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In Vitro Neurotoxicology

Methods and Protocols

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In Vitro Neurotoxicology

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Preface

In recent years, the need to develop acceptable alternatives to conventional animal testing for neurotoxicity and developmental neurotoxicity has been increasingly recognized. Hence, efforts are being directed toward the development of alternative models, utilizing not only mammalian cells in culture, but also nonmammalian model systems, such as zebrafish or *C. elegans*. These in vitro testing procedures have the purposes of investigating mode and/or mechanism of action of chemicals, particularly related to early, upstream events in the neurotoxic process, and of screening chemicals of unknown toxicity, to flag compounds for further in vitro and in vivo neurotoxicity studies.

This volume provides a series of cellular, biochemical, and molecular methodological protocols in the area of in vitro neurotoxicology, with an emphasis on mammalian cell culture systems. In the first section, methodologies for preparing several cellular systems of variable complexity amenable for in vitro neurotoxicological studies are presented. The second section deals with methods to measure cellular death and major mechanisms that may be involved. The third section presents methods for assessing mechanisms of nervous system cell toxicity related to impairment of cell signaling while the final section illustrates additional methods for assessing important nervous system processes, such as cell proliferation, neuritogenesis, and synaptogenesis.

This collection of methods would be useful to anyone interested in assessing or characterizing the potential neurotoxicity of environmental contaminants, drugs, or other chemicals.

The volume would be of interest to graduate students, postdoctoral fellows, and faculty in academia, as well as to research scientists in governmental laboratories, and in the chemical, food, and pharmaceutical industries.

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Chapter 1

In Vitro Neurotoxicology: An Introduction

Lucio G. Costa, Gennaro Giordano, and Marina Guizzetti

Abstract

This introductory Chapter provides a brief overview of the field of neurotoxicology and of the role played by in vitro approaches in investigations on mechanisms of neurotoxicity and of developmental neurotoxicity, and in providing suitable models for neurotoxicity screening.

Key words: In vitro models, In vitro endpoints, Mammalian cell cultures

1. Introduction

Neurotoxicology studies the adverse effects of chemical, biological, and physical agents on the nervous system, at maturity or during development. Adverse effects can be any modification to the structure and/or function of the nervous system. A proposed definition of adverse effect is "any treatment-related change which interferes with normal function and compromises adaptation to the environment" (1). Most morphological changes, such as a neuronopathy (a loss of neurons), an axonopathy (a degeneration of the nerve axon), a myelinopathy (a loss of the cells surrounding the axon), or other gliopathies, can be considered adverse, even if changes are transitory and reversible. Neurochemical changes, even in the absence of structural damage, can also be considered adverse, even if they are reversible, as they lead to impaired function.

A large number of compounds are neurotoxic. In their book "Experimental and Clinical Neurotoxicology, Spencer et al. (2) list 372 substances for which evidence of neurotoxicity exists, based on animal studies or human observations. In a recent review, Grandjean and Landrigan (3) list 201 chemicals known to

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be neurotoxic to humans. The list includes metals, pesticides, solvents, several industrial chemicals, as well as natural substances.

An aspect of neurotoxicology that receives much attention is the interplay between exposure to neurotoxicants and age. Certain neurotoxicants are believed to contribute to some of the neurodegenerative disorders that occur primarily with aging. For example, exposure to certain neurotoxicants (pesticides and metals) may contribute to the etiology of Parkinson's disease (4). Additionally, most human neurotoxicants are developmental neurotoxicants (3). Indeed, the developing nervous system appears to be exquisitely sensitive to the adverse effects of chemical exposure, either in utero or during childhood, leading to severe impairment or subtle effects. Examples in this regard are methylmercury and ethanol, or lead and polychlorinated biphenyls, respectively.

Neurotoxic effects can be detected in standard toxicity testing (acute toxicity, subchronic and chronic toxicity, developmental and reproductive toxicity) which are required by regulatory agencies for commercialization of most chemicals. In addition, specific guidelines exist to further investigate the potential neurotoxicity of chemicals (5, 6). These tests are carried out in vivo, usually in rodents, and include clinical observations, a functional observational battery, measurements of motor activity, and neuropathological examination of nervous tissue. If positive results are found, and if concerns for potential neurotoxicity may be provided by the compound's chemical structure and/or by specific metabolites, additional testing may be carried out, such as specialized behavioral testing for learning and memory, measurements of nerve conduction velocity, neurochemical measurements, etc. (7).

In response to the issue of sensitivity of the developing nervous system, developmental neurotoxicity guidelines have also been developed (8, 9). Exposure to the test chemicals is from gestational day 6 to postnatal day 10 or 21 to the mother, thus ensuing exposure in utero and through maternal milk. Tests involve measurements of developmental landmarks and reflexes, motor activity, auditory startle test, learning and memory tests, and neuropathology. As for neurotoxicity testing, developmental neurotoxicity tests has proven useful in identifying compounds with neurotoxic potential.

In the past several years, toxicologists have recognized the need to develop alternatives to standard in vivo animal testing, to reduce problems related to increasing costs and time required for such testing, to address efforts aimed at reducing the number of animals used for toxicity testing (7, 10), and to respond to recently enacted legislations [e.g., REACH (Registration, Evaluation, and Authorization of Chemicals)]. Significant efforts have been devoted to the development of in vitro alternative models which could aid investigators in identifying and characterizing potential neurotoxicity of chemicals.

2. In Vitro Neurotoxicity Testing in Mammalian Cells

In vitro testing procedures utilizing mammalian cells have usually two main purposes: (a) to investigate modes and mechanisms of action of chemicals, particularly related to upstream events in the neurotoxic process and (b) to screen new chemical entities to flag out those with neurotoxic potential which will undergo further in vitro and/or in vivo studies. Both aspects are discussed in following sections.

There are a number of advantages, and some disadvantages, in using in vitro approaches in neurotoxicology. One of the advantages is that cells in culture represent a relatively uniform environment and that exposure parameters can be strictly controlled. Only small amounts of the test chemicals are needed, and systemic effects (e.g., metabolism) are bypassed (this may also be a disadvantage if the ultimate neurotoxicant is a metabolite). In vitro systems are available from various animal species, usually rodents, and also humans. Some in vitro methods are amenable to high-throughput screening, an important issue if several thousand compounds need to be tested. Though many in vitro systems utilize primary cultures from rodents, the overall number of animals is significantly reduced; furthermore, immortalized cell lines are also available. Finally, compared with in vivo approaches, in vitro methods allow a saving of time and money. There are, however, disadvantages in using in vitro methods. The adult nervous system is protected by the blood–brain barrier, a feature that is absent in in vitro cells in culture. Furthermore, in vitro systems lack integrated functions, and compensatory mechanisms cannot be determined. Last, but not least, because of the complexity of the nervous system and the several cells present (neurons, astrocytes, microglia, oligodendrocytes/Schwann cells, and stem cells), a single test cannot cover all targets or mechanisms (11, 12).

Several issues need to be considered when exploring potential in vitro models for neurotoxicity and developmental neurotoxicity. There is the initial issue on whether one should use, for example, neurons or glial cells. For both cell types, immortalized cell lines (e.g., human SH-SY5Y neuroblastoma cells or rat glioma C6 cells) or cells in primary cultures (e.g., rat cerebellar granule cells or cortical astrocytes) may be used. To capture important cell–cell interactions, coculturing system can be utilized (e.g., neurons and astrocytes), or more complex systems, such as aggregating brain cell cultures or brain slices. Each model has its advantages and disadvantages (7). For example, a cell line provides a defined and homogenous population of cells (usually clonal) derived from tumors or using oncogene-containing retroviruses. Cell lines are easy to grow, divide rapidly, are available from several animal species including humans, and can be induced to differentiate with

appropriate agents. However, transformed cell lines may not exhibit the same phenotype as primary cells or may represent a subpopulation of cells. Cell lines may have increased genetic instability with increasing number of passages, neurites may not represent true axons or dendrites, and, of course, cell–cell interactions are missing.

There are also a number of other issues to be considered. For example, is it always better to use human cells (cell lines) if they are available? Are primary cells in culture a better model than cell lines of brain cells in situ? In case of primary cultures, which brain area should they be isolated from? Are more complex systems, such as brain neurospheres, better in vitro models, as they capture interactions among several cell types? There are no clear answers to these and similar questions. Thus, while selection of the appropriate model can be driven by existing knowledge or by specific hypotheses in the case of mechanistic studies, it remains a primary concern for application to screening purposes.

3. In Vitro Systems for Mechanistic Studies

In vitro systems are highly amenable and extremely useful for mechanistic studies and as such, they continue to be used extensively in neurobiological, neuropharmacological, and neurotoxicological research. Again, given the complexity of the nervous system, no single in vitro preparation can provide a complete model for neurotoxicity. However, depending on the knowledge on the neurotoxicity of a certain compound, and of the specific questions that are being asked, different cellular systems or preparations can be utilized. There are indeed hundreds of examples in which different cell culture models have been successfully utilized to investigate specific mechanisms of action of neurotoxicants. In vitro test systems are amenable to biochemical, molecular, electrophysiological, and morphological examinations. In addition, all "omics" technologies (i.e., transcriptomics, proteomics, and metabolomics) can be easily and effectively utilized in in vitro systems.

In the context of mechanistic in vitro neurotoxicology some examples of possible investigations are: (a) Assessment of neurotoxicant-induced cell death (e.g., the marine neurotoxin domoic acid causes apoptotic neuronal cell death by mechanisms that involve oxidative stress (13)); (b) Study of the mechanisms involved in the inhibition of cell proliferation (e.g., inhibition of DNA synthesis in astrocytes by ethanol through an interaction with phospholipase D (14)); (c) Measurements of alterations of signal transduction pathways leading to changes in cell function (e.g., interactions of polychlorinated biphenyls

and of polybrominated diphenyl ethers with protein kinase C and calcium homeostasis (15)); (d) Study of the modulation of neurotoxicity by cell–cell interactions (e.g., protective role of astrocytes toward neurons upon exposure to a pentabrominated diphenyl ether mixture (16), or astrocyte-mediated impairment of differentiation of hippocampal neurons caused by manganese (17)); and (e) Investigations on alterations of inhibitory or excitatory circuitries (e.g., effects of trimethyltin on GABAergic interneurons in the hippocampal slice (18)), and many others.

Though there is no doubt that in vitro systems play the most relevant role in mechanistic neurotoxicology, extrapolation of in vitro findings to in vivo effects still requires additional important considerations. An important one relates to the concentrations used in in vitro studies, and their relationship to in vivo target doses (19). Another one is the role of metabolism and pharmacokinetics (Is the compound effectively detoxified? Is the ultimate neurotoxicant a metabolite?) (10, 20). Other considerations relate to permeability of a compound through the blood–brain barrier, to the ability of the brain to compensate for mild injury, etc. Notwithstading these and other caveats, important for extrapolating the in vitro findings to in vivo situations (21), in vitro systems represent nevertheless very useful models to investigate mechanisms of neurotoxic effects.

4. In Vitro System for Neurotoxicity Screening

As mentioned earlier, in vitro systems may also be used as possible tools to provide rapid, reliable, and relatively inexpensive screening of compounds for potential neurotoxicity and/or developmental neurotoxicity. These initial screening approaches should be seen as a Tier 1 evaluation, to be followed, in the case of positive findings, by further in vitro and in vivo studies. Paramount to this goal is that in vitro screening approaches have very low incidence of false positives, and in particular, of false negatives. In addition, they need to have good predictive value, i.e., good correlation with in vivo data, and need to be sensitive, relatively simple, rapid, and possibly amenable to high-throughput applications, economical, and versatile (7, 12).

When the goal is to utilize in vitro methods for neurotoxicological screening, all issues discussed in the previous section gain significant relevance, as one as to decide, without any knowledge on the biological activity of the compound, which cell type to use, the degree of model complexity, and, in particular, which endpoint(s) to measure (12). It is often stated that a good approach would be that of examining general cellular processes, such as cell viability,

cell proliferation, differentiation of precursor cells, or elongation of neurites. However, each approach requires careful considerations. For example, one study concluded that the assessment of cell viability and of possible underlying mechanisms (e.g., energy status, oxidative stress, and cytoskelatal modifications) in one neuronal cell line (PC12 cells) does not allow distinguishing cytotoxicity from neurotoxicity (22). In another study, in which cell viability and proliferation were assessed, the resulting false positive/false negative rate was high (25%) (23). A more comprehensive approach may consist in utilizing a battery of cell types, including nervous system and non-neuronal cells; this has shown to be promising in identifying neurotoxicants, but only a few compounds of known neurotoxicity were tested (24).

Neurite outgrowth has been proposed as an important endpoint when screening for potential developmental neurotoxicants (25). However, though this approach appears to be very promising, and is amenable to high-throughput screening, initial results indicate that the rate of false positives (25%) and false negatives (37.5%) is still too high (26). Additional studies have investigated in vitro metabolomics and electrophysiological approaches for possible neurotoxicity screening (27, 28); both approaches look promising, but would require the testing of a substantial number of compounds, and careful considerations on how to differentiate between pharmacological and neurotoxic effects.

In case of biochemical approaches, there are examples suggesting that limited mechanistic knowledge may lead to the use of in vitro methods to screen for a particular neurotoxicity. For example, organophosphorus (OP) compounds, a major class of insecticides owe their acute neurotoxicity to inhibition of the enzyme acetylcholinesterase (AChE). Some OPs can also cause a delayed polyneuropathy, which is unrelated to their inhibition of AChE, and is attributed instead to irreversible inhibition of another esterase, NTE (neuropathy target esterase) (29). Knowledge of the two targets for acute toxicity (AChE) and delayed neurotoxicity (NTE) has allowed the use of an in vitro system, utilizing human neuroblastoma cells, to screen OPs for their potential of inducing delayed polyneuropathy (30). Another example is that of the use of cerebellar granule neurons from transgenic mice to investigate neurotoxicant-induced oxidative stress. Mice-lacking GCLM (the modifier subunit of glutamate cysteine ligase, the first and rate-limiting enzyme in the synthesis of glutathione) have very low glutathione content, and as such, are more susceptible to the toxic effects of chemicals that cause oxidative stress. This simple in vitro system may be exploited to screen chemicals for their ability to induce oxidative stress (12).

While, as said, in vitro cell culture systems have a prominent role in mechanistic neurotoxicology, the situation is less satisfactory

with regard to their use for neurotoxicological screening purposes. Indeed, despite the development of several models and tests of potential usefulness, the lack of validation to determine the rate of false positives/false negatives, and the degree of inter-laboratory variability, has hampered so far the further use and application of such alternative approaches.

5. What You Will Find in this Volume on In Vitro Neurotoxicology

The present volume is aimed at providing a series of methods and protocols for use in in vitro neurotoxicological research. The first section deals with model systems, i.e., various types of in vitro preparations that may be used to test, for mechanistic or screening purposes, known or unknown neurotoxicants. Chapters are devoted to either a single cell type in culture (e.g., neurons, astrocytes, microglia, and stem cells) or more complex systems such as cells in coculture, rat brain aggregates or human neurospheres, and brain slices. One chapter also describes methods to establish in vitro models for the blood–brain barrier.

In the second section a number of endpoint related to cell death and possible underlying mechanisms are presented. Methods to measure cell death and apoptosis are first described, followed by chapters on measurements of major mechanisms underlying cell death, i.e., oxidative stress, glutathione homeostasis, and energetic failure of the cell.

The third section focuses on intracellular signals that have been associated with neurotoxic damage. These include calcium overload, alterations in glutamate homeostasis, modulation of the nitric oxide system, activation of mitogen-activated protein kinases and other kinases, interference with signal transduction pathways, such as protein kinase C and phospholipases, and the involvement of the proteasomal system.

The final section provides methods and protocols to measure additional endpoints relevant to neurotoxicity and developmental neurotoxicity. Protocols are described for measurements of neurite outgrowth with an emphasis on high-throughput/high-content applications, assessments of cell proliferation, measurement of cell swelling, and assessment of synaptogenesis. A chapter deals with protocols related to cholesterol homeostasis in the developing brain, while a final chapter provides mathematical models for investigating interactions among chemicals in vitro.

While it is recognized that this volume does not cover all possible models and endpoints that can be used to investigate neurotoxicity in vitro, the reader should find a number of protocols and methods which will allow the testing of specific hypotheses and will provide possible models for screening purposes.

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Part I

Model Systems

Chapter 2

Primary Neurons in Culture and Neuronal Cell Lines for In Vitro Neurotoxicological Studies

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Abstract

Primary neuronal cultures and neuronal cell lines derived from rodents are widely used to study basic physiological properties of neurons, and represent a useful tool to study the potential neurotoxicity of chemicals. While short-term culturing of neurons can be a very straightforward process, long-term cultures of relatively pure neuronal populations require more effort. This chapter describes methods and protocols to isolate and culture primary neurons obtained from the rodent cerebellum (cerebellar granule cells), the hippocampus, and the striatum. Furthermore, culturing of rat pheochromocytoma (PC-12) cells is also described, as these cells represent a useful and widely used cell line for in vitro neurotoxicological studies.

Key words: Primary cultures, Neuronal cell lines, Cerebellar granule cells, Hippocampal neurons, Striatal neurons, PC-12 cells

1. Introduction

Since the beginning of the past century, neuroscientists and biologist have devoted considerable efforts to developing in vitro methods to culture nerve cells (1). These in vitro systems have played an important role in the understanding of basic physiological processes of nervous system cells, and can also be used to analyze the neurotoxic effects of chemicals and their underlying mechanisms.

Overall, two different models of neuronal cells in culture can be used: primary neuronal cultures and immortal cell lines (1, 2). Neurons in primary culture are prepared directly from animal brain tissue and do not divide in culture. After isolation and plating, neurons form synapses and become electrical active, acquiring a neuronal phenotype, and ultimately dye. In contrast, immortal

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cell lines undergo a period of rapid division (log phase growth) after plating and, as they approach confluence, they start to differentiate. In choosing an in vitro cell culture model based on primary cultures, one should consider the fact that neurons from different areas of the nervous system have different characteristics and properties. In this chapter, we present methods for isolating and culturing neuronal cells from the cerebellum, the hippocampus, and the striatum, three brain areas which are often targets for neurotoxicants. Cerebellar granule cells are interneurons in the granular layer of the cerebellar cortex and represent the most numerous neuronal population in the cerebellum. The high purity of cerebellar granule cell cultures makes these neurons particularly suitable for in vitro studies. Cultures of embryonic rat hippocampal neurons have found particular favor in basic neurobiology (e.g., in studies of voltage-sensitive and ligand-gated ion channels, synaptic transmission, and neurite development), and are also relevant for neurotoxicology. Subheading 3.3 provides a simple and reliable culture protocol for culturing primary striatal neurons, which can be used for morphometric and electrophysiological analyses, when seeded at very low density, while higher density allows biochemical and molecular approaches.

Immortal cell lines (such as PC-12 cells and several neuroblastoma lines) have also been extensively used to evaluate the neurotoxic potential of chemical compounds. Cell lines offer the advantage of a homogeneous population of cells, but being transformed cells, they have characteristics which are different from primary cells. In this chapter, we describe a protocol to culture PC-12 cells, a rat pheochromocytoma-derived cell line, which provides an excellent model for studying chemical disruption of processes associated with neuronal differentiation, and the synthesis, storage, and release of neurotransmitters.

2. Materials

2.1. Primary Cerebellar Granule Neurons

1. Autoclaved surgical instruments: one large scissor, one small scissor, two small forceps, a scalpel blade, a spatula, a small angled scissor, 35- and 100-mm Petri dishes, and sterile glass Pasteur pipettes.
2. Autoclaved paper filters (No. 3).
3. 1 M HEPES [4-(2-hydroxyethyl)-L-piperazine ethanesulfonic acid] stock solution: dissolve 283.3 g of HEPES in 1 L of double-distilled water. Use diluted 1:100. Store at 4°C and wear gloves when handling.
4. Poly-D-lysine hydrobromide (PLYS) stock solution: dissolve 10 mg of PLYS in 1 mL of high-quality distilled water. Dilute to 200 μg/mL with water and sterilize by filtration

through a 0.2-μm filter. Divide stock in aliquots and store at –20°C.

5. Hank's balanced salt solution (HBSS) can be purchased from Invitrogen or prepared as a 10× stock solution as follows: 1.4 g/L $CaCl_2$, 4.0 g/L KCl, 0.6 g/L KH_2PO4, 1.0 g/L $MgCl_2 \cdot 6H_2O$, 1.0 g/L $MgSO_4 \cdot 7H_2O$, 80 g/L NaCl, 0.35 g/L $NaHCO_3$, 0.9 g/L $Na_2HPO_4 \cdot 7H_2O$, 10 g/L glucose, and 0.1 g/L phenol red (see Note 1).
6. Sodium bicarbonate ($NaHCO_3$) solution: 1.4 g $NaHCO_3$ in 100 mL of double-distilled water. Sterilize by filtration through a 0.2-μm filter. Add approximately 2.5 mL (0.35 g/L) of this sterile solution to HBSS (see item 7 below) just before use (see Note 2).
7. Complete, calcium- and magnesium-free Hank's balanced salt solution (CMF-HBSS): add HEPES stock solution (final concentration 10 mM) to CMF-HBSS (see Note 3).
8. Complete Neurobasal medium (CNM): add 4 mM (final concentration) Glutamax, 2% (final concentration) B27 or B27 minus antioxidant (AO), 25 mM (final concentration) KCl, 10 μg/mL gentamicin, to Neurobasal medium.
9. 1 M KCl stock solution: dissolve 74.55 g in 1 L of double-distilled H_2O, then sterilize by filtration through a 0.2-μm filter. Divide stock in aliquots, and store at room temperature.
10. B27: a supplement from Invitrogen (3, 4) containing the following 27 components (concentrations are not published): biotin, L-carnitine, corticosterone, ethanolamine, D(+)-galactose, glutathione (reduced), linoleic acid, linolenic acid, progesterone, putrescine, retinyl acetate, selenium, T3 (triiodo-L-thyronine), DL-α-tocopherol (vitamin E), DL-α-tocopherol acetate, bovine albumin, catalase, insulin, superoxide dismutase, and transferrin.
11. B27 minus AO (without antioxidants): it has the same composition as B27 without the following components: glutathione (reduced), catalase, and superoxide dismutase.
12. Deoxyribonuclease (DNAse) I (Sigma) stock solution: 4 mg DNAse in 1 mL of CMF-HBSS. Do not vortex the solution since this enzyme is very sensitive to mechanical mixing. Sterilize by filtration through a 0.2-μm filter. Use diluted 1:100. Divide stock in aliquots and store at –20°C (see Note 4).
13. Papain solution: 2 mg papain in 1 mL of CMF-HBSS. Prepare the solution fresh at the beginning of the procedure, and leave it at 37°C in a water bath for a minimum of 30 min. After this period, a precipitate may be still present, as papain has a low solubility in water. Dissolve this precipitate by inverting the tube, then sterilize by filtration through a 0.2-μm filter right before the incubation (see Note 5).

14. 4% (w/v) Trypan Blue stock solution: a liquid, sterile-filtered, cell culture tested solution prepared in 0.81% sodium chloride and 0.06% potassium phosphate, dibasic.
15. 5 mM 1-β-arabinofuranosylcytosine (Ara-C) stock solution: add 12.1 mg Ara-C to 10 mL HBSS. Divide in aliquots, and store at −20°C, protected from light. This solution is stable for up to 1 year. Solutions stored at room temperature in physiological buffer are stable for only 24 h. Sterilize by filtration through a 0.2-μm filter.

2.2. Hippocampal Neuronal Cultures

1. Complete Neurobasal medium (CNM): add 4 mM (final concentration) Glutamax, 2% (final concentration) B27, 10 mM (final concentration) glucose, 10 μg/mL gentamicin to Neurobasal medium. No KCl is required for hippocampal neuronal cultures.
2. 2.5 mM glucose stock solution.

2.3. Striatal Neuronal Culture

Complete serum-free Neurobasal medium (CNM) supplemented with B27.

2.4. PC-12 Cells

1. Complete Dulbecco's Modified Eagle's Medium (C-DMEM): 10 g/L glucose, 5% iron-supplemented calf serum, 5% horse serum, and 1% pen/strep antibiotic.
2. 1 M Ethylenediaminetetraacetic acid (EDTA) stock solution: add 186.1 g EDTA to ~900 mL double-distilled H_2O and stir vigorously. Place 20 g NaOH pellets into a 50-mL centrifuge tube. Every few minutes, add a few NaOH pellets to the EDTA solution while stirring. When the EDTA goes into solution, stop stirring, and adjust pH using NaOH stock solution. Sterilize by filtration through a 0.2-μm filter.
3. 10 M Hydrogen peroxide (NaOH) stock solution: add 40 g NaOH to 100 mL of double-distilled water and sterilize by filtration through a 0.2-μm filter. Store at room temperature.
4. The rat pheochromocytoma PC-12 cell line can be purchased from ATCC.

