversely, complete renewal negatively affected cell survival, with vacuolization and cell lysis indicative of neuronal death (not presented).

Cultured neurons at different time-points were then analyzed by electron microscopy. Analysis at DIV 7 and DIV 14 revealed many structures as seen in *ex vivo* neurons, including well-defined cell bodies, nuclei, mitochondria, endoplasmic reticulum, and lysosomes (Fig. 2A, B), and the same organelles were observed at DIV 21 and DIV 28 (data not shown). Interestingly, at the same DIVs numerous mitochondria observed along microtubules suggested the existence of mitochondrial transport within neuronal processes as shown at DIV 21 in Fig 2C, D. For example, because of its caliber, its smooth limiting membrane, the presence of microtubules, the neurite shown in