3. Methods

3.1. Preparation of Primary Cerebellar Granule Neurons from 6- to –8-Day-Old Mouse or Rat Pups (See Note 6)

Anatomically, the cerebellum has the appearance of a separate structure attached to the bottom of the brain, located underneath the cerebral hemispheres. The cerebellum is a region of the brain that plays an important role in motor control. It is also involved in some cognitive functions, but its function in movement is the most clearly understood. The cerebellum does not initiate movement, but it contributes to coordination, precision,

and accurate timing. Because of this fine-tuning function, damage to the cerebellum does not cause paralysis, but instead produces disorders of fine movement, equilibrium, posture, and motor learning. The cerebellum is characterized by the presence of five major types of neurons: Purkinje cells, granule cells, and three types of interneurons (Golgi cells, stellate cells, and basket cells). Cerebellar granule cells are among the smallest neurons in the brain. They are also easily the most numerous neurons in the brain: in humans, estimates of their total number average around 50 billion, which means that about 3/4 of the brain's neurons are cerebellar granule cells (5). Their cell bodies are packed into a thick layer at the bottom of the cerebellar cortex. Granule cells use glutamate as their neurotransmitter, and therefore, exert excitatory effects on their targets.

The small size and incredible abundance of granule neurons make it the easiest cell to obtain in large numbers allowing long-term cultures and the possibility to perform a variety of biological and biochemical assays (6). In addition, it is relatively easy to obtain pure populations of cerebellar granule cells (>95%).

1. Before starting the culture preparation, dishes should be coated with water containing PLYS (concentration in the range of 100–200 μg/mL) overnight in the incubator at 37°C. PLYS solution should be previously sterilized by filtration through a 0.2-μm filter.
2. After the overnight incubation, wash the dishes twice with double-distilled water, and once with HBSS, to eliminate water left by the first two washes. Use dishes as soon as possible.
3. Sacrifice 6- to 8-day-old rat or mouse pups by decapitation (see Note 7) using a large scissor. Place the head on a 100-mm Petri dish provided with a filter paper on top.
4. Holding the skull with a large forceps, cut the skin along the mid line of the head. Then remove the skin using a forceps to expose the skull.
5. Cut the skull along the mid line using a small scissor. Start the cut inserting a fine scissor gently under the edge of the skull. Make two additional coronal cuts from the mid line of the skull.
6. Pull the skull off with the small spatula or using a fine forceps and expose the brain and the cerebellum.
7. Pull the brain out inserting the small spatula edge gently under the base of the brain. Remove the brain and the cerebellum gently on the filter paper. Drop a few drops of ice cold CMF-HBSS on the brain to make sure it is kept moist.
8. Using a fine scalpel blade, separate the cerebellum from the rest of the brain, then cut the cerebellum across its base, severing the connections coursing through the cerebellar peduncles.

9. Remove the meninges by rolling the cerebellar tissue on the filter paper (meninges will stick to the paper). Transfer the cerebellum (with a soft white appearance) to a 35-mm Petri dish filled with CMF-HBSS. Keep the 35-mm dishes on ice during the dissection. Chilling the tissue retards the action of proteolytic enzymes. As an alternative, meninges can be removed with the aid of a dissection microscope.
10. Collect all isolated cerebella (from 8 to 10 pups) in 35-mm Petri dishes filled with cold CMF-HBSS.
11. Mince the tissue into small chunks with a fine-curved scissor. Transfer the tissues to a 15-mL polypropylene conical tube using a disposable pipette. The polypropylene is favored since cells do not adhere readily to this surface.
12. Wait for the tissue to settle for 2 min, and then remove the liquid in excess by aspiration.
13. Add the papain solution to the tissue. Add DNAse stock solution (dilution 1:100) and 5 mM (final concentration) $MgCl_2$ (see Note 4).
14. Digest the cerebellum in a water bath at 37°C for 30 min. 10 min after starting the digestion invert the tube containing the tissue (only once, by hand).
15. At the end of digestion, sediment the tissue at $300 \times g$ for 3 min at 4°C. Carefully remove the supernatant containing papain. Wash the cerebellar tissue once with CMF-HBSS.
16. Gently triturate cerebellar tissue ten-times with a glass Pasteur pipette to dissociate larger aggregates.
17. Allow aggregates, which may still present in the solution, to sediment for 2 min.
18. Remove and filter the supernatant (cell suspension) through a 40-μm filter into a 5-mL sterile tube.
19. Add 5 mL of CNM to the pellet and gently triturate the tissue (ten-times) with a 5-mL pipette with a P1000 tip.
20. Allow aggregates to sediment for 2 min.
21. Remove and filter the supernatant (cell suspension) through a 40-μm filter into a 50-mL sterile tube.
22. Centrifuge the cell suspension at $300 \times g$ for 10 min at 4°C.
23. Aspirate the supernatant and gently resuspend the cell pellet in 10 mL CNM.
24. Add 50 μL of cell suspension and 50 μL of trypan blue stock solution to a sterile Eppendorf tube. Mix gently using a 200-μL pipette and wait a few seconds.
25. Count cells using a hemocytometer, and seed them at a density of 6×10^5/mL in CNM.

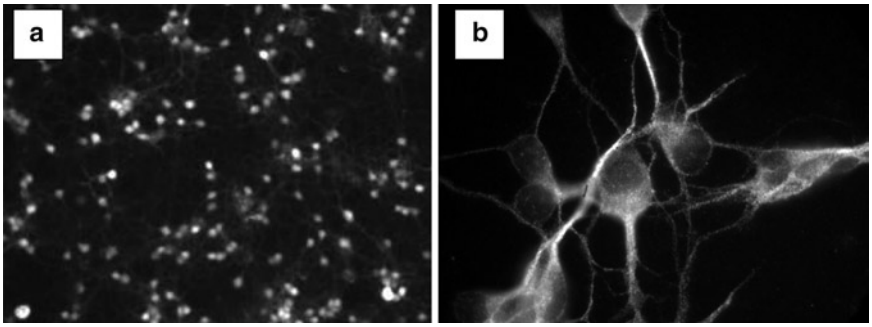

Fig. 1. Cerebellar granule neurons in culture. Cells are shown at 10 days after plating at high density, which provides a high protein yield. Neurons were fixed and stained using a primary antibody raised against β III tubulin. At this stage, cells are round-shaped, with a small cell body occupied mostly by the nucleus. (**a**) and (**b**) show a ×10 and a ×100 magnification, respectively. Some astrocytes (<5% of cells), with long-branched processes, can be found in these cultures, especially if they are not treated with AraC a few days after plating.

26. Incubate cells for 60 min in a cell culture incubator.
27. Remove medium containing nonadherent cells and cell debris. Wash plates twice with HBSS and leave them in CNM for 4 days.
28. On day 4, remove the medium, wash the plates with CMF-HBSS, and leave the plates in CNM (minus AO) (7) (see Note 8). If the presence of astrocytes represents a problem (this can be verified with a microscope), replace the medium with fresh CNM (minus AO) containing 2 μM AraC.
29. On day 8, replace half of the medium with fresh CNM (minus AO).
30. Cerebellar granule neurons should be used 10–13 days after seeding (see Fig. 1). If long-term cultures are needed, half of the medium should be changed twice a week.

3.2. Preparation of Hippocampal Neuronal Cultures

The hippocampus is a fascinating area of the brain, which possesses a very distinct anatomical organization with very unique and remarkable neuronal activities. For example, in 1973, a phenomenon characterized by changes in synaptic responsiveness induced by brief strong activation and lasting for hours, days, or longer was first described (8). This phenomenon was soon referred as long-term potentiation (LTP) and is a candidate mechanism for memory.

The hippocampus contains different populations of neurons. Pyramidal neurons are present in both CA1 and CA3 region of the hippocampus, and represent the most abundant neuronal population, accounting for about 90% of the total number of the neurons. The presence of a variety of interneurons has also been reported, but they seem to be much less in number compared with pyramidal cells. A third type of neurons (granule neurons) is

in the dentate gyrus. Hippocampal pyramidal neurons have a characteristic and well-defined shape with a triangular-shaped soma, or cell body, after which the neuron is named. Other key structural features of the pyramidal cell are a single axon, a large apical dendrite, multiple basal dendrites, and the presence of dendritic spines (9). In addition, hippocampal pyramidal neurons establish connections with each other and with interneurons. Thus in culture, where afferent fibers are missing, hippocampal neurons make synaptic connection with one another preserving the same features.

The development of pyramidal neurons in rats and mice begins at gestational day 15 and is completed before the day of birth, while the generation of dentate granule cells occurs postnatally. Therefore, the time of the preparation (prenatal vs. postnatal) can determine the neuronal composition of the culture. Hippocampal neuronal cells in culture, when grown at low density, allow the investigation of individual cell morphology. In these conditions, neurons require the presence of appropriate growth factors which have not been completely identified. Therefore, long-term survival of low-density hippocampal neuronal cultures is not routinely possible. Most protocols aimed to culture central nervous system neurons depend on high cell density, to allow the proliferation of glial cells, which in turn support neuronal survival and development. However, new culture media based on serum-free supplement formulations (4) allow the development and the survival of central nervous system neurons in the absence of glial cells.

1. Coat plates overnight with 100 μg/mL PLYS (diluted with sterile water and filtered). If microscopic techniques, which are difficult to apply to cells growing in plastic culture dishes, are to be used, neurons should be plated on glass coverslips coated with PLYS.
2. After the overnight incubation, wash dishes (or glass coverslips) twice with sterile water and once with HBSS. Leave the last wash in the plates to ensure that no water is left behind (as water is toxic to neurons).
3. Place the papain and the DNAse stock solutions in the water bath until needed. Minimize the time, making sure that all needed materials are ready before starting the procedure. The dissection should not take more than 2.5 h.
4. Hippocampal neuronal culture can be prepared from rat fetuses obtained at gestational day 20; in this case, the culture will consist mostly of pyramidal cells. 0.5 Day-old-pups can be used to generate hippocampal granule cells.
5. Euthanize the pregnant rat using carbon dioxide. Wipe the abdomen with 70% ethanol before making any incision. To prevent possible contaminations, cut and remove the skin

away, then cut the abdominal wall. Remove the uterus and place it in a 100 mm-Petri dish. Perform all the following steps under a laminar flow hood.

6. Decapitate fetuses with a scissor, and place the heads in a Petri-dish containing CMF-HBSS.
7. During the dissection, the brain is visualized best if kept in dish (filled with cold CMF-HBSS) under a dissection microscope illuminated from above.
8. Open the skull using the protocol described in Subheading 3.1 (steps 4–6) of this chapter.
9. Using a spatula, remove the entire brain and place it on sterile filter paper. Keep brain moist to prevent the tissues from dying.
10. Separate the two cerebral hemispheres from the brainstem.
11. Cut the two cerebral hemispheres along the midline using a sharp scalpel. Lay each hemisphere on its side.
12. With the diencephalon side facing up, place the spatula between the diencephalon and the cerebral hemisphere and spread the diencephalon away from the cerebral hemisphere to see the hippocampus.
13. Being careful not to damage the hippocampus, separate the hemisphere completely from the diencephalon using a fine curved-tip forceps. The pole of the hemisphere can be identified by the olfactory bulb. When viewed from this position the hippocampus is a well-defined C-shape area, which is contiguous to surrounding area of the brain delineated by blood vessels.
14. If parts of the thalamus remain attached to the hippocampus, gently remove them using a fine forceps.
15. Remove meninges and choroid plexus rolling the tissue on filter paper.
16. Collect all isolated hippocampi in a 35-mm Petri dish filled with cold CMF-HBSS.
17. Mince the tissue into small chunks with the fine-curved forceps. Transfer the tissue to a 15-mL polypropylene conical tube using a disposable pipette. The polypropylene is favored since cells do not adhere readily to this surface.
18. Wait the tissue to settle for 2 min and aspirate the liquid in excess.
19. Add the following to the tissue: papain solution, DNAse stock solution (dilution of 1:100), and $MgCl_2$ (5 mM final concentration).
20. Digest hippocampi in a water bath at 37°C for 30 min. 10 min after starting the digestion, mix the tube containing the tissue once by hand.

21. At the end of the digestion, sediment the tissue at 300 × *g* for 3 min at 4°C. Carefully remove the supernatant containing papain. Wash the hippocampal tissue once with CMF-HBSS.
22. Gently triturate the hippocampal tissue ten times with a Pasteur pipette to dissociate larger aggregates.
23. Allow aggregates, which are still present in the solution, to sediment for 2 min.
24. Remove and filter the supernatant (cell suspension) through a 40-μm filter into a 50-mL sterile tube.
25. Add 5 mL CNM to the pellet and gently triturate the tissue (ten times) with a 5-mL pipette with a P1000 tip.
26. Allow aggregates, which are still present in the solution, to sediment for 2 min.
27. Remove the supernatant (cell suspension) and filter through a 40-μm filter into a 50-mL sterile tube.
28. Centrifuge the cell suspension at 300 × *g* for 10 min at 4°C
29. Add 10 mL CNM to the cell pellet and gently resuspend using a 10-mL pipette.
30. Add 50 mL of cell suspension and 50 mL of trypan blue stock solution in a sterile Eppendorf tube. Mix gently using a 200-μL pipette. Wait a few seconds.
31. Count cells using a hemocytometer, and seed them at a density of $0.3–0.5 \times 10^5$/mL in CNM.
32. Incubate hippocampal neurons for 60 min at 37°C in a tissue culture incubator.
33. After 60 min, remove medium containing nonadherent cells and cell debris. Wash plates once with HBSS and leave cells in CNM for 7 days.

3.3. Preparation of Striatal Neuronal Cultures

The striatum is a subcortical area of the brain, which represents the major component of the basal ganglia and processes the inputs involved in voluntary movements. More than 90% of neurons in the striatum are the so-called medium spiny neurons, characterized by the presence of fine spines on the dendrites which use GABA as a neurotransmitter. All cortical areas send excitatory glutaminergic projections to specific portions of the striatum. The striatum also receives excitatory inputs from the intralaminar nuclei of the thalamus, dopaminergic projections from the midbrain, and serotoninergic input from the raphe nuclei. Depletion of dopamine in the striatum, as seen in Parkinson disease, may lead to impaired movements.

When compared with the complexity of the vivo situation, primary striatal neuronal cultures represent a simplified in vitro system. A disadvantage is represented by the lack of the striatal architectural organization which is no longer present. The in vitro

striatal cultures, described below, are prepared at a time point (gestational day 18–19) when the majority of the neurons in the striatum have been generated. Furthermore, maintenance of striatal cultures in serum-free medium conditions reduces the proliferation of nonneuronal cells, allowing to culture a relatively pure population of neurons. These cultures can be used for morphometric and electrophysiological analyses, when seeded at very low density, while higher density allows biochemical and molecular approaches.

1. Fetuses at embryonic day 18–19 (from rats or mice) are used. In alternative, striatal neuronal cultures can also be prepared from 0.5- to 1 day-old rodents.
2. Euthanize the pregnant animal using carbon dioxide, and clean the skin with 70% ethanol.
3. Carefully remove the uterus to avoid contamination with dirty skin, and place it in a clean 100-mm Petri dish.
4. Decapitate fetuses under a dissection microscope and transfer them to a 35-mm Petri dish containing CMF-HBSS.
5. Using a pair of angled/straight Dumont forceps, anchor the brain and peel away the skin using a pair of microsurgery straight-tip forceps.
6. Gently cut the skull using a pair of microsurgery scissor, pull the brain out, and place it in a new 35-mm Petri dish containing complete CMF-HBSS.
7. With the ventral side of the brain up, cut a coronal section using an angled microscissor. The section is made cutting the brain interiorly along the level of the optic chiasm, and posteriorly along the midpoint of the hypothalamus.
8. Isolate the section from the brain, and carve out the striatum (putamen, caudate nucleus) using a microdissecting knife or an angled microsurgery scissor (see Note 9).
9. Transfer tissue to a new 35-mm dish containing cold complete CMF-HBSS.
10. Mince striatal tissue in 1-mm size pieces using a curved scissor.
11. Transfer tissue to a 15-mL conical tube and wait 2 min to allow it to settle to the bottom of the tube.
12. Carefully remove the extra liquid using vacuum aspiration.
13. Add the following to the tissue: freshly made-papain solution (1 mL for 3–4 pups) 40 μg/mL (final concentration) DNase solution, and 5 mM (final concentration) $MgCl_2$.
14. Incubate tissue for 30 min in a 37°C water bath.
15. At the end of the incubation, centrifuge the tissue at 300 × *g* for 3 min at 4°C, then remove the supernatant.

16. Resuspend the tissue in 5 mL of complete CMF-HBSS.
17. Centrifuge tissues at 300 × *g* for 3 min at 4°C.
18. Remove the supernatant, and resuspend the tissue in 5 mL CNM.
19. Triturate the tissue using a glass Pasteur pipette, making sure to pass the tissue gently through the pipette. Avoid applying excessive strength and avoid the formation of bubbles. Ten strokes will achieve a cell suspension without the presence of large chunks.
20. Wait 2 min to allow the tissue debris to settle at the bottom of the 15-mL tube, then remove the supernatant (containing the cell suspension) using a 5-mL pipette.
21. Filter the cell suspension through a 40-μm filter into a 50-mL sterile tube.
22. Add 5 mL CNM to the 15-mL tube containing the debris, and triturate the remaining tissue using with a 5-mL pipette with a P1000 tip. Ten strokes are normally sufficient to disaggregate all the tissue.
23. Filter cell suspension through a 40-μm filter into the 50-mL sterile tube used in step 22.
24. Centrifuge the cells suspension at 300 × *g* for 10 min at 4°C.
25. Discard the supernatant and resuspend the pellet in 5 mL CNM.
26. Add 50 μl of cell suspension and 50 μl of trypan blue stock solution in a sterile Eppendorf tube. Mix gently using a 200-μl pipette. Wait a few seconds.
27. Count cells using a hemocytometer, and seed them at a density of $0.3–0.5 \times 10^5$/mL in CNM.
28. Incubate neurons for 2 h at 37°C in a tissue culture incubator.
29. After 2 h, remove medium containing nonadherent cells and cell debris. Wash plates once with HBSS and leave cells in CNM for 7 days.

3.4. Culture of PC-12 Cells

The PC-12 cell line was derived from a rat pheochromocytoma, an adrenal medullary tumor (10). This cell line has since been widely utilized for studies of different aspects of neuronal and endocrine cell physiology. Differentiation (arrested proliferation and neurite outgrowth) of PC-12 cells into a neuronal phenotype by nerve growth factor (NGF) treatment was described in the first report on these cells (10). PC-12 cells have also become an important model for studies of the mechanisms of regulated neuroendocrine secretion. The ability to grow PC12 cells in continuous culture with a well-defined secretory cell phenotype presents

advantages for cell biological and biochemical studies of the secretory pathway. PC12 cells release, depending on the conditions: dopamine, norepinephrine, and acetylcholine, and provide an excellent model for studying chemical disruption of processes associated with neuronal differentiation, synthesis, storage, and release of neurotransmitters.

1. PC-12 cells are cultured in complete DMEM, in a 5% CO_2 atmosphere.
2. Remove the growth medium from cultures that are 80% or less of maximal density, and rinse the culture dish with Hanks' basal salt solution containing 10 mM HEPES.
3. Add EDTA to the flask (5 mL for a 75 cm^2 T-flask), and incubate the dish for less than 5 min in a 37°C incubator to loosen adherent cells.
4. Dislodge the loosened cells by agitating the dish and adding growth medium (5 mL for a 75 cm^2 T-flask).
5. Triturate the cell suspension up to ten times by passage through a 5-mL pipette fitted with a 1,000-µL tip (see Note 10).
6. Transfer cells in a 15-mL conical tube and centrifuge them for 5 min at $300 \times g$ at 4°C.
7. Count cells (e.g., using trypan blue) and plate them in new dishes containing fresh medium.

4. Notes

1. Primary neurons grow well in a pH range of 7.2–7.5. Phenol red is commonly used as a pH indicator. It is red at pH 7.4 and becomes orange at pH 7.0, yellow at pH 6.5, lemon yellow below pH 6.5, reddish-pink at pH 7.6, and purple at pH 7.8.
2. In the medium, the pH level is mainly determined by the dissolved amounts of carbon dioxide (CO_2, present in the gas phase) and by HCO_3^- ions. The CO_2 tension regulate the concentration of dissolved CO_2 which in turn produces H_2CO_3, which dissociates according to the following equation:

$$H_2O + CO_2 \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$$

HCO_3 tends to reassociate, since it has a low-dissociation constant, leaving the medium acid. The effect of increased atmospheric CO_2 is to decrease the pH of the medium; this can be neutralized by increased the HCO_3^- concentration until equilibrium is reached at pH 7.4.

3. HEPES has no nutritional benefit to cells. It is added to the medium only for extra buffering capacity when cell culture requires extended periods of manipulation outside of a CO_2 incubator. The level of HEPES in cell culture media may vary from 10 to 25 mM.
4. Deoxyribonuclease (DNAse I), first crystallized from beef pancreas, is an endonuclease, which ensures the digestion of any DNA that might be released by damaged or dying cells. Since DNAse I is activated by bivalent metals, the addiction of $MgCl_2$ is necessary to ensure the correct digestion of the tissue.
5. Proteolytic enzymes are widely used for dissociation of cells. With some tissues, papain has proved less damaging and more effective than other proteases. Huettner and Baughman (11) describe a method using papain to obtain high yields of viable, morphologically intact cortical neurons from postnatal rats. Papain is used with fetal, as well as postnatal, brain regions to provide maximal dissociation and viability of neurons.
6. In preparing a cell suspension of maximum purity, four important issues should be considered. First, a high purity on the proteolytic enzyme; second, a gentle triturating of the cells; third, the time of cell manipulation, which should be kept to a minimum; and fourth, the density of cell plating. For cerebellar granule cells, a range between 500 and 800 cells per microliter is optimal. Plating the cells at high densities gives maximal cell survival.
7. The age of the tissue from which the cells are obtained is a critical factor in the culture. The general rule is to obtain cells from the developmental period of interest. The optimal ages for granule cell cultures are postnatal days 6–8, a time when all the cerebellar cell types are present in vivo. Purkinje cells should be harvested at gestational day 18 through postnatal day 1, before the granule cell population start to develop.
8. For studies of oxidative stress (seeking to isolate the effects of pro- or antioxidant compounds), it may be preferable that antioxidants (other than ones under investigation) be excluded from the media. Moreover, because antioxidants are believed to function by pre-accumulation of intracellular stores before the onset of insult, simply removing antioxidants at the time of experimental treatment may not substantially diminish antioxidant protection, and therefore may not be a preferred solution for studies of pro-oxidative effects (7).
9. It should be noticed that because the position of the striatum, such dissection is time-consuming and challenging to perform. Particular care should be exerted to completely exclude the presence of surrounding tissues.

10. PC-12 cells require special attention as they tend to form cell aggregates that are associated with irreversible changes in cell physiology, which include a loss of secretory responsiveness. To prevent the progressive accumulation of cell aggregates through multiple passages, cell suspensions are triturated extensively prior to replating.

Acknowledgments

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Chapter 3

Determination of Metal Interactions with the Chaperone Hspa5 in Human Astrocytoma Cells and Rat Astrocyte Primary Cultures

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Abstract

Molecular chaperones assist the folding of nascent proteins during translation into their correct conformations. Neurotoxic metals such as copper (Cu) and lead (Pb) may produce a deficiency in chaperone function that compromises protein secretion and exacerbates protein aggregation, potentially promoting neurodegenerative diseases that exhibit protein aggregation. Because astrocytes function as depots in the brain for certain metals, including Cu and Pb, the interaction of metals with chaperones in these cells is of interest. Furthermore, Pb and Cu bind strongly to the molecular chaperone heat shock 70 kDa protein Hspa5, also known as glucose-regulated protein 78 (Grp78) or immunoglobulin-binding protein (BiP). This chapter describes methods for expressing fluorescent chimeric proteins in astrocytes and astrocytoma cells in order to examine the metal-induced cytosolic redistribution of Hspa5, as well as associated effects on the secretion of interleukin-6 (IL-6).

Key words: Astrocytes, Copper, Lead, Chaperones, Protein aggregation, Hspa5, Grp78

1. Introduction

Molecular chaperones are highly conserved proteins that assist the folding of nascent proteins into their correct conformations as they are translated by the rough endoplasmic reticulum. Several chaperones were initially discovered such as heat-shock proteins and stress proteins because they are induced by environmental stress. These proteins also play a critical role in cell survival and recovery after stress. A deficiency in chaperone function may underlie neurodegenerative diseases that exhibit protein accumulation

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or misfolding, including Alzheimer's disease and Parkinson's disease (1). In particular, neurotoxic metals such as copper (Cu) and lead (Pb) are hypothesized to produce a deficiency in chaperone function that compromises protein secretion and exacerbates protein aggregation, potentially promoting neurodegenerative diseases that exhibit protein aggregation (2). Because astrocytes function as depots in the brain for certain metals, including Cu and Pb (3), the interaction of metals with chaperones in these cells is of interest. We have shown that astrocytes accumulate Pb and Cu and that Pb and Cu bind strongly to the molecular chaperone heat shock 70 kDa protein Hspa5, also known as glucose-regulated protein 78 (Grp78) and immunoglobulin-binding protein (BiP) (4).

This chapter describes methods for expressing a fluorescent Hspa5 chimeric protein in CCF-STTG1 human astrocytoma cells and rat astrocyte primary cultures, and imaging its cellular distribution in CCF-STTG1 cells treated with Cu or Pb by fluorescence microscopy. Because one of the proteins chaperoned for secretion by Hspa5 from astrocytes is interleukin-6 (IL-6) (5, 6), we also describe methods for coexpressing fluorescent Hspa5 and IL-6 proteins in rat astrocyte primary cultures. Lastly, we describe a functional application of these techniques in which we demonstrate that Pb treatment induces IL-6 retention and reduces its secretion by astrocytes.

2. Materials

2.1. Cell Line Culture (see Note 1)

1. Cryopreserved human CCF-STTG1 astrocytoma cell line purchased from American Type Culture Collection (ATCC).
2. Cryopreserved human SH-SY5Y neuroblastoma cell line purchased from ATCC.
3. RPMI-1640 Medium, HEPES Modification. Add 5 mL of 100 mM sodium pyruvate to 500 mL of RPMI-1640 Medium containing 10% fetal bovine serum (FBS) to prepare pyruvate- and serum-containing RPMI-1640 Medium (complete RPMI-1640 medium). Add 5 mL of 100 mM sodium pyruvate to 500 mL of RPMI-1640 medium without FBS to prepare serum-free RPMI-1640 medium. These media are used to culture CCF-STTG1 cells.
4. Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12) supplemented with 10% FBS. This medium is used to culture SH-SY5Y cells.
5. Trypan Blue Solution: 0.4% in 0.81% sodium chloride and 0.06% potassium phosphate dibasic, sterile-filtered, cell culture tested.

6. Puck's solution, pH 7.2: 1 L solution contains 8.0 g NaCl, 0.4 g KCl, 0.1 g Na_2HPO_4, 2.38 g HEPES, and 1.0 g dextrose.
7. Puck's solution with EDTA, pH 7.2: 1 L contains 8.0 g NaCl, 0.4 g KCl, 0.1 g Na_2HPO_4, 2.38 g HEPES, 1.0 g dextrose, 0.29 g tetra sodium ethylenediamine tetraacetic acid.
8. 1.7-mL safe-seal DNase- and RNase-free microcentrifuge tubes.
9. 1, 5, 10, and 25-mL sterile disposable polystyrene serological pipettes in individual sleeves.
10. Autoclaved disposable Pasteur pipettes in a canister.
11. 4-well Lab-Tek Chambered Coverglass (Nagle Nunc Intl.).
12. T-25 cell culture flasks.

2.2. Astrocyte Primary Culture (See Note 1)

1. Sprague Dawley rat pups, aged 1–3 days postnatal (see Note 2).
2. Waymouth's MD 705/1 medium without serum; Waymouth's MD 705/1 medium supplemented with 10% FBS; and Waymouth's MD 705/1 medium supplemented with 10% FBS and 1% Penicillin–Streptomycin–Neomycin solution (PSN) (see Note 3).
3. PSN solution: sterile-filtered cell culture tested, formulated to contain ~5,000 U penicillin, 5 mg streptomycin, and 10 mg neomycin/mL.
4. 10× Trypsin–EDTA solution: 5.0 g porcine trypsin and 2 g EDTA.4Na/L of 0.9% sodium chloride, sterile-filtered, cell culture tested.
5. DMEM/F12 medium, Trypan Blue Solution, Puck's solution as described in Subheading 2.1 above (see items 4–7).
6. 70% Ethanol.
7. Disposable pipettes, microfuge tubes, and Lab-Tek Chambered Coverglasses as described in Subheading 2.1 above (see items 8–11).
8. T-75 cell culture flasks.
9. Sterile brain dissection kit consisting of a steel sterilization tray with steel lid (about 9 in. × 5 in. × 2 in. deep) packed as follows from the bottom up: four layers of 4 in. × 4 in. × 4-ply gauze sponges, two fine curved-tip dissecting forceps with serrated tips (3¾ in.), another four layers of gauze, a thin steel weighing spatula with flat 3/8 in wide blade and two straight-bladed iris scissors, four more layers of gauze, one large scissors (6 in.), four more layers of gauze, one large forceps wrapped in gauze, and a final layer of gauze. The lid is placed on the tray and the kit is wrapped twice with aluminum foil and taped with autoclave tape. Autoclave the kit up to 1 day

before cell cultures will be prepared, oven dry the kit, and store at room temperature.

10. 35-mm Diameter tissue culture dishes.
11. 50-mL sterile polypropylene disposable centrifuge tubes, conical tip, and flat top cap.
12. Coarse mesh tissue collection beaker prepared by laying a 7×7 in square of #130 polyamide nylon mesh filter (TETKO, Inc., Switzerland) across the mouth of a 500-mL glass beaker and securing it with a heavy rubber band. Wrap the beaker and filter twice in aluminum foil and autoclave, oven dry the unit, and store in the same manner as the dissection kit.
13. Fine mesh tissue collection beaker prepared as above, except with a square of #33 or 35 polyamide nylon mesh (TETKO, Inc., Switzerland).
14. 4′,6-Diamidino-2-phenylindole (DAPI), 5 mg/mL stock solution in water.
15. SH-SYSY conditioned medium (see Note 4).

2.3. Immunocytochemistry for Glial Fibrillary Acidic Protein

1. Mouse monoclonal anti-glial fibrillary acidic protein (GFAP) (Sigma).
2. Rabbit polyclonal anti-mouse IgG–tetramethyl rhodamine isothiocyanate (TRITC) conjugate (Sigma).
3. 1× Phosphate-buffered saline (PBS): add 11.5 g Na_2HPO_4, 2 g KH_2PO_4, 8.0 g NaCl, and 0.2 g KCl to 1 L dd H_2O and adjust pH to 7.4 with NaOH or HCl.
4. 4% Paraformaldehyde in PBS, pH 7.4.
5. Tris-buffered saline (TBS) washing buffer, pH 7.5: 50 mM Tris–HCl, 150 mM NaCl.
6. TBS Tween20 (TBST) blocking buffer, pH 7.5: 5% dried nonfat milk in buffer consisting of 50 mM Tris–HCl, 150 mM NaCl, and 0.1% Triton X-100.

2.4. Construction of Chimeric Proteins

1. pEGFP-N3 vector plasmid with kanamycin resistant gene (BD Biosciences Clontech).
2. pDsRed2-N1 vector plasmid with kanamycin resistant gene (BD Biosciences Clontech).
3. TRIzol® Reagent (monophasic solution of phenol and guanidine isothiocyanate, Invitrogen).
4. DH5α competent *Escherichia coli* cells.
5. Kanamycin powder in monosulfate.
6. Luria–Bertani (LB) broth: weigh 25 g of powdered LB broth, Miller, add the broth to 1 L double-distilled water in a 2-L glass flask, cover the mouth of the flask with aluminum foil,

autoclave at 120°C and 1.5 bar for 20 min, add kanamycin to a final concentration of 25 μg/mL after the temperature has dropped down to room temperature, and refrigerate. LB broth can be stored for up to 3 months refrigerated.

7. Luria–Bertani (LB) agar plates: weigh 40 g of powdered LB agar, Miller, add the agar to 1 L double-distilled water in 2-L glass flask, cover the mouth of the flask with aluminum foil, autoclave at 120°C and 1.5 bar for 20 min, add kanamycin to a final concentration of 25 μg/mL after the temperature has dropped down to 55–60°C, pipette 20 mL agar into each 50 100-mm diameter bacteria culture plates, and refrigerate the plates for up to 3 months.
8. SOC bacterial growth medium: add 20 g tryptone, 5 g yeast extract, 2 mL of 5 M NaCl, 2.5 mL of 1 M KCl, 10 mL of 1 M $MgCl_2$, 10 mL of 1 M $MgSO_4$, and 20 mL of 1 M glucose to 900 mL of double-distilled H_2O. Adjust to 1 L with distilled H_2O. Sterilize by autoclaving. Divide into 5 mL sterile vials. Store frozen at –20°C.
9. Chloroform (≥99%, PCR reagent grade, amylenes as stabilizer, DNase- and RNase-free).
10. Isopropyl alcohol (≥99%, molecular biology reagent grade, DNase- and RNase-free).
11. Absolute alcohol (≥99.8%, molecular biology reagent grade, DNase- and RNase-free).
12. Copper sulfate, 99%, ReagentPlus® grade: prepare a 10 mM stock solution in double-distilled H_2O, filter-sterile with a 0.2-μm pore size syringe filter and store at 4°C.
13. Lead acetate (≥99.8%, American Chemical Society reagent grade). Prepare a 1.0 mM stock solution in double-distilled H_2O, filter-sterile with a 0.2-μm pore size syringe filter and store at 4°C.
14. 10× Tris/Boric acid/EDTA (TBE) buffer: 1 L of 10× stock solution contains 157.5 g Tris base, 27.8 g Boric acid, 50 mL of 0.5 M EDTA, pH 8.3.
15. Ultra Pure DNA grade agarose.
16. Diethylpyrocarbonate (DEPC)-treated water. Prepare RNase-free water in a fume hood by adding 0.5 mL DEPC to 1 L of double-distilled water in a glass bottle and mixing. Autoclave the treated water next day at 120°C and 1.5 bar for 20 min to inactivate the DEPC. Treated water may be stored at room temperature for up to 3 months.
17. DEPC-treated 75% ethanol. Prepare freshly before use by mixing 25% volume of DEPC-treated water with 75% absolute ethanol.

18. SuperScript™ First-Strand Synthesis System for RT-PCR kit (Invitrogen).
19. Taq DNA polymerase (Promega).
20. dNTPs: dGTP, dTTP, dATP, and dCTP, 100 mM each in separate vials. Dilute in DEPC-treated water to 1 mM when needed for PCR. We typically prepare 200 µL at a time. Both the stock and dilution are stored at –20°C.
21. Rat Hspa5 PCR sense primer with *Hin*dIII site (the underlined portion indicates the *Hin*dIII restriction site and bold letters show the start codon): 5′-ATAGTCAAGCTTAG**ATG**AAGT TCACTGTGG-3′ (see Note 5).
22. Rat Hspa5 PCR anti-sense primer with *Bam*HI site (the underlined portion indicates the *Bam*HI restriction site): 5′-ATAGTCGGATCCTGATGTATCCTCTTCACC-3′.
23. Rat IL-6 PCR sense primer with *Hin*dIII site (the underlined portion indicates the *Hin*dIII restriction site and bold letters show the start codon): 5′-ATAGTCAAGCTT**ATG**AAGTTT CTCTCCGCAAG-3′.
24. Rat IL-6 PCR antisense primer for IL-6-EGFP chimera with *Bam*HI site (the underlined portion indicates the *Bam*HI restriction site): 5′-ATAGTCGGATCCTAAGGTTTGCCGA GTAGACC-3′.
25. Rat IL-6 PCR antisense primer for IL-6-DsRed2 chimera with *Bam*HI site (the underlined portion indicates the *Bam*HI restriction site): 5′-ATAGTCGGATCCAAGGTTTGCCGA GTAGACC-3′.
26. *Bam*HI restriction enzyme.
27. *Hin*dIII restriction enzyme.
28. T4 DNA ligase.
29. 1 kb and 100 bp DNA ladders.
30. DNA agarose gel extraction kit.
31. Plasmid miniprep kit.
32. 100 × 15-mm disposable petri dishs for bacterial culture.
33. 1% Agarose gel.
34. Ethidium bromide, molecular biology reagent grade, saturated stock solution in double-distilled H_2O, and final concentration of 10 µg/mL (see Note 6).

2.5. Transfection and Image Collection

1. Lipofectamine™ 2000 transfection reagent (Invitrogen) (see Note 7).
2. Inverted fluorescence microscope.
3. Bio-Rad Radiance 2000MP laser scanning instrument.

2.6. Detection of IL-6-EGFP Release

1. Nitrocellulose membrane.
2. Bio-Rad dot-blot apparatus.
3. Mouse monoclonal antibody against EGFP (Sigma).
4. Goat anti-mouse IgG conjugated with a horse radish peroxidase (Sigma).
5. Multiwell 24-well tissue culture plate treated by Vacuum Gas Plasma, polystyrene nonpyrogenic, individually packaged.

3. Methods

Protein distribution, aggregation, retention, and secretion can be investigated in living cells in culture with a combination of proteomics and imaging techniques. In this section, we describe how to construct and express three fluorescent chimeric proteins: Hspa5-EGFP and IL-6-EGFP, which fluoresces green, and IL-6-DsRed, which fluoresces red and can be coexpressed in the same cell with Hspa5-EGFP. The main protein of interest in this chapter with respect to mechanisms of Cu and Pb neurotoxicity is Hspa5, because both metals bind strongly to this protein. IL-6 is a secreted protein that is chaperoned in astrocytes by Hspa5 (5, 6), thus providing a functional test for perturbations of Hspa5 functions by metals. Two cell culture models will be employed: the CCF-STTG1 human astrocytoma cell line and rat primary astrocyte cultures. Both cell culture models have been widely used for studying mechanisms of neurotoxicity (7). Indeed, most of the known functions of astrocytes were initially discovered in primary astrocyte cultures, which express many characteristics of developing astrocytes in vivo. In addition, astroglial cell lines that reliably replicate some of the properties of normal astrocytes are often useful for neurotoxicity studies because they are easily obtained and maintained. CCF-STTG1 cells, which were established from astrocytoma grade IV in a 68-year-old woman, express astroglial properties, such as the ability to generate Ca^{2+} waves in response to stimulants (8) and the secretion of apoE in response to cytokines (9).

3.1. Astroglial Cell Line Culture

1. Thaw one vial of cryopreserved CCF-STTG1 astrocytoma cells within 1 min in a 37°C water bath and transfer to a T-25 flask containing 5 mL of complete RPMI-1640 medium. Begin changing the medium three times per week 2 days after initial seeding.
2. After cells reach 70–80% confluence by visual inspection under a phase contrast microscope, aspirate the medium with a sterile Pasteur pipette and detach the cells by incubating for

5 min at 37°C with 1 mL of Puck's solution with EDTA. Stop the action of the EDTA by adding 5 mL of complete RPMI-1640 medium.

3. Passage the cells to a new T-25 flask at 1:10 dilution with complete RPMI-1640 Medium to establish stock cultures.

3.2. Astrocyte Primary Culture and Assessment of Purity

1. In a Class II biological safety cabinet, remove brains from four 1- to 3-day-old rat pups as follows. Pups should be separated from the dam shortly before their use for cell culture. Open the dissecting kit inside the hood. Handle gauze and instruments sterilely with the large forceps that were packed at the top of the dissecting kit. Spray the head and neck of one rat pup with 70% ethanol. Quickly decapitate it with large scissors, collecting the head on sterile gauze. Open the scalp down the midline with the first pair of small scissors and pull it away with gauze, maintaining sterility of the skull. Rest the blades of the scissors in a small beaker containing 70% ethanol between uses. Open the skull down the midline with the second pair of small scissors and make one transverse incision on each side of the calivarium to make the brain accessible. Remove the brain with a thin steel spatula and place it in a 35-mm tissue culture dish containing 2 mL of Waymouth's MD 705/1 medium without FBS. Repeat until four brains are collected in each dish.
2. Use curved dissecting forceps to peel off meninges and to remove cerebral hemispheres from the brains. Collect the eight hemispheres in a fresh 35-mm dishes containing 2 mL of Waymouth's MD 705/1 medium without FBS.
3. Aspirate the hemispheres from the dish with a 10-mL disposable sterile tissue culture pipette and place in a sterile 50-mL centrifuge tube containing 15 mL of the same serum-free medium.
4. Triturate five times with a 10-mL pipette.
5. Add 0.2 mL of 10× trypsin to the tube and triturate three times with 10-mL pipette. Immediately add 25 mL of Waymouth's MD 705/1 medium containing 10% FBS to cells and mix thoroughly to inhibit the trypsin.
6. Filter the above cell suspension through the coarse tissue filter (#130 mesh) to remove large cell clumps and lipid globules.
7. Transfer the filtrate with a sterile pipette to a sterile 50-mL centrifuge tube and centrifuge the cell suspension at $200 \times g$ force for 5 min in a swinging bucket rotor. Remove the supernatant and resuspend the cells in 30 mL of Waymouth's medium containing 10% FBS.

8. Filter the cell suspension through the fine tissue filter (#33 or #35 mesh) to separate the fibroblasts and blood cells from the astroglia.
9. Add an adequate amount of Waymouth's medium containing 10% FBS and 1% PSN to achieve a final seeding volume. We seed primary astrocyte cultures from newborn pups at one cerebral hemisphere per T-75 flask in 10 mL of medium.
10. Incubate cultures in a 5% CO_2 water-jacketed, humidified incubator at 37°C.
11. After 4 days (day 4), feed the cultures with PSN-free Waymouth's MD 705/1 medium containing 10% FBS, and thereafter maintain the cultures in PSN-free medium. Between day 4 and 10, cultures are fed once with Waymouth's MD 705/1 medium containing 10% FBS.
12. After another 6 days (day 10), switch cultures to DMEM/F12 medium containing 10% FBS. Alternatively, feed the cultures with one 1:1 v:v mixture of fresh DMEM/F12 medium containing 10% FBS and SH-SY5Y-conditioned medium (see Note 4).
13. Between day 15 and 21, aspirate the medium with a sterile Pasteur pipette detach cultures from the T-75 flasks by incubating at 37°C for 3–5 min in 2 mL Puck's solution with EDTA to which 100 μL of 10× trypsin–EDTA has been added (i.e., a 1:20 dilution of the 10× stock trypsin). Stop the action of the trypsin and EDTA by adding 10 mL of DMEM/F12 medium with 10% FBS.
14. Stain an aliquot of the cell suspension with trypan blue and count dye-excluding (live) cells with a hemocytometer. Seed cells in 4-well Lab-Tek Chambered Coverglass unit (0.5 mL/well) at a density of 100,000 live cells/mL.
15. Three days after seeding in coverglass chambers, confirm the purity of astrocyte cultures by immunofluorescence staining for GFAP. Rinse the cells in the coverglass chambers twice with 0.5 mL/well cold (<10°C) PBS to reduce the activity of proteases. Fix the cells with 0.5 mL/well 4% paraformaldehyde in PBS at room temperature for 10–20 min. Wash the cells with 0.5 mL of PBS three times at room temperature, allowing each wash to remain on the cells for 5–10 min each wash. Remove the last wash and add 0.2 mL 5% nonfat-dried milk in TBST to each well for 1 h at room temperature to block nonspecific binding. Remove the blocking solution, add 0.2 mL of mouse GFAP monoclonal antibody diluted 1:2,000 in TBST-containing 5% nonfat-dried milk to each well, and incubate for 1 h at room temperature or at 4°C overnight. Wash the cells with 0.5 mL of TBS 3–5 times at

room temperature, allowing each wash to remain on the cells for 5–10 min. Remove the wash, add 0.2 mL rabbit polyclonal anti-mouse IgG–TRITC conjugate diluted 1:1,000 in TBS containing 5% nonfat dried milk in TBS to each well, and incubate in the dark for 1 h. After this step, procedures should be carried out under dim light. Wash the cells with 0.5 mL/well of TBS 3–5 times at room temperature for 5–10 min each wash.

16. In the same immunostained specimen, stain nuclei with DAPI (1 μg/mL) in TBS at room temperature for 1–3 min under dim light.
17. Visualize fluorescence images of GFAP via GFAP signals via the red channel (545 mm excitation and 590 mm emission) and DAPI signals via the cyan channel (355 mm excitation and 475 mm emission) on an inverted fluorescence microscope (Fig. 1). Cultures should be 90–95% GFAP-positive.

3.3. Preparation of Amplified Rat Hspa5 cDNA

1. Seed detached astrocytes, Subheading 3.2, step 13, in one T-25 flask with 5 mL of DMEM/F12 Medium containing 10% FBS and culture at 5% CO_2 and 37°C for 1 week, changing the medium every other day. The cultures should reach 70–80% confluence during this time.

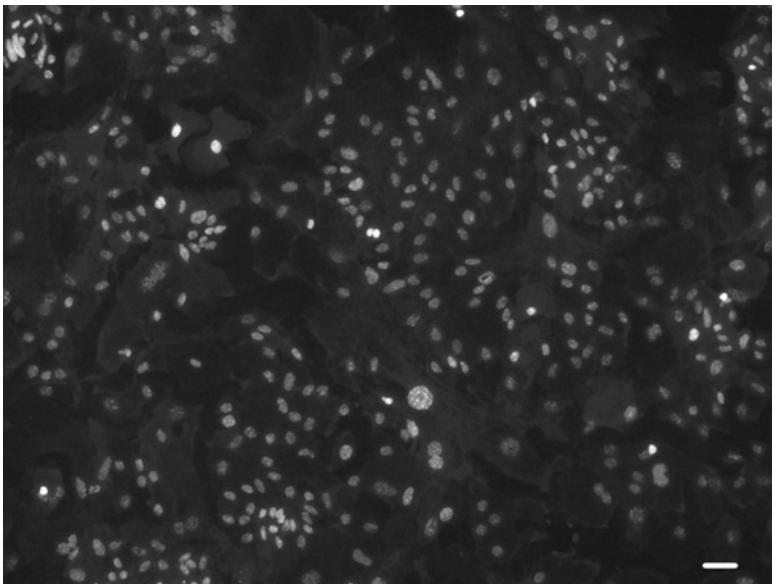

Fig. 1. Immunocytochemical identification of rat primary astrocytes. Cultures in 4-well Lab-Tek Chambered Coverglass were immunostained by mouse monoclonal anti-GFAP (1:2,000) as primary antibody and rabbit polyclonal anti-mouse IgG–TRITC conjugate (1:1,000) as secondary antibody for the identification of GFAP expression (*gray*). The nuclei (*bright*) were stained by DAPI (1 μg/mL). The image was captured on an inverted Olympus fluorescence microscope. *Scale bar*: 50 μm.

2. Aspirate the medium and immediately add 1 mL of TRIzol reagent to the flask to lyse cells.
3. Transfer the lysate to a DNAse and RNAse-free microcentrifuge tube (1.7 mL).
4. Incubate the lysate for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes.
5. Add 0.2 mL of chloroform to the lysate and cap the tube securely.
6. Shake the tube vigorously by hand for 15 s and incubate the lysate at room temperature for 2–3 min.
7. Centrifuge the lysate at 12,000 × *g* force for 15 min at 2–8°C.
8. Transfer the aqueous phase (~0.6 mL) to a fresh microcentrifuge tube.
9. Add 0.5 mL of isopropyl alcohol to the aqueous phase, mix, and incubate the mixture at room temperature for 10 min.
10. Centrifuge at 12,000 × *g* force for 10 min at 2–8°C to collect RNA.
11. Discard the supernatant and add 1 mL of DEPC-treated 75% ethanol.
12. Vortex and centrifuge at 10,000 × *g* for 5 min at 2–8°C to collect RNA.
13. Discard the ethanol and briefly air-dry the RNA pellet for 5–10 min. It is important not to let the RNA pellet dry completely as this will dramatically decrease RNA solubility. Do not dry the RNA pellet by centrifugation under a vacuum.
14. Add 50 μL of DEPC-treated water to dissolve the RNA pellet.
15. Determine the RNA concentration by the measure of OD260 in DEPC-treated water on a spectrophotometer. Both OD260 and OD280 should be measured to verify that the OD260/OD280 ratio ranges between 1.8 and 2.0. A ratio below 1.6 suggests that the RNA is only partially dissolved or impure. A ratio above 2.0 suggests that the RNA is degraded. The RNA concentration can be calculated as follows: 40 μg/mL × OD260 × dilution.
16. Reverse transcribe the RNA into cDNA with First-Strand Synthesis System for RT-PCR kit according to the manufacturer's protocol. We use 4 μg RNA in the reverse transcription.
17. Amplify rat Hspa5 cDNA by PCR with rat Hspa5 cDNA primers (see Note 8).
18. Separate PCR products on a 1% agarose gel in a horizontal gel electrophoresis apparatus with 1× TBE. Stain the gel with 10 μg/mL EB to visualize DNA.

19. Collect the approximately 2 kb cDNA band of amplified rat Hspa5 cDNA, which can be located on a UV light box by measuring against the 1 kb DNA ladder (see Note 9).
20. Extract rat Hspa5 cDNA with a DNA agarose gel extraction kit according to manufacturer's directions.
21. Determine the DNA concentration by measuring the OD260 on a spectrophotometer. The cDNA concentration can be calculated as follows: 50 μg/mL × OD260 × dilution.
22. Confirm the identity of the rat Hspa5 cDNA by DNA sequencing.

3.4. Preparation of Hspa5-EGFP Chimeric Plasmids

1. Digest the extracted rat Hspa5 cDNA and the pEGFP-N3 plasmid DNA, respectively, with *Bam*HI and *Hin*dIII restriction enzymes (see Note 10).
2. Separate the digested Hspa5 cDNA and the pEGFP-N3 plasmid DNA on a 1% agarose gel with 1× TBE by gel electrophoresis. Stain the gel with EB.
3. Collect the digested Hspa5 cDNA and pEGFP-N3 DNA agarose bands and extract them with DNA agarose gel extraction kit according to manufacturer's protocol.
4. Determine the DNA concentrations of Hspa5 cDNA and pEGFP-N3 plasmid DNA by measuring the OD260 on a spectrophotometer.
5. Ligate the digested Hspa5 cDNA with the digested pEGFP-N3 plasmid DNA with T4 DNA ligase (see Note 11).
6. Transform DH5α competent cells with Hspa5 cDNA and pEGFP-N3 ligates as follows. Add 2.5 μL of the Hspa5 cDNA and pEGFP-N3 plasmid DNA ligates to 50 μL DH5α competent bacterial cells in 2-mL vial, thawed on ice, and mix gently with a pipette tip. After heat shock at 42°C for 40–60 s in water bath, immediately transfer the mixture to an ice bath for 5 min. Add 400 μL of SOC medium pre-warmed at 37°C. Culture the bacteria at 37°C for 1 h on an orbital shaking incubator at 225 rpm for in preparation for the selection of positive Hspa5 clones.
7. Innoculate a total of 450 μL of transformed DH5α competent (150 μL cells per plate) into three LB agar plates containing kanamycin (25 μg/mL) at 150 μL of transformed bacterial cells. Incubate the plates overnight at 37°C in a bacterial incubator and inspect the bacterial colonies the next day.
8. Randomly select 15 colonies (the colonies that can grow on a kanamycin agar plate may be either empty pEGFP-N3 plasmids or Hspa5 cDNA-ligated plasmids) for identification of Hspa5 positive colonies. Screen the Hspa5-positive colonies by PCR analysis (see Note 8, cDNA is replaced by colonies).

9. Further screen Hspa5-EGFP chimera-positive plasmids by DNA sequencing as follows. Select three colonies based on PCR screening and inoculate each colony in 4 mL of LB broth containing kanamycin (25 μg/mL). Culture at 37°C overnight on an orbital shaker at 225 rpm. The next day, extract the plasmids with a QIAprep Spin Miniprep kit according to manufacturer's protocol (Qiagen) and measure plasmid concentrations. Confirm the identity of the correct Hspa5-EGFP chimeric clones by DNA sequencing.

3.5. Preparation of Amplified Rat IL-6 cDNA, IL-6-EGFP Chimeric Plasmids, and IL-6-DsRed2 Chimeric Plasmids

1. Amplify rat IL-6 cDNA by PCR with rat IL-6-EGFP cDNA primers according to the protocol described in Subheading 3.3.
2. Separate PCR products on a 1% agarose gel in a horizontal gel electrophoresis apparatus with 1× TBE. Stain the gel with EB.
3. Collect the approximately 600 bp band amplified rat IL-6 cDNA bands, which can be located on a UV light box by measuring against the 100 bp DNA ladder.
4. Follow procedures in Subheading 3.4 to prepare IL-6-EGFP chimeric plasmids.
5. Amplify rat IL-6 cDNA by PCR with rat IL-6-DsRed2 cDNA primers according to the protocol described in Subheading 3.3.
6. Follow procedures in Subheading 3.4 to prepare IL-6-DsRed2 chimeric plasmids.

3.6. Transfection of CCF-STTG1 Cells with Hspa5-EGFP Plasmids or IL-6-EGFP Chimeric Plasmids

1. Prepare subcultures from stock cultures of CCF-STTG1 cells that are 70–80% confluent as follows. Detach the cells as described in Subheading 3.1, step 2 and count an aliquot of cells stained with trypan blue with a hemocytometer. Seed the cells in 4-well Lab-Tek Chambered Coverglass unit (0.5 mL/well) in complete RPMI-1640 medium at a density of 200,000 live (dye-excluding) cells/mL and culture the cells at 5% CO_2 and 37°C.
2. Within the next 24 h, dilute 0.8 μg of the Hspa5-pEGFP or IL-6-EGFP plasmids in 50 μL serum-free RPMI-1640 medium in a 1.7 mL DNase-free microcentrifuge tube for each well of the coverglass chanbers. Controls should be transfected with the pEGFP-N3 plasmid to test the effects of transfection on viability.
3. Dilute 2 μL of the Lipofectamine™ 2000 transfection reagent in 50 μL serum-free RPMI-1640 medium in a 1.7 mL DNase-free microcentrifuge tube for each well.
4. After 5 min at room temperature, mix the diluted plasmid DNA with the diluted transfection reagent by transferring one to another tube.

5. Incubate the mixture for 20 min at room temperature to form plasmid DNA–transfection reagent complexes.
6. Add 100 µL of plasmid DNA–transfection reagent complexes to each well and culture the cells at 5% CO_2 and 37°C.
7. After 24–48 h, image EGFP-expressing cells with a Bio-Rad Radiance 2000MP laser scanning instrument using an excitation wavelength of 488 nm and an emission wavelength of 515 nm (Figs. 2 and 3).

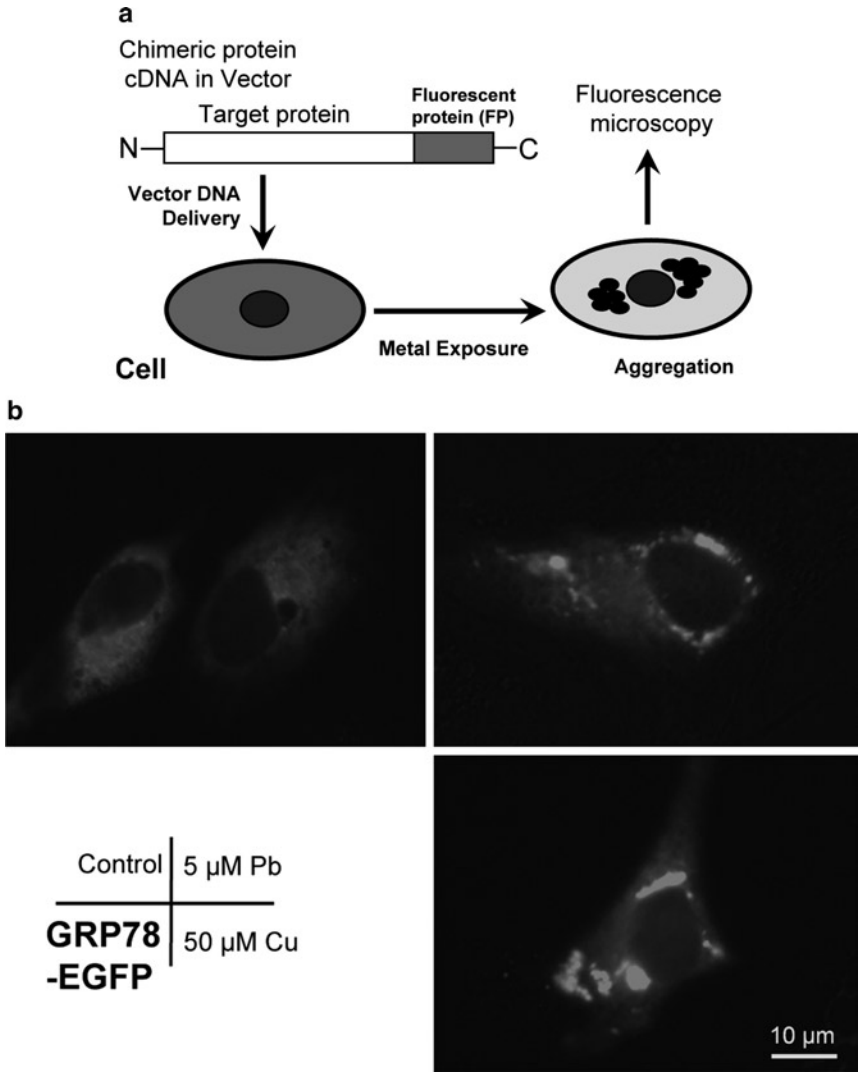

Fig. 2. Metal-induced Hspa5 redistribution in human astrocytoma cells. *Top*: Diagram of a fluorescence approach used to examine the compartmentalization of Hspa5 in metal-treated cells. *Bottom*: Representative micrographs of transiently transfected CCF-STTG1 astrocytoma cells that have been exposed to 5 µM lead acetate or 50 µM copper sulfate for 10 h. The clumping of the fluorescent chimeric protein is observed in the Pb- or Cu-treated cells expressing Hspa5-EGFP chimera but not in cells expressing EGFP (data not shown), suggesting that metals interact with Hspa5, *scale bar*: 10 µm (Micrograph adapted from Qian Y.C. et al., Neurotoxicology, 26, 267–275, 2005, with permission).

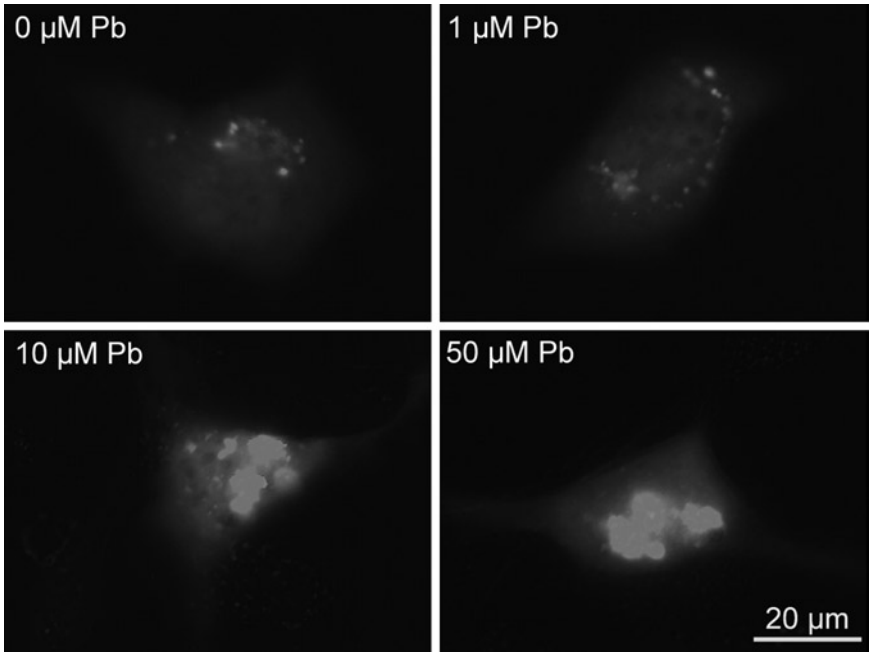

Fig. 3. Pb-induced IL-6 aggregation in human astrocytoma cells. Representative micrographs of transfected cells that have been exposed to 0, 1, 10, and 50 µM lead acetate for 18 h. The clumping of the fluorescent chimeric protein is observed in the lead-treated cells expressing IL-6-EGFP chimera but not in cells expressing EGFP (data not shown). *Scale bar*: 20 µm.

8. After 24–48 h, the transfected cells may be treated with metals to test the effects of metals on the intracellular distribution and function of the fluorescent proteins (see Note 12, Figs. 2 and 3).
9. Image Hspa5-EGFP or IL-6-EGFP chimera-expressing cells with an inverted fluorescence microscope.

3.7. Transfection of Astrocytes with IL-6-EGFP Chimeric Plasmids

1. Prepare subcultures of primary astrocytes as follows. Detach the cells as described in Subheading 3.2, step 13 and count an aliquot of count an aliquot of cells stained with trypan blue with a hemocytometer. Seed cells in 4-well Lab-Tek Chambered Coverglass unit (0.5 mL/well) in DMEM/F12 medium containg 10% FBS at a density of 200,000 live cells/mL and culture the cells at 5% CO_2 and 37°C.
2. Follow procedures in Subheading 3.6 from steps 2–9 to transfect astrocytes with IL-6-EGFP chimeric plasmids, except that DMEM/F12 medium should be used instead of RPMI-1640.

3.8. Cotransfection of Astrocytes with Hspa5-EGFP and IL-6-DsRed2 Chimeric Plasmids

1. Subculture primary astrocytes into 4-well Lab-Tek Chambered Coverglass unit as described in Subheading 3.7, step 1.
2. Within the next 24 h, dilute 0.6 µg of the Hspa5-pEGFP and 0.6 µg IL-6-DsRed2 plasmids in 50 µL serum-free DMEM/

F12 medium in a 1.7 mL DNase-free microcentrifuge tube for each well.

3. Dilute 2 μL of the transfection reagent in 50 μL serum-free DMEM/F12 medium in a 1.7 mL DNase-free microcentrifuge tube for each well.
4. After 5 min at room temperature, mix the diluted plasmid DNA with the diluted transfection reagent by transferring one to another tube.
5. Incubate the mixture for 20 min at room temperature to form plasmid DNA–transfection reagent complexes.
6. Add the 100 μL of plasmid DNA–transfection reagent complexes to each well and culture the cells at 5% CO_2 and 37°C.
7. After 24–48 h, image EGFP-expressing cells using an excitation wavelength of 488 nm and an emission wavelength of 515 nm and DsRed2-expressing cells using an excitation wavelength of 595 nm and an emission wavelength of 615 nm with a Bio-Rad Radiance 2000MP laser scanning instrument (Fig. 4).

3.9. Assay of IL-6-EGFP Release from Astrocytes

1. Subculture primary astrocytes into 24-well plates as described in Subheading 3.7, step 1 at a density of 100,000 live cells/mL, 1 mL per well.
2. Transfect the cells with IL-6-EGFP plasmids as described in Subheading 3.8.

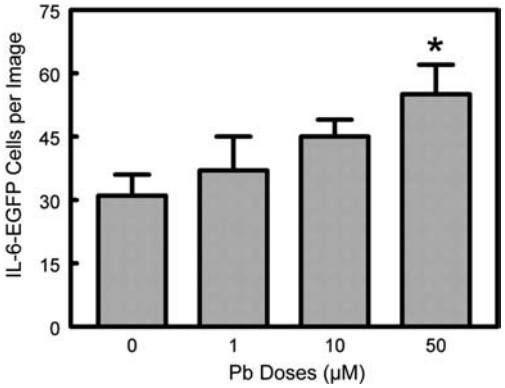

Fig. 4. Pb-induced IL-6-EGFP retention in rat astrocytes. Number of cells expressing IL-6-EGFP on each blindly captured image was counted with AlphaEaseFC software and data were expressed as cells per image and represent mean ± SE ($n = 10$, $^*p < 0.05$ differs from the control). Based on previous findings that Pb binds strongly to Hspa5 (reference), this finding implies but does not prove that Pb impairs the chaperoning function of Hspa5 for IL-6. To support such as conclusion, we obtained additional data, reported elsewhere, that IL-6 secretion is reduced in cells depleted of Hspa5 and that Pb does not bind to IL-6. (Adapted from Qian Y.C. et al., Am J Physiol 293, C897–C905, 2007, Am. Physiol. Soc., used with permission).

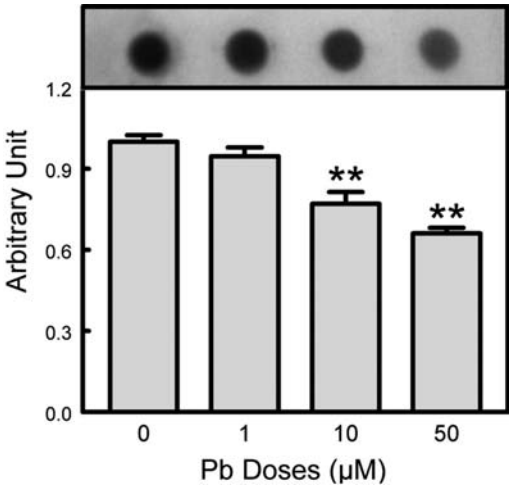

Fig. 5. Pb-induced decrease of IL-6 release from astrocytes. Astrocytes were transfected with IL-6-EGFP plasmids for 24 h and then treated with 0, 1, 10, and 50 μM Pb acetate for another 24 h. Extracellular IL-6-EGFP signals were detected by dot-blot analysis with EGFP antibody and quantified with ImageJ ($n=5$; $^{**}p<0.01$ differs from the control). (Adapted from Qian Y.C. et al., Am J Physiol 293, C897–C905, 2007, Am. Physiol. Soc., used with permission).

3. Harvest the medium (1 mL per well) 48 h after transfection.
4. Load medium onto a nitrocellulose membrane (Bio-Rad) in a Bio-Rad dot-blot apparatus connected to a vacuum pump.
5. Detect extracellular IL-6-EGFP proteins on the membrane with a monoclonal antibody against EGFP as a primary antibody (1:1,000) and goat anti-mouse IgG conjugated with a peroxidase as a secondary antibody (1:2,500).
6. Visualize signals on Kodak X-Omat film by the use of NEN western blot chemiluminescent reagent mixture (NEN Life Science, MA).
7. Quantify the signal intensities with ImageJ software, version 1.43 (Fig. 5).

4. Notes

1. All cell culture reagents are tissue culture grade and filtered sterilized with 0.2 μM pore size filters. Materials and reagents from other manufacturers than those listed might work, but those identified in this chapter are known to work. All solutions and dilutions should be prepared in water that has a resistivity of 18.2 MΩ).
2. An Animal Use Protocol (AUP) for rodent primary cultures of astrocytes should be approved by appropriate institutional animal care and use committee.

3. The serum lot should be tested for its ability to support proliferation of astrocytes. We routinely test three serum lots and then purchase sufficient serum for 1–2 years that is stored and held in reserve by the supplier.
4. SH-SYSY conditioned medium is collected from 70–80% confluent SY5Y cells 2 days after the culture cells have been fed with DMEM/F-12 medium containing 10% FBS. The conditioned medium is filtered through a 0.2-μm pore diameter filter prior to being used. In our hands, primary astrocyte cultures prepared as described in this protocol show a decline in the expression of GFAP evident at about 25 days, suggesting dedifferentiation. However, astrocytes cultured in 50% DMEM/F-12 medium and 50% SH-SY5Y cell-conditioned medium containing 10% FBS maintain a constant level of GFAP expression. We, therefore, use SH-SY5Y conditioned medium for experiments in which a mature astrocyte phenotype is desired.
5. PCR primers can be obtained from many suppliers. We order the primers described in this chapter from Integrated DNA Technologies, Inc.
6. EB is toxic at high concentrations. To avoid weighing the powder, we prepare a saturated stock solution in double-distilled H_2O by adding excess EB to water and incubating overnight to allow the EB to dissolve. Excess EB settles to the bottom of the bottle. The solution above the precipitate has a saturated concentration of 10 mg/mL. The stock solution may be stored for many months at room temperature. Dilute the stock 1:1,000 in TBE buffer (final concentration 10 μg/mL) to stain DNA in agarose gels.
7. A transfection reagent is a cationic lipid formulation that provides a high transfection efficiency for plasmids or DNA oligos into adherent and suspension cell lines. Lipofectamine™ 2000 supplied by Invitrogen is known to work in the applications we describe, though other reagents may also be suitable.
8. PCR reaction for rat Hspa5 cDNA amplification (with the expected rat Hspa5 cDNA PCR product being 2 kb):

One PCR reaction component	
10× PCR buffer	2.5 μL
25 mM $MgCl_2$	2.0 μL
2.5 mM dNTPs mix	2.0 μL
Taq enzyme	0.25 μL
10 μM sense primer	1.25 μL
10 μM anti-sense primer	1.25 μL

cDNA (0.2 μg/μL)	1.25 μL
Sterile water	14.5 μL
Total	25 μL

PCR cycles: 94°C (2 min) – {[94°C (15 s) – 56°C (15 s) – 68°C (2 min)] × 25 cycles} – 72°C (7 min).

9. We collect the cDNA with a 200-μL Eppendorf pipette tip which is cut so that the inner diameter of the tip is the same thickness as the band on the agar gel. This tip is used like a hole punch and inserted into the band at all points throughout its horizontal width to collect the cDNA in the tip.
10. Restriction enzyme (RE) digest based on 20 μL reaction:

Sterile water	15 μL	
RE 10× buffer	2.0 μL	
Hspa5 cDNA (0.2 μg/μL)	2.0 μL	
Or pEGFP-N3 plasmid (0.5 μg/μL)		
*Bam*HI	0.5 μL	
*Hin*dIII	0.5 μL	
Total volume	20 μL	at 37°C for 90 min

11. Ligation of the digested rat Hspa5 cDNA with the digested pEGFP-N3 plasmid DNA:

Digested pEGFP-N3 plasmid DNA	100 ng
Digested rat Hspa5 cDNA	120 ng
Ligase 10× buffer	1 μL
T4 DNA ligase	0.5 μL
Nuclease-free water to final volume of	10 μL

12. As shown in Fig. 1, the three terminus of Hspa5 cDNA is ligated in frame to the five terminus of EGFP cDNA and the C terminus of Hspa5 protein is conjugated to the N terminus of EGFP to form an Hspa5-EGFP chimera. Human CCF-STTG1 astrocytoma cells expressing Hspa5-EGFP chimera were treated with 5 μM lead acetate or 50 μM copper sulfate in RPMI-1640 medium containing 10% FBS. Images were captured at various times on an inverted fluorescence microscope

with an excitation wavelength of 488 nm and an emission wavelength of 515 nm. Metals bind to or disrupt the function of Hspa5-EGFP chimeric proteins alter the cytosolic compartmentalization of Hspa5-EGFP.

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Chapter 4

Microglia Cell Culture: A Primer for the Novice

Anke Witting and Thomas Möller

Abstract

Microglial cells are the resident immune cells of the central nervous system. Progress in the recent decade has clearly established that microglial cells participate or even actively drive neurological disease. Much of our current knowledge has been generated by investigating microglial cells in cell culture. The aim of this chapter is to give the uninitiated a basic and adaptable protocol for the culturing of microglial cells. We discuss the challenges of microglial cell culture and provide a collection of tips which reflect our 25+ years of collective experience.

Key words: Microglia, Cell culture, Medium, Serum, Growth factor, M-CSF, GM-CSF, Transfection, Endotoxin, Cell yield

1. Introduction

1.1. Microglia

This chapter only provides a brief introduction into microglial cells as a point of reference. For more detailed information on the topic, the reader is respectfully referred to a number of excellent and comprehensive reviews on microglial biology (1–7). Microglia are the resident immune cells of the CNS. They resemble peripheral tissue macrophages and are the primary mediators of neuroinflammation (1, 8). Studies in the last two decades have demonstrated the involvement of microglia in many acute and chronic neurological diseases (2, 9). In the healthy adult brain, microglia exist as so-called "resting" or "surveilling" microglia, characterized by a small cell body with fine, ramified processes and minimal expression of surface antigens. Upon CNS injury, these cells are rapidly activated and participate in the pathogenesis of neurological disorders. They secrete various inflammatory molecules, including TNF-α, IL-6, and nitric oxide (8). When CNS cells die, microglia

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are further activated and become phagocytes. It is widely believed that substances released from damaged cells within the brain trigger microglial activation, consequently leading to the long-term changes of gene expression and reorganization of the cell phenotype (2, 8).

Activated microglia exert their effects on neurons and macroglia (astrocytes and oligodendrocytes) through the release of cytotoxic substances such as oxygen radicals, nitric oxide, glutamate, proteases, and neurotoxic cytokines, as well as cytoprotective agents such as growth factors, plasminogen, and neuroprotective cytokine (8). The effects of microglia are themselves modulated by astrocytes and neurons through cytokines and neurotransmitters, thus giving rise to complex interactions between microglia, neurons, and astrocytes. Evidence suggests that microglial cells play a central role in HIV encephalopathy and multiple sclerosis (10, 11). In addition to infectious or inflammatory diseases, there is accumulating evidence that microglia play a significant role in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) (12–15).

1.2. Microglia in Cell Culture: A Catch 22

EARLY evidence that microglial cells play a role in neurological diseases emerged from histological studies. With the advent of cell culture and its successful application to neurons and astrocytes, it was only a question of time until ingenious "glioscientists" developed protocols to isolate microglia. A seminal paper by Giulian and Baker in the mid-1980s describing the culture of microglial cells from neonatal mice jump started the field and with roughly 800 citations it might be the most cited primary research paper on microglial cells (16). Much has been learned from these cells in a culture dish; from their complement of receptors and ion channels, to their ability to proliferate and migrate, as well as their annotated genomic profile. However, one lingering concern has always been this: what happens to immune cells, which are supposed to monitor CNS integrity after you put a brain through a wire mash and bath the cells in 10% serum? The answer seems blatantly clear, the cells get activated. This has been ignored at the peril of the field for sometime, until it became clear that microglial cells in vitro not always (and this might be an understatement) resemble microglial cells in vivo. This controversial topic has been covered in recent years by several reviews and primary papers and is far outside the scope of this chapter (1, 17, 18). Advances in imaging technology and smart genetic approaches however have considerably improved our understanding of microglia in vitro (19–22) and the field is now embarking on reconciling the in vitro and in vitro findings.

It is clear that mechanistic studies will necessitate the in vitro approach for the time being. However, no self-respecting microgliologist will attempt to explain a complex neurological disease from data solely derived from a Petri dish.

1.3. Microglia Cell Culture: A Generic Protocol

While there might be as many specific microglial cell culture protocols as there are laboratories working with microglia, most protocols in use are based on the above-mentioned publication of Giulian and Baker (16). The overriding principle is to first generate mixed CNS cultures from late embryonic to postnatal day 5 brains and then repeatedly isolate microglial cells from these mixed cultures. However, other approaches exist, for example the isolation and (long-term) culture of adult microglia (23); the generation of larger amounts of microglia from the subventricular zone (24); or the derivation of microglia precursors from embryonic stem cells (25). Microglial cells have not only been isolated from mouse and rat, but also from fetal human tissue (26); adult human surgically resected tissue (27); human postmortem tissue (28), human retinal tissue (29); and porcine CNS (30) and even gold fish (31).

In this chapter, we provide a basic protocol for the initial mixed CNS cultures similar to what can be found in many CNS cell culture books (32). The described protocol for microglia isolation has several advantages. It will generate reasonable amounts of microglia; microglia can be kept for an extended time in culture to do stimulation experiments and lastly, in all its variations, it is time proven. While the overall properties of microglia generated this way seem to be the same, it is difficult to judge whether reported differences are species-dependent or cell culture protocol-dependent.

For many procedures, written instructions are similar to instruction on how to tie shoe laces. In such a case, a picture is worth a thousand words, and a video of the procedures is invaluable. The reader is referred to the Journal of Visualized Experiments at http://www.jove.com, which has several instructional videos on the general procedures on how to obtain CNS cell cultures. The added value in our protocol are the notes, which highlight some of the common challenges and are usually not spelled out in detail. We are almost certain that most of our esteemed colleagues will have a divergent opinion on one issue or the other. However, we would like to point out that for the most part there is no single right or single wrong in how to prepare microglial cultures. There are actually many right and unfortunately many wrong ways. We intend to provide one possible way which has successfully worked for us and warn of many wrong turns one can take along the arduous road to microglial cultures.

2. Materials

1. Dissecting microscope.
2. Sterile dissection tools (scalpels, scissors, forceps, etc.).
3. Hanks' Balanced Salt Solutions (HBSS) (see Note 1).
4. 0.25% Trypsin in HBSS (cell culture grade, not trypsin/EDTA).
5. Petri dishes, 15-ml tubes, 50-ml tubes, cell strainer (100 μm; BD Falcon).
6. DNase I (not the expensive molecular grade). A stock solution at 10 mg/ml in HBSS should be aliquoted and frozen.
7. Dulbecco's Modified Eagle's Medium (DMEM, high glucose, with glutamine) supplemented with 100 U/ml of penicillin and 100 μg/ml streptomycin (see Note 2).
8. DMEM/10% FBS: DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS) and 100 U/ml of penicillin and 100 μg/ml streptomycin (P/S) (see Note 3). Of note, one should avoid HEPES containing medium. HEPES is used to increase the pH buffer capacity of the medium once removed from the CO_2 atmosphere of the incubator. However, it as a negative influence on microglia yield in primary mixed CNS culture (see Note 1).
9. Poly-L-ornithine-coated flasks for primary mixed cultures: flasks are coated with 1 mg/ml poly-L-ornithine dissolved in tissue-culture water for at least 30 min at 37°C or over night at 4°C. The coating solution should cover the whole surface of the flasks. After the coating, flasks are washed two times with tissue culture grade water and one time with HBSS (see Note 4). For three brains, one 75 cm^2-coated cell culture flask will be needed, or for one brain, one 25 cm^2-coated cell culture flask. For higher throughput cultures, ratios can be scaled to 175-cm^2 culture flasks. The actual number of brains is flexible and can be empirically determined. However, it is imperative that for cultures which will be compared, the same number of brains is used. Otherwise differences in the growth phase will lead to nonlinear differences with might distort results.
10. Culture dishes or well plates for microglia cells are either coated with poly-L-lysine or poly-L-ornithine or specialized cell culture ware can be used (e.g., PRIMARIA from BD Falcon).

3. Methods

The extraction of the brains from the animals (P1–P5 mouse or rat) and the removal of the meninges should be preferably done in a sterile work bench. The "cleaner" the work at this step, the less likely is a (low-level) contamination in the later steps, which most likely will be cleared by the antibiotics in the medium (see Note 2). However, the presence of microbial products will lead to an undefined activation state of the cultures (see Note 5). Please note that the sterile work bench for animal preparation should be different from the actual cell culture hood. Avoid bringing animals into your cell culture facility. If there is not a sterile work bench for animal preparation, the preparation can be done on a laboratory bench, which gets sanitized beforehand and is preferably located in a low-traffic area.

1. Sterilize the surface of the bench and the dissection-microscope with 70% ethanol. Lay out the sterile dissection tools on a sterile surface (e.g., sterile Petri dish). To sterilize the preparation tools directly after each usage, a bead sterilizer is optimal; however, a beaker filled with 70% ethanol can work as well.
2. For the collection of brains, put Petri dishes and 15-ml centrifugation tubes filled with 10 ml HBSS on ice to prechill the solution. To keep the Petri dishes cold, while under the dissecting microscope a rectangular cold pack as frequently used for shipping has been proven useful. To increase contrast in the dissection microscope, a black sheet might be put between the cold pack and the Petri dish.
3. Decapitate animals, spray the head with 70% ethanol, and then place it in a sterile Petri dish. Please check institutional animal care and use rule for decapitation of animals. CO_2 narcosis prior to decapitation is not desirable, and usually omission can be justified.
4. Remove the skin from the skull and cut the skull on the right and left side from the entry of the spinal cord toward the eyes. For an easy removal of the brain, it is recommended to also cut the skull along the midline of the brain, which is easily visible as a meandering line in the skull. To cut only the skull and avoid cutting into the brain tissue, a scissor with one blunt blade is helpful. Once the incision is done alongside the base of the brain and along the midline the skull bone can be removed by gripping them with forceps and lifting up and outward. An alternative approach is to half the skull along the midline and carefully scoop out the hemisphere. However, this approach is only advisable for very young animals.

Extreme care has to be taken not to squish the brain with a dull blade. Nevertheless, if executed with care, this procedure can be very efficient and safe valuable time.

5. Remove the brain as a whole after cutting the olfactory nerves. Place the brain into a Petri dish filled with ice-cold HBSS. Once the brains are on ice (HBSS in a 50-ml Falcon tube), they can be stored for up to 4 h with little effect on glial cell viability. Nevertheless, for consistent results, it is advisable to perform the preparation as fast as possible.
6. Remove the olfactory bulb, cerebellum, and midbrain by tweezing them off with forceps. Remove the meninges under the dissection microscope: first cut the brain along the hemispheres, detach the meninges along the inner side of the hemispheres, and carefully pull off the meninges. They might come off in one piece; however, they may also rip and only come off in little pieces. It is very important to check for left over meninges and remove them carefully, as they might contaminate the culture with fibroblasts. This might not seem a big issue if one plans to isolate microglia from the mixed cultures. However, one needs to keep in mind that the fibroblast will release a different set of growth factors and cytokines into the mixed culture and therefore might lead to a different differentiation pattern of microglia than in cultures without fibroblasts. In general, the meninges are easily removed from a carefully disected brain. A brain bruised while extracted will have sticky meninges.
7. Transfer the meninges-free brain to a 15-ml centrifugation tube filled with 10 ml HBSS and store on ice. Up to six brains per tube can be pooled together.

   The next steps are performed in the cell culture hood.
8. Wash the brain tissue at least three times with 7 ml of HBSS. This step will remove contaminations such as blood, pieces of meninges, etc., and reduce microbial load by dilution. If the preparation took longer and was performed in an unsterile environment, more washes are recommended. To wash, remove the supernatant and add 7 ml of HBSS. Close the centrifugation tube and reverse it twice. When the brains have settled down, remove the supernatant, and repeat the next washing step as desired. After the last wash, leave 1–2 ml supernatant to cover the brains.
9. Trypsinize the brain tissue by adding 1 ml of 0.25% trypsin in HBSS and 50 µl of the DNase solution. Incubate the brains with trypsin and DNase for 5–10 min at room temperature. During the incubation agitate, the centrifugation tubes a few times to ensure good trypsinization. Several other enzyme preparations have been used such as collagenase or dispase.

Some protocols actually perform the dissociation step enzyme-free, and some combine a mincing step with enzymatic digestions. There are pros and cons for each approach. However, we have found that the procedure we describe is a good compromise between speed and consistent results.

10. Stop the trypsinization by adding 10 ml of DMEM/10% FBS. At this point, a jelly-like band might enwrap some of the tissue. This is DNA from broken up cells. This is more frequently the case, if a strong mechanical force is used, and can be countered by adding more DNAse. However, generally this should not be necessary, and it is usually a sign that either the trypsinization was too long or the DNase had expired.
11. Wash the tissue twice with HBSS as stated in step 9. After the last wash, keep 5 ml of supernatant. This amount of volume can be adjusted to match the number of brains used.
12. Homogenize the brains by pipetting with either serological or fire-polished Pasteur pipettes of decreasing opening diameters. Care has to be taken to avoid bubbles, as this will reduce cell viability and yield. A fine line has to been walked between pipetting often enough to break up the tissue and pipetting too often, thus killing cells because of the shear forces in the pipette tip. This is the most critical part of the preparation and is indeed more an art than a science. We recommend limiting the homogenization to only two fire-polished Pasteur pipettes for a maximum of five pipetting steps each time. It is better to err on the side of still having some undissociated tissue, than to homogenize too much and kill liberated cells. Again, if in this step jelly-like bands or clumps are observed, more DNase can be added.
13. Filter the cell suspension is through a 100-μm cell strainer. This step will remove larger undigested clumps. After transferring the cell suspension, the cell strainer can be washed with additional DMEM + 10% FBS to increase cell recovery from the cell strainer.
14. Centrifuge the cell suspension for 10 min at 200 × *g*. Discard the supernatant and resuspend the cell pellet in 10 ml of DMEM + 10% FBS + P/S. Add the cell homogenate to the flasks in an amount that correspond to three whole brains for one 75-cm^2 flask.
15. Incubate the cell culture flasks at 5% CO_2 and 37°C. The cultures will look rather cloudy because of a lot of debris in the supernatant.
16. Live cells will attach within hours. One considerable difference among different protocols is what happens next. One school of thought suggests removing debris after overnight incubation, arguing that the myelin in the debris is toxic to

the cells and should be removed as soon as all cells have safely attached. Others argue that leaving debris around will stimulate microglial proliferation. Both arguments are most likely correct. The important point, as actually in everything related to microglia culture, is consistency. If you decide to remove debris at, let us say DIV 2, then always remove it on DIV 2. Changing these seemingly unimportant, early parameters can lead to variable result later on.

17. After 24 h, wash the attached cells three times with PBS, then add 12 ml of DMEM + 10% FBS + P/S. After about 7 days in culture, an astrocyte monolayer with a few microglia on top will form. Frequency of medium change is also at variance among different protocols. We found that keeping the initial 12 ml of medium until DIV 7 yields good results. However, other laboratories immediately start with a 3–4 day medium change cycle. In general, the longer you maintain the cells in the same medium (without depletion of nutrients!), the more the cultures get conditioned by factors secreted from the astrocytic monolayer. This seems to have beneficial effects on microglial cell yield. One frequently employed alternative is the use of growth factors to increase microglia yield (see Note 6).
18. Depending on the details of the preparation (number of brains per flask, medium change frequency) 10–20 days after the preparation, a larger number of microglia appear on top of the astrocytic monolayer. In mouse-derived cultures, microglia usually remain attached to the astrocytes, in rat-derived cultures the microglia tend to float in the supernatant.
19. To collect the microglia cells from a primary culture flask, collect the loosely attached microglial cells by gently shaking the flasks for 30 min on an orbital shaker at 37°C. Alternatively, the flask can be tapped on the side with the flat hand with medium intensity for 5–10 times in a way that does not induce foaming of the medium. A quick inspection with a microscope will indicate whether microglial cells are successfully dislodged and can be collected from the supernatant. Similar to the trituration of the CNS, this process is more art than science and needs to be perfected by iteration. Too light shaking will cause microglial cells to stay attached, while too vigorous shaking will also dislodge oligodendrocytes. A further consideration is the need to strike a balance for current microglial yield and leaving enough cells remaining in the flask to repopulate the primary culture. While there is ongoing discussion whether the actual microglial cells proliferate or if the cell culture contains an actively proliferating microglial precursor, it is clear that removing too many cells will reduce the repopulation rate (see Notes 6 and 7).

20. Collect the supernatant in a centrifugation tube, and centrifuge for 10 min at 200 × *g*. Because microglial cells reattach very quickly, it is important to collect the supernantant immediately after shaking the flasks. Primary mixed CNS cultures are refed with 12 ml DMEM + 10% FBS and the flasks are placed back in the incubator. Microglial cells will continue to proliferate and the flasks can be shaken off again after 5–7 days. The yield of microglia will decrease significantly with every shake off and after the forth shake off the microglia yield will be usually very low.
21. After the centrifugation, carefully remove the supernatant. Resuspend the microglial cells in the pellet with a fire-polished Pasteur pipette in DMEM/10% FBS, and plate them into the appropriate cell culture dish or multi-well plate by adding the appropriate amount of cells (see Note 8) and the medium of choice (see Note 3). Cells can be used for stimulation experiments (see Notes 9 and 10) or can be transfected for overexpression or knock-down studies (see Note 11).

4. Notes

1. Many protocols use phosphate-buffered saline (PBS) or Dulbecco's Phosphate-Buffered Saline (DPBS) as basis for the digestion solutions. We prefer Hank's buffered salt solution (HBSS). While different in salts, the main difference is the presence of glucose in HBSS, therefore providing an energy source for the cells. Trypsinization is affected by the presence of divalent cations and the use of buffers without calcium and magnesium might increase trypsinization. On the other hand, optimal DNase activity requires buffers with calcium and magnesium. Again there is no right and wrong. However, being cognizant of these underlying mechanisms will allow for efficient troubleshooting.
2. The use of penicillin and streptomycin in cell cultures is standard operating procedure (SOP). It prevents the hostile takeover of the cultures by microbes carried in from the preparation or due to unsterile work practices. This SOP indeed seems like a good thing. However, even low levels of bacterial contamination will activate the microglial cells in culture. So, a contamination kept in check by antibiotics might give the impression of a healthy culture, while the cells are actually activated by bacterial products. This would indeed confound results. Therefore some laboratories, prefer to work without antibiotics. However, this is not without risk either. Low-level contamination could be cleared by the cells themselves (after

all, they are phagocytes) appear healthy but are activated by bacterial products as well. Additionally, cultures where a contamination could have been easily eliminated by antibiotics could be lost due to unchecked bacterial growth. Our approach is to work in the cleanest possible conditions, thus reducing the risk of contamination from the preparation, and the need to "clean" the cultures with antibiotics. If a given primary culture leads to experimental outcomes, which substantially differ from the data usually produced in the laboratory, a low-level contamination might be a potential explanation.

3. One important difference between microglia culture protocols, and most likely the one which might be at the root of the large body of divergent data on microglia responses to a given stimulus, is the medium microglial cultures are maintained in-culture medium with or without serum. Serum was the decisive factor which actually enabled the successful culturing of cells. It is the major source of nutrients. However, based on its biological nature, it is also the most variable component in a cell culture medium. The virtues of testing and banking serum lots are discussed at length in any basic cell culture hand book and will not be repeated here. And while the actual source, lot and repeated freeze/thaw cycles of a given FBS bottle will certainly influence the culture conditions, it is self-evident that the absence or presence of serum will have a much more profound effect on cells.

   The most commonly acknowledged reason to culture cells serum free is to synchronize cells and arrest them in G_0/G_1 phase (33). This is virtually true for all cells. However, there are other issues worth contemplating when culturing microglia. Microglia reside behind the blood brain barrier. The only time these cells are exposed to serum components is during break down of the blood brain barrier, for example, during stroke, trauma, or in active multiple sclerosis regions. Indeed several serum components have been identified as microglia-activating signals (1, 34–36). This could lead to an increased baseline activation of the cells. Proponents of serum in the medium would argue that cell culture is artificial in the first place and that there are no resting microglia in a culture dish anyway (see below for more detailed discussion on the microglial "activation state" in vitro). To our mind, there is no right or wrong in this discussion. As with everything we stressed before, consistency is the most important message. However, when interpreting own results in context of the literature it is very important to pay attention to this detail. For example, there are broadly diverging data on the sensitivity of microglial cells to lipopolysaccharide (LPS), the archetypical activator of toll-like receptor (TLR4). While it is now appreciated that not all LPS is created equally potent (see

Notes 5 and 9), it is now also well understood that serum contains LPS binding protein (LBP). LBP, together with CD14 on the cell surface, leads to optimal presentation of LPS to TLR4 and increases cell sensitivity to LPS by 2–3 orders of magnitude (37). Similarly, serum might contain cofactors for other agonists and/or receptor systems or rather unselectively serve as a priming signal for an unrelated trigger.

Because of the many potential pitfalls with serum-containing medium, many laboratories and commercial sources have developed serum-free alternatives. For microglia, approaches developed for peripheral macrophages have been successful adapted. While it is certainly possible to culture microglia in unsupplemented DMEM, cells do not survive long. Serum withdrawal is also a common way to induce autophagy (38) and the induction of autophagocitic pathways in serum starved microglial is a concern. Most laboratory-derived serum-free media start with DMEM, supplemented with serum albumin (bovine or human, usually 1 mg/ml), insulin, transferrin, and selenite. The latter three are readily available as an ITS supplement, making this a convenient and inexpensive alternative to commercial media.

Of the many commercial serum-free media, the Macrophage Serum-free Medium from Invitrogen and Mediatech's Cellgro COMPLETE™ Serum-Free/Low-Protein Medium are of note. While the former is specially formulated for macrophages both allow for ready growth of microglial cells without further supplementation. The compositions are proprietary, however, one can enter in an agreement with the respective supplier to get information on the ingredients (not concentration) of the media, (e.g., if one needs to determine the presence of a specific factor). We have had excellent experience with Invitrogen's Macrophage Serum-free Medium and it is our (pricey) medium of choice.

4. Polystyrol culture flasks or plates should be coated with substrates that facilitate the attachment of cells. Usually poly-D-lysine (MW 30,000–70,000; 70,000–150,000; >300,000) or poly-L-lysine (MW 70,000–150,000; 150,000–300,000) are used for the attachment of the primary mixed CNS cultures. When it is required that poly-lysine is not metabolized by the cells, poly-D-lysine is preferred. Instead of poly-D/L-lysin, poly-L-ornithine (MW 30,000–70,000) can be used. Our observation is that with a poly-ornithin more microglial cells float in the supernantant of the primary mixed cultures. Pure microglia cultures can be plated without coating on pretreated polystyrol flasks or specialty cell culture ware such as PRIMARIA™, (Becton Dickinson) or Cell$^+$ (Sarstedt).

Coating with laminin will increase the mobility of microglial cells and can be used for migration experiments. The cell culture material and coating will change interactions with integrins and induce changes in microglial phenotype. Again one needs to keep in mind that even small changes can have big effects on a given read out and avoid comparing apples and oranges.

5. LPS is a heat-stable, non-proteinacious bacterial cell wall component. LPS's synonym, endotoxin, derives from its ability to induce secretion of overwhelming concentrations of proinflammatory cytokines in vivo leading to severe vascular, hemodynamic and respiratory changes (39). It is found ubiquitously in the environment and can induce a wide spectrum of biologic activities (39–41). LPS is a pathogen-associated molecular pattern (PAMP) and many of its responses are mediated by Toll-like receptor 4 (TLR4) (42). As all myeloid cells, microglial cells are exquisitely sensitive to LPS. It is, therefore, of utmost importance to avoid endotoxin contamination in microglial cultures. Only certified endotoxin-free cell culture ware should be used. This also applies to centrifuge tubes, which are used to store microglia-activating agents, inhibitors and alike, which will come in contact with the cells. We routinely use single packed, certified endotoxin-free tubes.

   A common source of endotoxin is (an inexpensive batch of) serum. While most sera are "endotoxin tested," this does not equate to low levels of endotoxin. Many standard preparation sera only certify <1 EU/ml, which at 10% serum could in the worst case still reach about 0.1 EU/ml, enough to trigger a microglial response. Low endotoxin sera are certified to 0.005 EU/ml, a reasonably safe level. The second, and in our mind most common source of endotoxin, is derived from contaminated stimulation agents. Proteins recombinantly expressed in *Escherichia coli*, blood-derived products processed under suboptimal conditions or clean compounds contaminated by sloppy experimentators, are potential sources of endotoxin. Indeed, we showed that biologicals are frequently contaminated with endotoxin (43) and that a good number of microglial responses might not be due to the reported stimulus but endotoxin contamination. We routinely test compounds used in microglial experiments for endotoxin contamination with commercially available Limulus amebocyte lysate assays.

6. The yield of microglia from primary cultures, especially from mouse tissue, can be discouragingly low. Several laboratories have, therefore, developed protocols, which increase microglia numbers in primary mixed CNS culture by virtue of adding

growth factors and stimulating microglia (precursor) proliferation. Macrophage Colony-Stimulating Factor (M-CSF) and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) are the two most frequently used growth factors and can increase microglia cell yield substantially when used in a concentration range from 2 to 20 ng/ml (see below for potency). While both belong to the colony stimulating factor (CSF) family, they have very divergent effects on microglia. Cells treated with M-CSF will adapt more of a macrophage-like phenotype, while microglia treated with GM-CSF will adapt more of a dendritic cell-like phenotype (44). This is another source of potential variability in the reported microglial responses to a given stimulus. Depending on the use of growth factor (yes/no, M/GM-CSF), essentially distinctly differentiated cells are used. To further complicate things for the unsuspecting novice, several reports documented that low levels of M-CSF and GM-CSF (<1 ng/ml) have little effect on specific readouts (45, 46). This is certainly true. However, the growth factors that have "no effect" should not be extrapolated to other readouts not specifically investigated in these papers.

So, would it not be best to avoid growth factors at all? Our stand on this issue is rather pragmatic. Some experiments necessitate the use of a larger number of microglial cells. There, the use of growth factors can be essential to accomplish the experimental goal. Furthermore, we believe that using a growth factor and thereby forcing the cells to differentiate into a specific lineage restriction will further reduce variability. However, on the same token, it also reduces plasticity and thereby might prevent the cells to respond to a given stimulus. One always needs to keep the experimental setup and desired readout in mind. For example, M-CSF and GM-CSF have opposing effects on LPS-induced TNF-α or IL-10 release or the regulation of phagocytosis (47, 48). It is, therefore, necessary to familiarize oneself with the "side effects "of these helpful mitogens, to perform meaningful and interpretable experiments.

One note on the source of growth factors. We prefer recombinant proteins with certified low endotoxin levels (see Note 5 on endotoxin). However, not all recombinant proteins are expressed (and glycosylated) alike. We have found large variability in the mitogenic potency of the same growth factor from different suppliers. We recommend to use a reputable and most likely expensive source for the growth factors, as low potency will very quickly negate low cost per µg. We also strongly advise against the use of conditioned media such as from L929 fibroblasts as source for growth factors (M-CSF

in the case of L929). While conditioned media certainly work, they harbor two problems. The first, you get more than you bargain for (i.e., other factors releases by the conditioning cells). Second, there will be batch-to-batch variability.

One more issue to consider when using growth factors is the effect that high-dose growth factor treatment will have on the microglial cells in primary mixed culture. Cells are receiving a constant mitogenic, prosurvival signal. A sudden withdrawal of this signal, for example when transferring the cells to purified microglial cultures, could lead to starvation-induced cell death. We, therefore, usually maintain purified microglia cultures at 10% of the growth factor level we induced proliferation with in the primary mixed cultures.

7. One inevitable question is for how long will the primary mixed cultures produce microglial cells. This depends on the frequency of isolation and on how many microglial cells remain in the primary cultures. On average primary cultures are productive for 2–4 weeks, however, this can be extended by the use of growth factors (see Note 6). One important factor is again consistency. We strongly advice to use a set interval between collections. Factors released from the mixed cultures will accumulate in the medium and if variable intervals are used, different mediator concentrations might lead to different signaling states of the isolated cells and consequently variability in the readout.
8. Plating of highly enriched microglial cultures. Experiments on microglial cells are performed in many settings, for example, on class coverslips for live cell imaging or immunocytochemistry, in Boyden chambers for cell migration assays, or in 35-mm dishes for western blots. The most frequent use, however, is most likely the plating into 96-well plates for cell proliferation assays, ELISA, nitric oxide, and reactive oxygen species measurements. We recommend the use of an (electronic) repeater pipette for highly accurate plating. The cell density in a well of a 96-well plate can range from 5,000 cells (sparse) to 50,000 cells (confluent). We regularly perform our experiments at either 20,000 or 40,000 cells. As frequently mentioned above, consistency is the key to success and to ensure comparability between experiments. Microglial cells will attach to the plates (or coverslips) within minutes. Two factors will influence the necessary attachment time, the plating volume and the actual plate material. The volume for the obvious reason that the same number of cells will be more sparse in 200 µl and need more time to settle to the bottom of the plate than they would in only 100 µl plating volume. The actual material of the plate will determine surface interactions with the cells. For larger

plates such as the flasks used for primary culture, usually a coating with poly-l-ornithine provides a favorable surface charge for cell attachment. Coating 96-well plates, while possible, is cumbersome. We found that microglial cells attach very well to most plastic ware except from Corning. We prefer BD Falcon Primaria® cell cultureware, as microglia cells readily attach and display homogeneous morphology over extended periods of time. Indeed, the microglial ability to readily attach to the cell culture dishes can be used to increase culture purity. Washing the wells after microglial cells have attached, but before other contaminating cells such as oligodendrocytes or astrocytes attach can increase microglia purity >98%.

After attachment we routinely culture our cells for 24 h in the same medium as the primary mixed cultures. We then change to a serum-free medium (discussed above) for 24 h before we stimulate the cells. This additional medium exchange will remove factors released by microglia during the first 24 h induced by the isolation procedure and/or triggered by attachment to the culture plate. While some publication report baseline levels of factors such as TNF-α or nitric oxide, our cultures have none.

9. LPS is by far the most frequently used activator of microglial cells. It induces a robust proinflammatory activation in all macrophages including microglial cells. However, LPS is available from many bacterial strains and in many formulations. Because of its high potency in activating microglial cells, usually not much thought is spared on the source of LPS, and the cheapest LPS is obtained. It arrives in copious amounts, which could potentially last a researchers career. However, things are never as easy as they seem. Strain differences, storage conditions, and route of application all considerably affect microglial responses to LPS. We found that using a control standard endotoxin (CSE) commonly used for endotoxin level testing gives the most consistent and reproducible results. CSE comes with clear instructions on the handling, storage, and lifetime of the compound and has considerably improved our day-to-day and year-to-year reproducibility of specific experiments.

   On the topic of microglia activation, one should keep in mind that classical macrophage activation is two step process of priming [usually interferon (IFN)-γ] and trigger (e.g., LPS). In microglial cultures, plating alone can serve as a priming signal. However, in microglia, which are maintained at low activation levels by changing medium after attachment, an additional trigger might be necessary to induce a full blown inflammatory response.

10. Which activation state are cultured microglial in? A frequently made mistake, especially in the early days of microglial culture was to equate morphology with physiology. Amoeboid cells were called "activated" and when cells reached a more branched or ramified morphology they were deemed "resting". It should be self-evident to the alert reader what our viewpoint might be. To our mind, there are no resting microglial cells in vivo. Baseline-cultured microglial cells are cells with a generally low inflammatory profile and the capacity to be further activated. For the lack of a better word, we use the term "unstimulated" microglial cells. This also reflects the recently more appreciated paradigm of classically and alternatively activated macrophages (49) as cultured microglia not only have the capacity to transfrom into proinflammtory cells, but also into immunoregulatory/wound healing cells, secreting anti-inflammatory mediators.
11. Microglial cells such as all macrophage-like cells have been notoriously difficult to transfect. Transfection rates of 5–20% with standard methods have been the rule. Two recent developments have improved transfection efficiencies. The Lonza/Amaxa Small Cell Number kit can be used for the transfection of primary microglial cells with transfection rates of 70–80% with 60–80% viability. Very recently Polyplus Transfection brought the jetPEI™-Macrophage DNA Transfection Reagent to the market. It has a proprietary formulation which enables improved DNA uptake via mannose receptor. It requires culturing of the cells in GM-CSF to increase mannose receptor expression. However, if GM-CSF would already be used to expand microglia in primary cultures, this would have an additional positive downstream effect.

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Chapter 5

Neural Stem Cells for Developmental Neurotoxicity Studies

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Abstract

The developing nervous system is particularly susceptible to toxicants, and exposure during development may result in long-lasting neurological impairments. The damage can range from subtle to severe, and it may impose substantial burdens on affected individuals, their families, and society. Given the little information available on developmental neurotoxicity (DNT) and the growing number of chemicals that need to be tested, new testing strategies and approaches are necessary to identify developmental neurotoxic agents with speed, reliability, and respect for animal welfare. So far, there are no validated alternative methods for DNT testing. Recently, neural stem/progenitor cells have been proposed as relevant models for alternative DNT testing. In this chapter, we provide detailed protocols for culturing neural stem cells (NSCs), in vitro experimental models, including primary cultures of rat and human embryonic NSCs, rat and mouse adult NSCs, as well as the mouse NSC line C17.2 that we have implemented and successfully used for neurotoxicity studies.

Key words: Neural stem cells, Rodent, Human, Developmental neurotoxicity, Endpoints

1. Introduction

The developing nervous system is especially susceptible to toxicants, and chemical exposure during development may have long-term detrimental consequences that result in altered neurological functions (1, 2). The type of damage induced by developmental neurotoxicants can range from subtle to severe and, in addition to neurodevelopmental disorders, may result in an acceleration of age-related decline in the function and onset of neurotoxicity diseases (2).

Developmental neurotoxicity (DNT) induced by environmental toxic agents is a growing concern because of the limited information available on most of the chemicals and the increasing

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number of substances that we are exposed to. Relevant in vitro models to identify neurotoxic agents with speed, reliability, and respect for animal welfare are needed, with the ultimate goal to generate tests with higher throughput that can provide mechanistic data and possibly predict the levels of exposure that may cause adverse effects in humans.

Over the last few years, we have implemented and used neural stem cells (NSCs) for DNT studies (3–9). NSCs are derived from the developing central nervous system, and it is generally agreed that they exist in a variety of developmental time stages residing in designated stem cell niches that provide a controlled environment for proliferation and differentiation. In addition to the developing brain, NSCs are also present in discrete neurogenic areas of the adult brain, where they represent a unique cell population with neurogenerative capacity (10–13). Therefore, NSCs can be relevant for neurotoxicity studies and hazard identification not only in relation to the developing brain, but also to the adult nervous system.

In the following sections, we provide detailed protocols for culturing NSC primary cultures and immortalized cell lines, which are the in vitro models of current use in our laboratory for DNT studies. Rodent or human primary NSC cultures, grown either as dissociated adherent cells or in suspension as aggregates defined as neurospheres (Fig. 1), are suitable models, but require dissection skillness, in the case of rodent NSCs, and source availability in the case of human cells. Cell lines have the advantage of being easy to obtain, growing as a more homogeneous cell population. However, they may have different characteristics, for example, in terms of gene expression, as compared to primary NSCs.

To provide the highest level of predictability for neurotoxicity testing, it is important to check selected endpoints that can

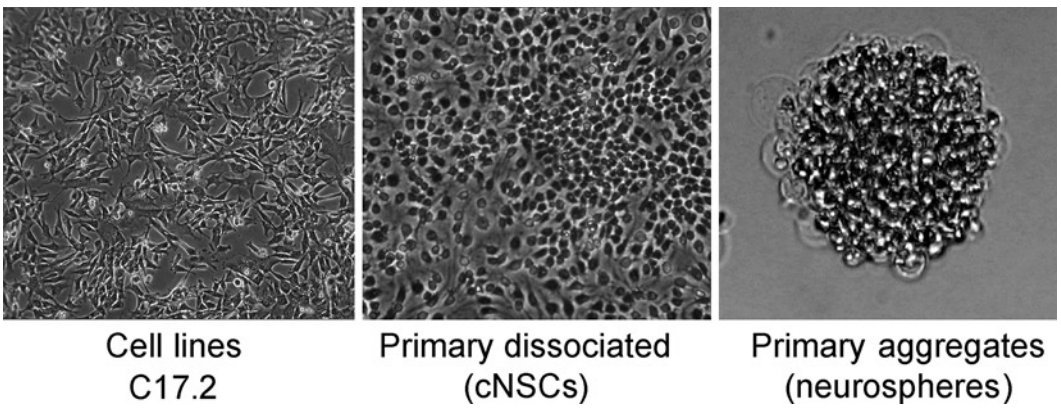

Fig. 1. Phase-contrast images of C17.2, cNSCs, and neurospheres are shown as representatives for NSC cell lines and primary cell cultures.

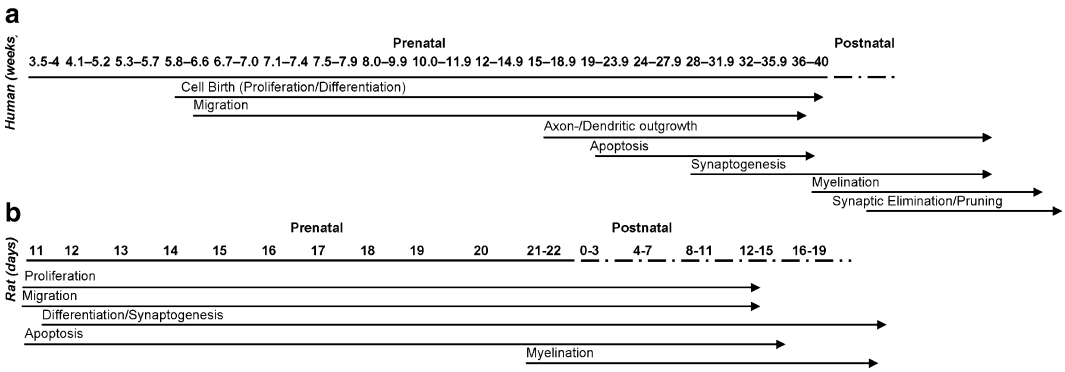

Fig. 2. Relative comparison of the human and rodent brain development. Estimated time periods are shown in relation to the developmental processes of human (**a**) and rat (**b**) brains (modified after refs. 1, 2).

provide information on critical developmental processes. Cell proliferation, migration, differentiation, cell death, synaptogenesis, neurite outgrowth, and myelination occur at well-coordinated time points according to a tightly regulated program to ensure the establishment of normal brain structure and function (Fig. 2). Although the sequence of events is comparable among species, developmental time scales between humans and rodents are considerably different, as illustrated in Fig. 2. Since these processes occur at well-defined developmental stages, the isolation age of neural cells is an important aspect to consider when using rodent and human in vitro models.

2. Materials

2.1. Primary Cell Culture of Rat Embryonic Cortical Neural Stem Cells (cNSC)

1. Medium: To 495 ml of DMEM/F12, add 5 ml of 10 mg/ml transferrin in DMEM/F12, 2.5 ml of 5 mg/ml insulin (dissolved in 10 mM NaOH), 1 M Putrescine, 500 µM Sodium selenite, and 100 µM Progesterone; filter sterile; add 5 ml Penicillin/Streptomycin; and keep at 4°C.
2. Hank's buffer: To 50 ml 10× HBSS, add 81.5 mM $NaHCO_3$ and 1 M HEPES; adjust the pH to 7.2; and store at room temperature.
3. Plates: Add 15 µg/ml poly-L-ornithine to each plate and incubate at 37°C for at least 2–3 h; remove poly-L-ornithine solution and wash three times with PBS; remove PBS and add 1 µg/ml Fibronectin; incubate at 37°C for at least 1 h; wash twice with PBS and remove the PBS; and add new PBS and incubate the plates in the cell culture incubator until seeding cells.

2.2. Primary Cell Culture of Rat or Mouse Adult Neural Stem Cells (aNSCs)

1. Solution 1 (S1) (total volume 500 ml): Add 50 ml 1× HBSS, 9.0 ml D-glucose, and 7.5 ml HEPES. Adjust the pH to 7.5.
2. Solution 2 (S2) (total volume 500 ml): 25 ml 1× HBSS and 154 g Sucrose. Adjust the pH to 7.5.
3. Solution 3 (S3) (total volume 500 ml): 20 g BSA, 10 ml 1 M HEPES solution, and 490 ml 1× EBSS. Adjust the pH to 7.5.
4. Dissociation medium: To 10 ml of S1, add 13.3 mg Trypsin, 7 mg Hyaluronidase, 2 mg Kynerenic acid, and 200 µl Dnase.
5. Neurosphere medium: To 47 ml of DMEM/F12, add 1 ml B27 supplement, 0.5 ml Penicillin/Streptomycin, and 0.4 ml HEPES.
6. Plates: Coat with 0.1 mg/ml Poly-D-Lysine for 2 h at 37°C.

2.3. Human Neural Precursor Cells (NPCs)

1. Proliferation medium: To 322 ml DMEM + Glutamax, add 161 ml F12 + Glutamax EGF 20 ng/ml EGF, 20 ng/ml FGF, 10 ml 50× B27, and 5 ml Penicillin/Streptomycin; keep at 4°C.
2. Differentiation medium: To 200 ml DMEM + Glutamax, add 100 ml F12 + Glutamax and 3 ml 100× N2 (a serum-free supplement recommended for in vitro differentiation of NSCs); keep the differentiation medium at 4°C.
3. TrypLE™ Express: A fast and gentle dissociation enzyme, stable at room temperature.
4. Plates: Incubate with 0.1 mg/ml Poly-D-Lysine for 2 h at 37°C; wash twice with sterile water; incubate with 5 µg/ml Laminin for 2 h at 37°C; wash twice with sterile water; add sterile PBS; and store at 4°C.

2.4. C17.2 Cell Line

1. Medium: To 500 ml DMEM (+ 4.5 g/L-glucose, + L-glutamine, and + Pyruvate), add 60 ml 10% fetal calf serum, 30 ml 5% horse serum, 0.25 µg/ml fungizone (kept at –20°C), 0.5 mg/ml Gentamycin (kept at room temperature), and 200 mM L-glutamine (kept at –20°C).
2. Differentiation medium: Same as the Subheading 2.4, item 1, except that fetal calf serum and horse serum should not be added.
3. Plates: Cells can be grown on uncoated dishes. When grown on glass coverslips, coat with 50 µg/ml Poly-L-lysine at 37°C for 2 h.

3. Methods

3.1. Neural Stem Cell Primary Cultures

3.1.1. Dissection of cNSCs

1. Sacrifice E14.5–15.5 timed-pregnant mother and take out the embryos and put them in Hank's buffer at room temperature.
2. Dissect the embryos from the amnion and separate the head.

3. Using a pair of tweezers, immobilize the head by placing one fork through the eye socket and caudally towards the neck. Peel back using the tweezers to expose the dorsal surface of the brain (see Note 1).
4. Free the brain from the skull, cut away the cerebellum, and separate the hemispheres of the cerebrum.
5. Cut away the olfactory bulb if present and immobilize the hemisphere by the most rostral point.
6. Cut away the developing thalamus/striatum (see Note 2).
7. Cut away the dorsal part of the developing cortex and transfer into Hank's buffer in a 15 ml falcon tube.
8. Allow the dissected cortices to settle to the bottom.
9. Aspirate the buffer from the cortices leaving 2 ml.
10. Dissociate the cortices by pipetting up and down for 15 times gently using a 1 ml pipette tip.
11. Add 10 ml of prewarmed DMEM/F12 medium (including the supplements) and mix gently twice with a 5 ml pipette.
12. Count the cells with trypan blue and seed 15,000 cells/cm^2.

3.1.2. Passaging cNSCs

1. Remove the medium from dish.
2. Add 5 ml Hank's buffer/100 mm dish and incubate for 5 min in culture incubator.
3. Remove 4 ml Hank's buffer from the dish and leave 0.5 ml.
4. Scrape cells gently with cell scraper.
5. Collect all cells in one dish and transfer them into a 50 ml falcon tube.
6. Dilute cells with 9 ml DMEM/F12 medium (including the supplements).
7. Count the cells using trypan blue.
8. Seed the cells at 2,500 cells/cm^2 in DMEM/F12 medium (including the supplements).
9. Wait until the cells have settled, and then add 10 ng/ml FGF.
10. Every 24 h, add 10 ng/ml FGF.
11. Every 48 h, change the medium and add 10 ng/ml FGF (see Note 3).

3.1.3. Differentiation of cNSCs

cNSCs have the capacity of differentiating into the major cell types found in the telencephalon, including pyramidal and interneurons, astrocytes and oligodendrocytes as well as smooth muscle cells (14). Upon mitogen withdrawal, cNSCs spontaneously differentiate into mainly neurons and astrocytes, but can also be differentiated specifically into astrocytes by treatment with interleukin-related compounds, such as ciliary neurotrophic factor

(CNTF), or oligodendrocytes by thyroid hormone (T3) treatment. Spontaneous differentiation of cNSCs is induced as follows:

1. When cells have reached the right confluency (about 5–7 days after the previous passaging), passage the cells as is in the Subheading 3.1.2.
2. Seed the cells at the density 500 cells/cm^2 and add 10 ng/ml FGF every day.
3. After 48 h, remove the old media and add media. DO NOT ADD FGF.
4. Change the media every 2 days, but do not add FGF.
5. Check the morphology. After about 1 week in culture, the cNSCs will appear more differentiated.

3.1.4. Dissection of aNSCs

1. Kill the adult rat/mice via cervical dislocation and take out the brain from the skull (see Note 4).
2. Put the brain into ice-cold PBS (see Note 5).
3. Under the dissection microscope, cut two-third of the cerebrum. Take the frontal part. Divide the two hemispheres into two parts.
4. Make visible the Lateral Ventricle (LV) wall.
5. Make a thin hole underneath the LV using a small scissor.
6. Cut out the LV and put into ice-cold PBS.
7. Dissolve the enzymes in 5 ml of solution 1 (15 ml falcon tube).
8. Place the enzyme mix at 37°C during fine dissections.
9. Sterile filter the enzyme mix and add DNase to the mixture.
10. Place ventricle walls in the enzyme mix and pipette very gently for 15 times using a blue pipette tip.
11. Incubate the ventricle walls in the enzyme mix for 20–30 min at 37°C and pipette carefully when half of the incubation time has gone.
12. Pipette the mix until most of the tissues dissolve.
13. Add 5 ml S3 to the tissue–enzyme mix.
14. Filter through a 70 μm cell strainer and centrifuge at 940 × *g* for 10 min.
15. Remove supernatant and resuspend cells in 10 ml ice-cold S2.
16. Centrifuge at 1,250 × *g* for 10 min.
17. Remove supernatant and resuspend cells in 2 ml ice-cold S3.
18. Fill a new 15 ml tube with 12 ml of ice-cold S3.
19. Gently apply the 2 ml cell suspension to the top of the new tube.

20. Centrifuge at 940 × *g* for 7 min.
21. Remove supernatant and resuspend cells in prewarmed neurosphere medium.
22. Seed all the cells in a 100 mm cell culture dish (Corning) in 15 ml neurosphere medium.
23. Add 10 ng/ml EGF.
24. Add 10 ng/ml EGF on day 3 without changing medium (see Note 6).

3.1.5. Passaging aNSC Neurospheres

1. Collect all neurospheres in a 15 ml falcon tube (see Note 7) and centrifuge at 940 × *g* for 10 min.
2. Resuspend the pellet in 1 ml TED and mix for 15 times with a yellow pipette tip and incubate for 10 min at 37°C in the water bath.
3. Mix gently using a yellow pipette tip and continue the incubation for 5–10 min at 37°C.
4. Mix gently for 10× using a yellow pipette tip.
5. Add 9 ml fresh medium and centrifuge at 940 × *g* for 10 min.
6. Discard supernatant and resuspend the pellet in 5 ml or 10 ml fresh medium, depending on the pellet's size.
7. Count the cells with trypan blue.
8. Seed cells at 80,000 cells/cm^2 in 15 ml medium and add 10 ng/ml EGF and 10 ng/ml FGF.
9. Place the cells inside the incubator and add 10 ng/ml EGF and 10 ng/ml FGF every third day (see Notes 6 and 8).

3.1.6. Passaging aNSC as Single Cells for Experiment

1. Coat plates with 0.1 mg/ml Poly-D-Lysine for 2 h.
2. Repeat steps 1–7 from the Subheading 3.1.5.
3. Add 1% filter-sterilized FCS to the medium. DO NOT ADD EGF OR FGF!
4. Seed 160,000 cells/cm^2 in the FCS-containing medium.
5. Let the cells grow overnight.
6. Gently discard the medium containing FCS and add 1 ml fresh medium containing 10 ng/ml EGF and 10 ng/ml FGF.

3.1.7. Differentiation of aNSCs

Upon the removal of EGF and FGF, cells can differentiate into neurons, astrocytes, and oligodendrocytes (15–19). Differentiation of adult neural stem cells (aNSCs) has been described by two methods: either as dissociated cells or as neurospheres. The latter is typically used to demonstrate that individual spheres are multipotent (20). For the spontaneous differentiation of dissociated

aNSCs, follow the protocol in the Subheading 3.1.6 and wait until the cells have differentiated sufficiently (about 1 week in culture).

3.1.8. Tissue Collection for Human NPCs

Human subcortical forebrain tissues are usually collected from gestation week 8–12 and dissected according to Akesson et al. (21). When human neural precursor cells (NPCs) are grown in culture, the medium should be changed every 2 days (see below), until the spheres are ready for passage (see Note 9).

1. Put 10 ml proliferating medium in a new plate.
2. Discard 10 ml of the old medium.
3. Use the rest of the old medium for transferring the spheres to the new plate (see Note 10).
4. Separate the spheres very well with a yellow pipette tip (see Note 11).

3.1.9. Passaging of Human NPCs

1. Transfer spheres in a dish that fits in the holder of the McIllwain tissue chopper.
2. Remove the medium.
3. Cut the spheres in smaller sections at a size of about 0.2 mm.
4. Add 1 ml medium and transfer the spheres in a new dish with fresh proliferation medium.
5. Separate the spheres very well with a yellow pipette tip (see Note 11).
6. Change the medium on the following day.
7. After 2 days, transfer the spheres in a falcon tube.
8. Centrifuge for 5 min at 940 × *g*.
9. Discard supernatant and add 0.5 ml TrypLE™ Express. Incubate for 6 min at 37°C in the water bath by shaking.
10. Add 6 ml proliferation medium.
11. Centrifuge for 5 min at 940 × *g*.
12. Add an appropriate amount (about 500 µl) of medium and resuspend the cell pellet mechanically to achieve a single cell suspension.
13. Count the cells with trypan blue and seed 50,000 cells/cm^2.

3.1.10. Differentiation of Human NPCs

Differentiation of human NPC cells can be initiated in spheres or single cell suspensions under the growth factor withdrawal. Therefore, human NPCs should be plated on poly-D-lysine/laminin-coated surfaces and grown in differentiation medium for up to 4 weeks. After 2–4 days, a decrease in NSCs can be observed.

3.2. Neural Stem Cell Lines

3.2.1. Passaging of C17.2 Cells

1. Remove the medium from the cell culture dish.
2. Wash with PBS.
3. Add 2 ml 1× TED/10 cm dish (Trypsin + EDTA) for 30 s.
4. Remove 1.5 ml TED after 30 s and wait for 1.5 min.
5. Add new medium and dissociate cells by pipetting.
6. Count the cells with trypan blue.
7. Seed the cells into a new dish at 4,000 cells/cm^2.

3.2.2. Differentiation of C17.2 Cells

Upon the removal of serum, C17.2 cells can differentiate into neurons, astrocytes, and oligodendrocytes (22). For differentiation assays, C17.2 cells are grown in serum-containing DMEM medium. Following the cell attachment, the medium is replaced with serum-free medium and the cultures are incubated for 1 day before performing experiments. Cell differentiation process can be evaluated by immunocytochemistry analysis of the neuronal marker β-III tubulin and the astrocyte marker Glial fibrillary acidic protein (GFAP). C17.2 cells grown in serum-containing conditions can also be differentiated by incubating the cultures in medium supplemented with, e.g., brain-derived neurotrophic factor (BDNF; 4 ng/ml), neurotrophin-3 (NT-3; 10 ng/ml), CNTF (10 ng/ml), and glial-derived neurotrophic factor (GDNF; 5 ng/ml) (23).

4. Notes

1. Autoclave the dissection tools 1 day before the dissection.
2. Looks white under the dissecting microscope and is located caudally and ventrally in the hemisphere.
3. Look for the morphological changes and at 80% confluency, passage the cells.
4. We recommend the preparation of aNSCs from the earlier age of adulthood (~8 weeks), since the yield of NSC number becomes lower with the higher age of the animal.
5. The brains can be kept in ice maximum for 2 h.
6. Flush up the spheres everyday which might have attached to the bottom of the dish.
7. Flush all the bottom of the plate with fresh medium to take all the spheres that might have been left.
8. After 3 days, aNSCs start to form neurospheres again.
9. The spheres are ready for passage when their diameter is larger than 0.7 mm. NPCs can also be passaged by either mechanical or enzymatic disruption of the sphere structure.

10. Some spheres are attached to the bottom of the old plate. These spheres should be discarded because they have started to differentiate, and hence are stuck to the bottom of the plate.
11. The spheres should be separated far away from each other before they are placed inside the incubator; otherwise, they will stick together and make a new big sphere.
12. Here, we mention briefly some specific endpoints for the detection of alterations in the developmental processes of NSCs due to chemical exposure (see also Tables 1 and 2).
    (a) *Proliferation* is measured by immunocytochemical staining against the proliferation marker Ki67 (see Fig. 3a), BrdU/EdU/Thymidine incorporation, or cell cycle analysis via FACS.
    (b) *Migration* can be easily evaluated in neurospheres, since single cells migrate out of the sphere when providing an extracellular matrix. Analysis can be performed microscopically by measuring the distance between the edge of the sphere and the furthest migrated cells with a phase-contrast microscope at four different positions per sphere.
    (c) *Differentiation* is mostly determined via immunocytochemical techniques using cell type-specific markers which are listed in Table 2. For analysis, changes in differentiation are determined by counting the number of different cell population in regard to the total cell number under a fluorescence microscope (see Fig. 3c–f).

Table 1
Endpoints and proposed NSC models for in vitro DNT

Species	Model system	Proliferation	Migration	Differentiation	Cell death
Rodent	C17.2			(22, 24, 25)	(4, 5, 7, 8)
	cNSCs			(5, 6)	(3, 5, 7–9)
	aNSCs				(4, 26, 27)
	Neurospheres	(28, 29)		(28, 29)	
Human	ReNcellCX	(30)			(30)
	HUCB-NSCs	(31, 32)	(32, 33)	(31–35)	(32)
	Neurospheres	(36)	(36–38)	(36, 38, 39)	(36, 38)

References for in vitro models and applied specific endpoints for DNT are shown. Abbreviations: *cNSCs* cortical NSCs, and *aNSCs* adult NSCs

Table 2
Cell type-specific marker proteins for the determination of different cell population

Differentiation stage	NSCs	Neurons	Astrocytes	Oligodendrocytes
Undifferentiated	Nestin			
Differentiating	(Nestin)	β(III)tubulin/Tuj1	GFAP, S100β	O4
Matured		NSE, MAP2	GFAP, S100β	CNPase, MBP

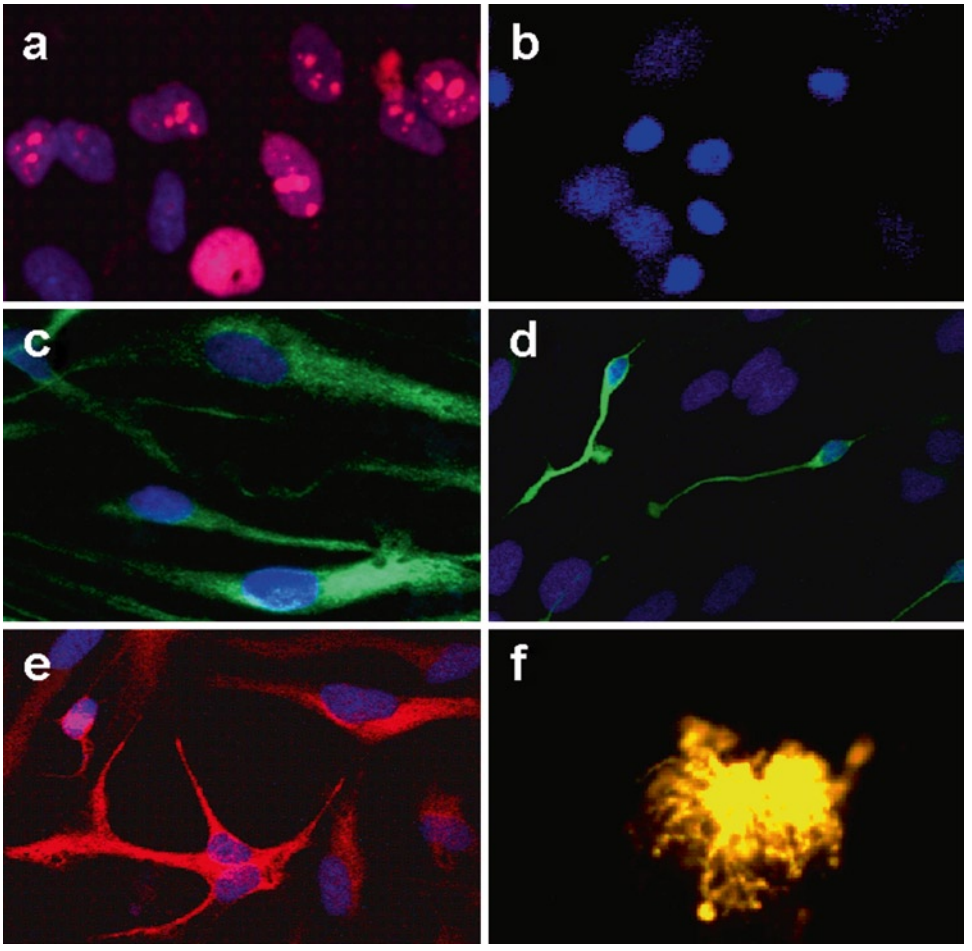

Fig. 3. Endpoints for in vitro DNT testing in NSC models. Alterations in proliferation, cell death, and differentiation are proposed as specific endpoints for DNT testing in vitro. Representative fluorescence pictures of proliferating cells stained against Ki67 (**a**), condensed apoptotic nuclei stained with Hoechst 33342 (**b**), nestin positive stem cells (**c**), Tuj1 positive early neurons (**d**), GFAP positive glia cells (**e**), and O4 positive oligodendrocytes (**f**). Nuclei are counterstained with Hoechst 33342.

When neurospheres are applied as in vitro model system, cell differentiation can be evaluated within the migration area around the sphere core or in cryosections.

(d) *Cell viability* measurements can be performed by determining the metabolic activity by MTT and CellTiter Blue assay or membrane integrity of NSCs by LDH leakage assay.

(e) *Apoptosis* can be evaluated by nuclear condensation (see Fig. 3b), TUNEL assays, immunocytochemical/immunoblotting techniques, and by enzymatic assays for evaluation of the activation of the key apoptotic enzymes. When neurospheres are applied as in vitro model system, occurrence of apoptotic nuclei can be evaluated within the migration area around the sphere core or in cryosections.

(f) *Neurite outgrowth* can be evaluated by: (1) the number of cells exhibiting neurites; (2) the number of neurites per cell; and (3) the length of neurites by determining the length of the longest neurite, total neurite length, length of segments, or average neurite length and branching.

NSCs represent a powerful and promising experimental system for the investigation and characterization of adverse effects on neural development induced by toxic agents and might provide relevant information for the assessment of risk of human exposure. Even if in vitro models provide only partial answers to the complex problems related to DNT, they are of great value in providing information on basic mechanistic processes that may lead to novel preventive and protective strategies.

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Chapter 6

Preparation, Maintenance, and Use of Serum-Free Aggregating Brain Cell Cultures

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Abstract

Serum-free aggregating brain cell cultures are free-floating three-dimensional primary cell cultures able to reconstitute spontaneously a histotypic brain architecture to reproduce critical steps of brain development and to reach a high level of structural and functional maturity. This culture system offers, therefore, a unique model for neurotoxicity testing both during the development and at advanced cellular differentiation, and the high number of aggregates available combined with the excellent reproducibility of the cultures facilitates routine test procedures. This chapter presents a detailed description of the preparation, maintenance, and use of these cultures for neurotoxicity studies and a comparison of the developmental characteristics between cultures derived from the telencephalon and cultures derived from the whole brain. For culture preparation, mechanically dissociated embryonic brain tissue is used. The initial cell suspension, composed of neural stem cells, neural progenitor cells, immature postmitotic neurons, glioblasts, and microglial cells, is kept in a serum-free, chemically defined medium under continuous gyratory agitation. Spherical aggregates form spontaneously and are maintained in suspension culture for several weeks. Within the aggregates, the cells rearrange and mature, reproducing critical morphogenic events, such as migration, proliferation, differentiation, synaptogenesis, and myelination. For experimentation, replicate cultures are prepared by the randomization of aggregates from several original flasks. The high yield and reproducibility of the cultures enable multiparametric endpoint analyses, including "omics" approaches.

Key words: Aggregating brain cell cultures, Development, Maturation, Neurotoxicity, Gliotoxicity, CNS, Three-dimensional cell culture, High content analysis

1. Introduction

The central nervous system (CNS) belongs to the critical target organs of xenobiotics and other potential toxicants because of the high vulnerability of this organ and the serious consequences adverse effects have for the entire organism. To the catalog of

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known neurotoxicants belong industrial solvents, reactive intermediates, and degradation products of industrial chemicals, pesticides, herbicides, plasticizers, heavy metals, psychoactive drugs, and sundry toxins and venoms of living organisms. Furthermore, many pharmaceuticals cause CNS symptoms as unwanted secondary drug effects. Almost as numerous as the noxious substances are the putative molecular CNS targets, which include specific membrane receptors, specific ion pumps, matrix and structural proteins, transporters, enzymes, and numerous constituents of vital biological pathways. In addition, some neurotoxic agents may exhibit selectivity for a specific developmental stage or a particular developmental event. The developmental, structural, and functional features of the CNS are known to be particularly complex due to the highly elaborate neuronal connectivity and the intimate physical, communicative, and metabolic interactions between all cell types present in the CNS, including neurons, oligodendrocytes, astrocytes, and microglial cells. In search of an in vitro model suitable for the detection and study of neurotoxicants, one should, therefore, opt for a culture system able to reproduce closely the developmental stages occurring in the CNS as well as the organ-specific structural and functional features.

The previous work has shown that mechanically dissociated embryonic rat brain cells reaggregate spontaneously, forming free-floating aggregates which develop into highly organized spheres that exhibit organotypic structures and functions. These rotation-mediated aggregating brain cell cultures show high reproducibility and robustness, owing to the initial mechanical dissociation of the embryonic brain tissue and the use of a serum-free, chemically defined culture medium (1). The aggregates are maintained as suspension cultures under continuous gyratory agitation in a CO_2 incubator. The cell types present in the original brain tissue are also found in the aggregates, including neural stem cells, neural progenitor cells, immature neurons and glial cells, and microglial cells. Most of the macroglia (astrocytes, oligodendrocytes) arise after culture initiation by the proliferation of precursor cells. Interestingly, the neuron:glia ratio in mature aggregates is about 2:3 similar to that found in rat whole brain (2) and in the human cortex (3). The tissue-specific environment within the aggregates enables the cells to interact in a physiological manner by physical contacts as well as by the exchange of soluble messengers and metabolites. Intrinsic factors enable extensive cellular differentiation and the formation of histotypic structures, such as the extracellular matrix, mature synapses, functional neuronal networks, and myelinated axons. The maturation process takes about 1 month. Mature aggregates contain a discrete population of undifferentiated stem/precursor cells beside the highly differentiated neurons and glial cells, a situation similar

to the adult brain in vivo. The cultures offer several advantages for their practical use, in particular high yield, robustness, high reproducibility, and their growth and development in a serum-free, chemically defined culture medium. This culture system, therefore, provides a suitable system for neurotoxicity testing, including developmental, acute, and chronic neurotoxicity.

The present chapter provides a detailed description of the preparation and handling of aggregating rat brain cell cultures as a complement to previous reviews describing their use in neurotoxicity studies (4–9). Furthermore, the developmental characteristics of aggregate cultures derived from the telencephalon (used most frequently in the past) are compared with those of cultures prepared from the whole brain (comprising telencephalon, mesencephalon, and rhombencephalon). For culture preparation, the principal steps comprise the dissection of the brain tissue and its mechanical dissociation into a single cell fraction by the sequential passage through nylon sieves of 200 and 100 μm pores. The cells are then pelleted, washed in Puck's D1 solution by centrifugation, and resuspended in cold culture medium (modified DMEM, serum-free). Aliquots of this cell suspension are then transferred to culture flasks and placed on a rotating platform. The reaggregation of the dissociated cells starts immediately after incubation. Initially, all reaggregated cells are immature, as evidenced by low or undetectable levels of cell type-specific differentiation markers. While most of the neurons are already postmitotic, the majority of the macroglia (astrocytes and oligodendrocytes) arise from glial precursor cells by proliferation during the first 2 weeks in vitro. Cellular maturation progresses during about 4 weeks, giving rise to highly differentiated histotypic cultures. Each flask contains some 1,200–1,500 aggregates. The high culture density contributes to the cellular stability and progress of maturation, but requires frequent media replenishment and occasional subdivision (split) to assure the adequate supply of metabolic substrates. For experimentation, replicate cultures containing some 200 aggregates are prepared by the randomization of aggregates from pools of several original flasks. The high yield and reproducibility of the cultures enable multiparametric endpoint analyses, such as quantitative RT-PCR (10), biochemical assays (11–14), immunocytochemistry (15), and metabolomics (16).

2. Materials

2.1. Animals

Timed-pregnant rats, SPF, Sprague-Dawley, 16 days in gestation, counting the day of mating as embryonic day 0 (E0). For rats mated overnight, the following day is counted as E1. For culture

preparation, embryos of rats mated during daytime are taken in the morning of E16; embryos of rats mated overnight are taken in the afternoon of E16. For more information about the staging and dissection of rat embryos, see Dunnett and Björklund (17). For rat housing, handling, and euthanasia, the existing specific guidelines have to be followed.

2.2. Special Equipment (see Note 1)

1. Incubator: 37°C ± 1°C; 90% ± 5% humidity; and 10% ± 1% CO_2/air (e.g., Heraeus, which can be heat-sterilized and accommodates the gyratory shaker listed below).
2. Gyratory shaker, 50 mm shaking diameter, i.e., a deviation of 25 mm to each side (e.g., Kühner gyratory shaker X-SL, driven by magnetic induction, tolerates high humidity and heat sterilization, and creates only little heat in the incubator).
3. Osmometer.
4. Culture vessels, i.e., 25- and 50-mL DeLong flasks or custom-made modified Erlenmeyer flasks derived from Schott Duran Erlenmeyer flasks of which the necks were made cylindrical to accommodate a cap with gas-permeable fit.
5. Caps for culture vessels, gas-permeable and autoclavable (e.g., Bellco KAP-UTS or Labocap).
6. Pyrex glass bottles (100 mL) with air-tight closures for media.
7. Filtration units, 0.2 μm, for sterile filtration of small volumes.
8. Dissecting tools, one set per dissecting person: Large scissors (1), small pointed scissors (1), large forceps (1), small curved forceps (1), Vannas scissors (1), and Dumont forceps (1).
9. Nylon-mesh filter bags, prepared from Nitex nylon monofilament (Sefar), with either 200 μm mesh opening or 100 μm mesh opening. The filter bags can be prepared from double sheets of nylon mesh by sealing and cutting them at once using a 40 W soldering iron. The 200-μm mesh sacs are 14 cm long and 3.5 cm large; the 100-μm mesh sacs are 5.5 cm long and 5 cm large.
10. Appropriate glass funnels around which the filter bags are fitted. For 200-μm mesh sacs, the funnels have a stem of 12 cm and an outside diameter of 2.5 cm (top) and 1.6 cm (bottom); for 100-μm mesh bags, the funnels have a stem of 4 cm and an outside diameter of 4.5 cm (top) and 2.5 cm (bottom). Only the dimensions of the second, shorter funnel are critical to fit into a 50-mL conical plastic tube.
11. Glass rods for mechanical dissociation, 0.6 cm diameter, 20 cm long, with blunt, fire-polished ends.

12. Conical plastic tubes, sterile, 15 and 50 mL, and suitable racks.
13. Serological plastic pipettes, 5 and 2 mL. For the sampling and transfer of aggregates, it is recommended to use plastic pipettes with a relatively wide orifice (e.g., pipettes from Costar) to avoid damage to the aggregate.
14. Facilities for the washing and sterilization of glassware (see Note 2).

2.3. Optional Equipment (see Note 3)

1. Dissecting block (optional) with wells for Petri dishes and perfused with cooling liquid.
2. Glass pipettes, 5 mL, for media replenishment.
3. Cotton plugger for glass pipettes (convenient if recyclable glass pipettes are used for media replenishment as a cost-reducing measure).
4. Pyrex glass jar (10 L) for the preparation of media, PBS, and Puck's solution D1.
5. Water purification system.
6. Peristaltic pump for media filtration.
7. Filtration units, 0.2 μm, for sterile filtration of large volume.
8. Pyrex glass bottles with air-tight closures, 500 mL, to store culture media and PBS.

2.4. Solutions and Media for Cell Culture (see Note 4)

1. Gentamicin stock solution, 500×: 12.5 mg/mL gentamicin sulfate. Sterilize by filtration and store at 4°C.
2. Concentrated salts for Puck's solution D1, 20×: 160 g/L NaCl, 8 g/L KCl, 1.5 g/L $Na_2HPO_4 \times 12\ H_2O$, 0.6 g/L KH_2PO_4, and 100 mg/L phenol red. Sterilize by filtration and store frozen in aliquots of 100 mL.
3. Concentrated glucose/sucrose for Puck's solution D1, 20×: 22 g/L D-glucose monohydrate and 400 g/L D-sucrose. Sterilize by filtration and store frozen in aliquots of 100 mL.
4. Puck's solution D1: Thaw equal volumes of the two 20× concentrated solutions for Puck's solution D1. After thawing, shake vigorously before use. To 450 mL of ultrapure sterile water, add 25 mL of 20× concentrated salts for Puck's solution D1 plus 25 mL of 20× concentrated glucose/sucrose for Puck's solution D1. Check osmolarity and adjust, if necessary, to 340 ± 5 mOsm. Store at 4°C. Shortly before use, adjust to pH 7.4 with sterile 0.2 N NaOH (color of solution should be "salmon" red, neither yellowish nor purplish) and add 1 mL of the gentamicin stock solution 500×.
5. Trace elements, 10^4-fold concentrated: Prepare the following nine aqueous solutions separately and store them at –20°C in a 1-mL aliquot: 2.5 mM Na_2SiO_3; 0.15 mM Na_2SeO_3;

0.05 mM $CdSO_4$; 0.1 mM $CuSO_4$; 0.05 mM $MnCl_2$; 0.005 mM $(NH_4)_6Mo_7O_{24}$; 0.0025 mM $NiSO_4$; 0.0025 mM $SnCl_2$; and 0.5 mM $ZnSO_4$.

6. Media supplements, 10^3-fold concentrated: Prepare the following five aqueous solutions separately, sterilize them by filtration, and store them at –20°C in aliquots of 5–10 mL: 0.02 mM hydrocortisone hemisuccinate; 0.03 mM triiodothyronine; 10 mM linoleic acid; 5 mg/mL insulin; and 1 mg/mL transferrin.
7. Concentrated mixture of vitamins A and E: Dissolve 114 mg vitamin A in a 0.2-mL absolute ethanol and mix this solution with 1.8 g of vitamin E. Store this mixture at –20°C protected from light.
8. Modified DMEM serum-free culture medium: Fill a 10-L glass jar (Pyrex) with about 8 L of ultrapure water and dissolve under gentle stirring (using a large magnetic stirring bar) DMEM powder mix for 10 L, containing high glucose (4.5 g/L) and glutamine, but no bicarbonate and no pyruvate. Add the following supplements under stirring: 1.35 g choline chloride, 20 mg L-carnitine, 2 mg lipoic acid, 13.6 mg vitamin B12, 1 mL of each of the nine trace elements 10^4-fold concentrated, and 37 g $NaHCO_3$. After the addition of bicarbonate, immediately adjust to pH 7.0–7.2 (color should be yellowish to salmon red) by bubbling CO_2 gas into the medium. Adjust the osmolarily of the medium to 340 ± 2 mOsm by adding the required volume of ultrapure water. Sterilize the medium by filtration using MediaKap-10 hollow fiber filters and a peristaltic pump. Store the medium in 500-mL Pyrex bottles with gas-tight closures at 4°C in the dark.
9. Complete culture medium: Shortly (2–3 days) before use, complement the culture medium by adding the following sterile supplements per 500 mL of medium: 0.5 mL of each of the five 10^3-fold concentrated media supplements, i.e., 0.02 mM hydrocortisone hemisuccinate, 0.03 mM triiodothyronine, 10 mM linoleic acid, 5 mg/mL insulin, and 1 mg/mL transferrin; 1 mL of gentamin stock solution 500×; 5 mL of vitamins BME 100-fold concentrated; and 0.05 mL of the concentrated mixture of vitamins A and E sonicated in 10 mL of medium and added to the medium by sterile filtration (0.2 μm filter; only a small fraction of the vitamins will pass the filter). Check the sterility of the final culture medium: From each bottle of complete medium, take one 4-mL aliquot and incubate it under normal culture conditions. Control the sterility by visual inspection after incubation for at least 1 day.

3. Methods

3.1. Dissection, Cell Isolation, and Culture Preparation

Cultures are prepared from brain parts (e.g., the telencephalon) or from the whole brain (i.e., telencephalon, mesencephalon, and rhombencephalon) of 16-day rat embryos (E16). The duration of the dissection and tissue collection should not exceed 2 h. All dissecting instruments and solutions should be sterile. From step 5 onwards, all work is done under strictly aseptic conditions. During steps 5–21, the tissue/cells remain at 0–4°C.

1. Sacrifice the pregnant rat (see Note 5).
2. Lay the sacrificed animal on its back on an adsorbent paper and rinse the abdomen with 95% alcohol.
3. Open the peritoneal cavity: Using the large forceps, grasp the skin about 1 cm above the genitalia. With the large scissors, cut through the skin and fascia, and extend the cut on both sides of the abdomen, until the entire peritoneal cavity lies open.
4. Remove the uterine horns containing the fetuses, using small curved forceps and small pointed scissors, and transfer them into a 50-mL plastic tube containing 25–30 mL of ice-cold Puck's solution D1.
5. Transfer the uterine horns to one of the Petri dishes placed on the cold dissecting block (or on ice if no dissection block is available) within a horizontal laminar flow bench.
6. Using the Vannas scissors and small forceps, separate the embryos from the uterus and from their amniotic sac and placenta, and transfer them to a fresh Petri dish containing Puck's solution D1.
7. Dissect the brain of each embryo: Fix the head of the embryo in a lateral position with the aid of the Dumont forceps, and make an extended lateral incision at the base of the brain using the Vannas scissors. Lift the brain through this opening using the blunt side of the closed Vannas scissors.
8. Using a scalpel, separate the telencephalon from the mesencephalon/diencephalon/rhombencephalon parts.
9. Collect the dissected brain parts either separately or together (depending on the part(s) used for culture preparation) in a 50-mL plastic tube filled with 40 mL of Puck's solution D1.
10. Repeat steps 1–6 for each pregnant rat and steps 7–9 for each embryo. The brain tissue collected from 50 embryos is the maximum amount to be kept in one tube. If necessary, use several tubes filled with 40-mL Puck's solution D1 to collect the dissected tissue, and replace the liquid periodically to avoid a drop of the pH.

11. At the end of dissection, rinse each batch of tissue 3× with about 40 mL of cold Puck's solution D1. Take these batches separately for the subsequent mechanical dissociation, using each time a fresh 200-μm mesh bag (see below).
12. For the dissociation procedure, place the 200-μm mesh bag (with a glass funnel fitted inside) in a 50-mL conical plastic tube containing 25 mL cold Puck's solution D1. Pour the tissue of one batch into the bag. Remove the funnel, and hold the upper ends of the closed bag against the outer side of the tube wall.
13. Using the glass rod, gently stroke downward, from the outside of the immersed bag, to squeeze the tissue through the mesh into the surrounding solution. At the end of this first passage, remove the bag and close the tube.
14. Repeat steps 12 and 13 for every batch of dissected brain tissue.
15. Filtration of the cell suspension: Take the 100-μm mesh nylon bag attached with tape to the short glass funnel, and place it on top of an empty 50-mL conical plastic tube. Using a 5-mL serological plastic pipette, transfer the cell/tissue suspension to the nylon bag, and let the suspension pass through the filter by gravity flow into the 50-mL tube. Toward the end of the filtration, add Puck's solution D1 up to a final volume of 50 mL.
16. Repeat step 15 for each batch of dissociated tissue.
17. Centrifuge the filtrate(s) containing the dissociated cells (300×*g*, 15 min at 4°C, with slow acceleration and deceleration).
18. Of each tube, remove the supernatant, and resuspend the pellet with cold Puck's solution D1 (start with 2.5 mL) by gentle trituration (five to six strokes up and down using a 5-mL plastic pipette, without foaming). Bring the volume to 50 mL with cold Puck's solution D1.
19. Centrifuge the cell suspension(s) (300×*g*, 15 min at 4°C, with slow acceleration and deceleration).
20. Of each tube, remove the supernatant and resuspend the cells in cold serum-free culture medium by gentle trituration. Transfer the resulting cell suspension to a plastic culture flask for further dilution.
21. Dilute the cell suspension with cold medium to the final volume (see Note 6).
22. Transfer 4-mL aliquots of the final cell suspension to 25-mL culture flasks, and place them onto the platform of a rotating (68 rpm) gyratory shaker in the CO_2 incubator (37°C, 10% CO_2/90% humidified air).

23. Accelerate the gyratory agitation to 70 rpm in the evening of the day of culture preparation (DIV 0) and to 74 rpm the next day (DIV 1).
24. At DIV 2, transfer the cultures to 50-mL culture flasks, either by pipetting or by pouring, and add 4 mL of fresh prewarmed medium. If the latter (quicker) procedure is chosen, pour the content of each flask along the wall inside a fresh flask, and then in the same way an additional 4 mL of fresh prewarmed medium so that no aggregates remain attached on the wall of the fresh culture flask. Continue with the transfer of other cultures, but do not exceed 30 min. Thereafter, the cultures should be allowed to equilibrate for at least 1.5 h before a further series is transferred into the same incubator. Accelerate the gyratory agitation at the end of the transfer to 78 rpm, and at DIV 4 to 80 rpm, the maximum speed kept throughout culture maintenance and experimentation.

3.2. Media Replenishment

For the maintenance of aggregating brain cell cultures, regular replenishment of the culture media is required, beginning at DIV 5.

Media are replenished every third day from DIV 5 to DIV 14 and every other day in the following. Media replenishment, i.e., the replacement of 5 mL of media supernatant (of a total of 8 mL) with fresh prewarmed serum-free culture medium, is done in a sterile hood with vertical laminar air flow according to the following protocols:

1. Fill the amount of fresh medium required for replenishment into a 100-mL sterile glass bottle and warm it in a water bath at 37°C for 10 min.
2. Put 5 flasks of cultures on a slanted support, and transfer them to the laminar flow bench. Using a 5-mL pipette, remove 5 mL of media supernatant, and replace it by a slightly larger volume (5.2 mL, to compensate evaporation) of fresh medium. [Note: To avoid contamination, use always a fresh pipette with fresh medium. Furthermore, the rim of the caps should be shortly immersed in 100% ethanol, taking care that no ethanol enters when closing the flask].
3. Return the flasks to the incubator and continue with another group of five cultures. Do not exceed 30 min of replenishment, until the cultures are allowed to equilibrate for at least 1.5 h before a further series of the same incubator is replenished.

3.3. Subdivision of Cultures

Cultures are subdivided (split) at DIV 20 because of the greatly increased metabolic activities of the now more mature cells. Depending on the duration of culture maintenance, additional subdivisions might be required, as indicated by increasing acidity

(yellowish color) of the culture medium. For subdivision, the cultures are split into two equal parts as follows:

1. Put the required number of 50-mL culture flasks into the laminar flow hood and label them.
2. Add to each flask 4 mL of cold culture medium and preequilibrate in the incubator under gyratory agitation for at least 1 h.
3. Fill the amount of fresh medium required for replenishment (step 5) into a 100-mL sterile glass bottle and warm it in a water bath at 37°C for 10 min.
4. Transfer half of one culture (4 mL) to one of the preequilibrated flask: With a 5-mL plastic pipette, quickly resuspend the aggregates by short aspiration and brisk expulsion of the supernatant medium, then quickly take a 4-mL aliquot, and transfer it to the fresh flask.
5. Replenish the original flask by adding 4 mL of fresh prewarmed medium.
6. Put the flasks back to the incubator and continue with the next series. The duration of subdivision should not exceed 30 min. Thereafter, the cultures should be allowed to equilibrate for at least 1.5 h before a further series is put into the same incubator.

3.4. Preparation and Use of Replicate Cultures for Experimentation

Due to their free-floating nature, aggregate cultures can easily be pooled from individual cultures and divided into aliquots for the preparation of replicate cultures. For routine applications, an average of six to seven replicate cultures are prepared from each original flask, each replicate culture containing some 200 aggregates (for typical values, such as total amounts of proteins and RNA per culture flask, see Note 7). For each experiment, a maximum of 48 replicate cultures are prepared conveniently, according to the following protocols:

1. At the day of experimentation, put the required number of small (25-mL) culture flasks into the laminar flow hood and label them.
2. Add to each flask 3 mL of cold culture medium, and preequilibrate in the incubator under gyratory agitation for at least 1 h.
3. Pool (e.g., in a 50-mL plastic tube) the aggregates from the original cultures.
4. Transfer the aggregates in a 1-mL aliquot from the pool to the preequilibrated flasks, in a series of 6–12. Using a 2-mL plastic pipette, quickly resuspend the aggregates by short aspiration and brisk expulsion of the supernatant medium, then quickly take a 1-mL aliquot, and transfer it to the first

flask in the row. Continue this way with the next flasks, until all 6 (12) flasks have received an aliquot of aggregates. Make sure that the sequence of numbering corresponds to that of aliquoting.

5. Put the flasks back into the incubator and continue the same way with the next series, until all flasks have received an aliquot.
6. Equilibrate the replicate cultures for at least 90 min before starting the experiment (e.g., before their exposure to neurotoxicants).
7. Maintain the cultures under standard culture conditions (i.e., at 37°C in 10% CO_2/90% humidified air and under 80 rpm gyratory agitation). If the duration of the experiment exceeds 3 days, media have to be replenished regularly.

3.5. Harvest of Cultures for Quantitative RT-PCR, Biochemical Assays, and Immunocytochemistry

The high yield and the free-floating character of aggregate cultures are suitable for multidisciplinary high-content analysis by quantitative RT-PCR, biochemical assays, and immunocytochemistry. For typical analytical endpoints and developmental characteristics of aggregating brain cell cultures, see Note 7.

For qRT-PCR and biochemical analyses, the harvest is carried out in ice, wearing disposable gloves, and using sterile materials and solutions. The aggregates of individual flasks/replicates are washed in ice-cold PBS and stored frozen according to the following protocols:

1. Take one replicate at a time, and transfer (pour) the aggregates into a 15-mL plastic tube placed in ice.
2. Let the aggregates settle by gravity sedimentation and remove the supernatant by suction.
3. Resuspend the aggregates with 5 mL of ice-cold PBS, let the aggregates settle, and remove again the supernatant.
4. Repeat step 3 once more (second wash). A third wash is necessary for cultures subjected to metabolic radiolabeling prior to the harvest.
5. Store the pelleted aggregates at −80°C.

For analyses by qRT-PCR (10), the total RNA is extracted of each replicate culture using a kit from Qiagen.

For biochemical assays, the aggregates of each replicate are sonicated in 150–400 µL of 2 mM potassium phosphate buffer pH 6.9 (three pulses of 3–4 s duration at 30 W using a microtip). The homogenate is then divided into aliquots for the different assays and stored at −80°C for analysis. Several useful methods for the determination of biochemical parameters have been described, e.g., for the measurement of enzymatic activities, protein and DNA content (11), thymidine incorporation (13), and 2-deoxyglucose uptake (14).

For immunocytochemistry, the harvest is started at RT and performed according to the following protocols:

1. Transfer (pour) the aggregates of three to four replicate cultures (at least 600 aggregates) into a 15-mL plastic tube at RT.
2. Let the aggregates settle by gravity sedimentation and remove the supernatant by suction.
3. Resuspend the aggregates with 5 mL of prewarmed (30–37°C) PBS, let the aggregates settle, and remove again the supernatant.
4. Repeat step 3 once more (second wash).
5. With a 2-mL plastic pipet, transfer the aggregates to a gelatin capsule (Feton International, Brussels, no. 00) and let the aggregates settle.
6. With a Pasteur pipet, remove the supernatant as quickly as possible (the capsule rapidly starts to liquefy) and quickly cover the pellet of aggregates with cryoform (Cryomatrix, Shandon Scientific, Runcorn, Cheshire, the UK).
7. Hold the capsule at the upper end with tweezers and dip it immediately into isopentane cooled with liquid nitrogen. The frozen capsule is transferred to dry ice and then stored at –80°C.

Cryostat sections are prepared from unfixed aggregates. The frozen capsules are transferred from –80°C to –20°C the day before cutting on the cryostat. The gelatin capsule is removed with the use of a razor blade. The cryosections (10 μm) are fixed for 10 min in 4% paraformaldehyde/PBS at RT, washed in PBS, and processed the same day or the next day (in the latter case, sections are kept overnight at 4°C) for immunocytochemistry (4, 10, 15).

4. Notes

1. Special equipment is required mainly for the dissection and mechanical dissociation of the fetal brain tissue and for the initiation and maintenance of the aggregate cultures under continuous gyratory agitation. In addition, facilities are required for the recycling of glassware (see Note 2). The equipment and procedures given in the present protocol work best in our hands, but alternative solutions are possible if the following constraints are taken into account: (a) The mechanical dissociation of the embryonic brain tissue into a single cell fraction must be complete, but sufficiently gentle to provide a single cell suspension with a maximum of viable cells (at least 50% viability as judged by the exclusion of trypan

blue, a basic test procedure not described here). An alternate mechanical dissociation method was described by Cole and de Vellis (18). (b) During the initial phase of cell aggregation, the geometry of the culture vessels is critical for a reproducible vortex and, hence, for the formation of aggregates of comparable size. Therefore, the culture vessels to be used for culture initiation have to be uniform. Because we found considerable variations between DeLong flasks of different batches, we use Duran Erlenmeyer flasks of which the neck was modified by a glass blower to accommodate a gas-permeable cap. The vortex depends on the geometry of the flasks and the speed of agitation. To find the optimal vortex with a new batch of flasks, it is recommended to vary, if necessary, the volume of medium per culture vessel, not the speed of agitation. If during agitation, the aggregates concentrate too much in the center of the flask, the volume has to be decreased until they cover about two-third of the bottom surface. During the early culture phase, the aggregates are very sticky. Therefore, continuous agitation is important to avoid adhesion between aggregates, and care has to be taken to avoid prolonged contact while changing their media for the first time (DIV 5). The final aggregates should be even in size, with an average diameter of about 500 μm. (c) The culture vessels need to be equipped with closures that permit gas exchange. While the CO_2 is important for media buffering, optimal O_2 levels are necessary because of the high metabolic activity of more mature cultures. This also requires continuous agitation of the aggregates (they should not remain immobilized for more than 10 min).

2. Glassware to be recycled (e.g., culture vessels and glass pipettes) is washed and subsequently sterilized. Used culture glassware should never be allowed to dry before it is cleaned, but stored immersed in a special detergent solution (e.g., neodisher LM-10, 5% v/v). In the dishwasher, a special alkaline cleaning agent free from detergents (e.g., neodisher FT) is used for washing and a neutralizer (e.g., neodisher Z) to remove alkaline residues. The glassware is then further rinsed with water and finally with ultrapure water. After drying, the glassware is sterilized in the autoclave (except for cotton-plugged 5-mL glass pipettes used for media replenishment, which are sterilized in dry air for 2.5 h at 220°C).
3. Except for the dissecting block, the optional equipment is proposed for economic reasons. The high culture density requires frequent media replenishments, and therefore a considerable amount of culture medium and 5-mL pipettes.
4. Protocols are given for the preparation of all listed solutions and media, although it may be possible to purchase some of them.

It is important that all solutions are made with ultrapure water. The shelf life of the DMEM culture medium is only about 2 months because of its high glutamine content, forming ammonia by degradation. After completion, the serum-free culture medium is used within 7–10 days.

5. Sacrifice of rats is made by decapitation for two reasons: (a) we find that decapitation of the conscious rat with an appropriate guillotine causes less stress to the animals than injection of anesthetics and (b) we want to avoid the presence of anesthetics in the brain of fetuses. In any case, it is important to follow the ethical guidelines in vigor. If decapitation is done, clean (rinse) the place of decapitation for each animal to be sacrificed.
6. For routine work, the final volume of the cell suspension is calculated by the number of embryos (brains) used per batch of cell suspension. Taking only the telencephalon, the number of flasks to be obtained equals the number of dissected brains divided by 1.7; hence, the final volume will be the number of embryos divided by 1.7 and multiplied by the final volume per flask. Taking the whole brain, the number of flasks will be the number of embryos times 1.2 and the final (total) volume of cell suspension will be the number of embryos multiplied by 1.2 and by the final volume per flask. Thus, each flask will contain an initial cell number of about 2.5×10^7 cells, and for the same number of embryos, twice the whole brain cultures can be obtained compared to telencephalon cultures. For routine work, cell counting is not required. The method is sufficiently robust to permit considerable variations in cell number without affecting the fundamental properties of the cultures. Nevertheless, it might be useful (particularly for beginners) to determine the cell number and viability. To this end, an aliquot (0.1 mL of 50 mL of cell suspension) is taken after the completion of step 18 in the isolation procedure, diluted at least 10×, and counted using either a hemocytometer or an electronic cell counter. Cell viability, determined by the exclusion of trypan blue (not described here), should be about 50%.
7. Typical values and developmental characteristics of aggregating cell cultures are shown for cultures derived from embryonic rat telencephalon (Table 1) and from embryonic rat whole brain (Table 2). In the original cultures (taken for the preparation of replicate cultures), the average amount of total protein per flask increases in telencephalon cultures from 3.5 mg (DIV 7) to 9.6 mg (DIV 28) and in whole brain cultures from 5.5 mg (DIV 7) to 9.1 mg (DIV 28). The average amount of total RNA per flask increases in telencephalon

Table 1
Characterization of the histotypic cellular maturation in aggregating cell cultures derived from 16-day embryonic rat forebrain [a]

Parameters assayed	DIV 7	DIV 14	DIV 21	DIV 28
Enzymatic activity[b]				
GAD [μU/mg protein]	365 ± 51	697 ± 126	1,084 ± 78	1,482 ± 115
ChAT [μU/mg protein]	30 ± 4	81 ± 10	138 ± 11	243 ± 16
GS [mU/mg protein]	3.8 ± 0.5	34.0 ± 3.7	49.0 ± 3.2	51.5 ± 2.8
CNP [mU/mg protein]	180 ± 15	1,290 ± 110	1,660 ± 150	1,510 ± 80
Gene expression[c]				
Nestin	1.00 ± 0.15	0.37 ± 0.02	0.58 ± 0.03	0.28 ± 0.02
DCX	1.00 ± 0.16	0.65 ± 0.11	0.37 ± 0.03	0.18 ± 0.01
NF-H	1.00 ± 0.22	1.37 ± 0.31	3.81 ± 0.26	4.20 ± 0.41
VGLUT1	1.00 ± 0.06	1.76 ± 0.17	5.09 ± 0.31	6.07 ± 0.30
TH	1.00 ± 0.05	0.94 ± 0.09	1.16 ± 0.26	1.12 ± 0.06
GFAP	1.00 ± 0.04	1.74 ± 0.14	20.5 ± 1.0	18.0 ± 1.7
MBP	1.00 ± 0.08	13.5 ± 1.8	21.7 ± 3.7	7.30 ± 0.55
MOG	1.00 ± 0.06	42.4 ± 9.1	317 ± 31	168 ± 0

[a] Cultures were prepared from telencephalon. The day of culture preparation is taken as day 0 (DIV 0)
[b] The activities of glutamic acid decarboxylase (GAD), choline acetyltransferase (ChAT), and glutamine synthetase (GS) were measured by radiometric methods; 2′,3′-cyclic nucleotide 3′-phosphohydrolase (CNP) by a photometric method (11)
[c] Gene expression was measured at the mRNA level by qRT-PCR (10). For each gene, the values were expressed as fold change relative to the cultures at DIV 7 (set equal to 1). Doublecortin (DCX), neurofilament high molecular weight (NF-H), vesicular glutamate transporter 1 (VGLUT1), tyrosine hydroxylase (TH), glial acidic fibrillary protein (GFAP), myelin basic protein (MBP), and myelin oligodendrocyte glycoprotein (MOG)

cultures from 84 μg (DIV 7) to 123 μg (DIV 28) and in whole brain cultures from 103 μg (DIV 7) to 141 μg (DIV 28). Both culture types show similar developmental patterns, i.e., a decrease in parameters representing immature cells (nestin mRNA , DCX mRNA) and an increase in markers of the differentiated phenotype (cell type-specific enzyme activities and mRNA content of structural and functional constituents). The two culture types show a similar developmental increase in the marker of neuronal cytoskeleton NF-H, as well as in the markers of glutamatergic neurons (VGLUT1, highly expressed in cerebral cortex and hippocampus, ref. 19), GABAergic neurons (GAD), cholinergic neurons (ChAT), astrocytes (GS, GFAP), and oligodendrocytes (CNP, MBP, MOG). Only tyrosine hydroxylase (TH) mRNA is clearly (5.4-fold) higher in whole brain cultures as compared to telencephalon cultures, in accordance with the known brain location of the catecholaminergic neurons. Beside

Table 2
Characterization of the histotypic cellular maturation in aggregating cell cultures derived from 16-day embryonic rat whole brain[a]

Parameters assayed	DIV 7	DIV 14	DIV 21	DIV 28
Enzymatic activity[b]				
GAD [μU/mg protein]	293 ± 7	605 ± 64	983 ± 50	1,428 ± 78
ChAT [μU/mg protein]	20 ± 2	41 ± 3	81 ± 3	164 ± 14
GS [mU/mg protein]	4.6 ± 0.4	28.8 ± 2.7	46.7 ± 3.5	49.0 ± 1.9
CNP [mU/mg protein]	130 ± 30	1,070 ± 120	1,550 ± 180	1,630 ± 90
Gene expression[c]				
Nestin	1.00 ± 0.03	0.33 ± 0.01	0.35 ± 0.00	0.24 ± 0.01
DCX	1.00 ± 0.05	0.70 ± 0.02	0.34 ± 0.00	0.18 ± 0.03
NF-H	1.00 ± 0.04	1.69 ± 0.02	3.02 ± 0.06	4.24 ± 0.12
VGLUT1	1.00 ± 0.04	1.88 ± 0.01	4.00 ± 0.22	7.55 ± 0.55
TH	1.00 ± 0.03	1.81 ± 0.23	1.77 ± 0.03	1.71 ± 0.25
GFAP	1.00 ± 0.02	4.38 ± 0.11	18.6 ± 0.9	18.8 ± 1.7
MBP	1.00 ± 0.04	34.6 ± 0.2	33.9 ± 1.4	15.2 ± 1.4
MOG	1.00 ± 0.19	131 ± 14	700 ± 2	556 ± 167

[a]Cultures were prepared from whole brain, comprising telencephalon, mesencephalon, and rhombencephalon. The day of culture preparation is taken as day 0 (DIV 0)
[b]The activities of glutamic acid decarboxylase (GAD), choline acetyltransferase (ChAT), and glutamine synthetase (GS) were measured by radiometric methods; 2′,3′-cyclic nucleotide 3′-phosphohydrolase (CNP) by a photometric method (11)
[c]Gene expression was measured at the mRNA level by qRT-PCR (10). For each gene, the values were expressed as fold change relative to cultures at DIV 7 (set equal to 1). Doublecortin (DCX), neurofilament high molecular weight (NF-H), vesicular glutamate transporter 1 (VGLUT1), tyrosine hydroxylase (TH), glial acidic fibrillary protein (GFAP), myelin basic protein (MBP), and myelin oligodendrocyte glycoprotein (MOG)

the highly differentiated brain cells, a small population of nestin-expressing cells with low mitotic activity persists, indicating the presence of immature cells with the characteristics of adult neural stem cells. Many of the parameters shown in Tables 1 and 2 were found to be useful as endpoints for the assessment of organ-specific neurotoxicity. For routine testing, measurements of the mRNA expression of a series of representative genes by quantitative RT-PCR proved to be most efficient (20).

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Chapter 7

Neurospheres as a Model for Developmental Neurotoxicity Testing

Ellen Fritsche, Kathrin Gassmann, and Timm Schreiber

Abstract

Developmental neurotoxicity (DNT) of environmental chemicals is a serious threat to human health. So far, DNT testing is performed in animals. Such in vivo testing is time-consuming, expensive, and uses large numbers of animals. Moreover, species differences (rat–human) bear the problem of extrapolation. Thus, alternative tests are needed to provide faster and cheaper methods for DNT testing.

Neurospheres are free-floating three-dimensional structures consisting of neural progenitor cells. Within the "neurosphere assay," basal processes of brain development, such as proliferation, migration, differentiation, and apoptosis, are mimicked. These processes can be disturbed by chemicals, and thus predict DNT. Therefore, we consider this cell system as a promising tool for DNT in vitro testing. The methods to determine the effects of chemicals on DNT-specific endpoints are described in this chapter.

Key words: Developmental neurotoxicity, Neurosphere, Neural progenitor cell, In vitro, Brain development, Human, Mouse

1. Introduction

In 1992, Reynolds, Weiss, and colleagues (1, 2) demonstrated for the first time that cells from the central nervous system (CNS) of adult and embryonic mice can be isolated and propagated in culture. In the presence of epidermal growth factor (EGF), such neural progenitor cells (NPCs) give rise to cell agglomerations that are termed "neurospheres." Furthermore, neurospheres can also be generated from single NPCs (3) indicating that they have the potency for self-renewal.

Nestin-positive NPCs proliferate in culture. Moreover, they have the ability to migrate and differentiate into neurons (beta(III) tubulin$^+$ cells), astrocytes (GFAP$^+$ cells), and oligodendrocytes (O4$^+$ cells; Fig. 1). As neurospheres are primary, three-dimensional cell systems, which are self-organized and contain cells which are

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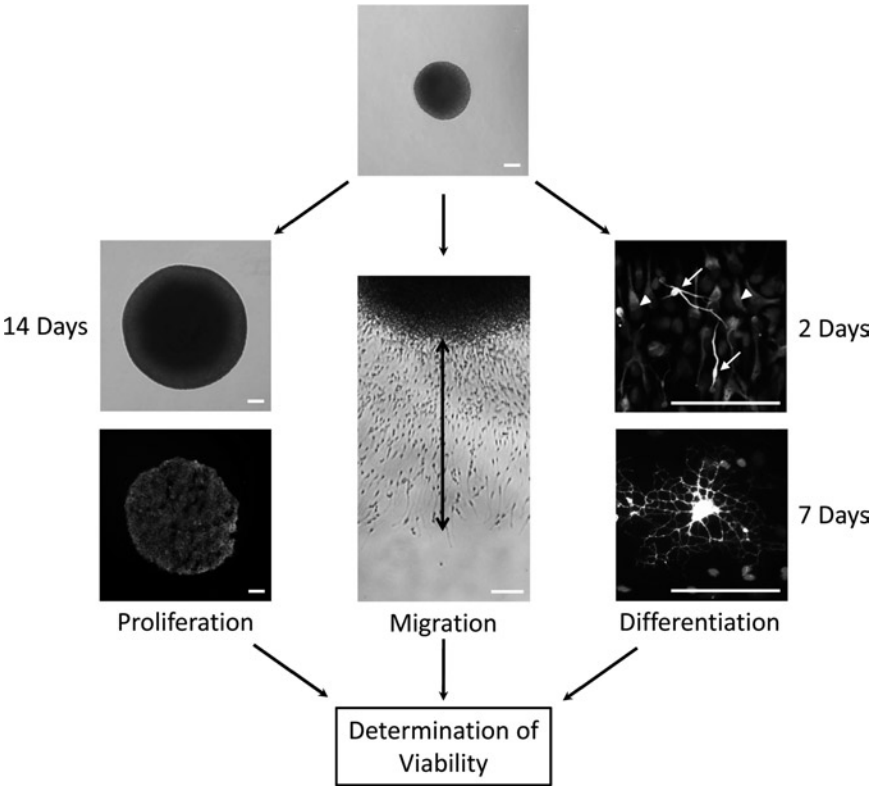

Fig. 1. The neurosphere assay. Proliferation is accessed via increase in single sphere diameter or indirectly via increase in metabolic activity. Immunocytochemical staining of cryostat sections indicates the presence of Nestin and GFAP in proliferating spheres; nuclei are stained with Hoechst instat of DAPI. After mitogen withdrawal and in the presence of an extracellular protein matrix, NPCs migrate radially out of the sphere. Migration distance over time is measured as indicated by the *arrow*. Specific immunocytochemical staining of NPCs indicates the presence of β(III)-tubulin (*arrow*) and GFAP (*arrow head*) after 2 days and O4 after 7 days; nuclei are stained with Hoechst instat of DAPI. Viability of proliferating and differentiating neurospheres is assessed to differentiate between general toxicity and specific developmental neurotoxicity of compounds. Scale bars: 100 μm.

the building blocks of the developing brain, they represent a valuable in vitro model that mimics basic processes of brain development (4). Therefore, we believe that they are a useful tool for testing chemicals for their abilities to interfere with these processes: proliferation, migration, differentiation, and apoptosis (4–7).

2. Materials

All procedures should be performed in a laminar-flow culture hood equipped with a microscope unless indicated otherwise. All cultures are maintained in a humidified incubator at 37°C and 7.5% CO_2, and all media are warmed in a 37°C water bath prior to use.

2.1. Murine NPC Isolation

1. Minimal Essential Medium (MEM; Gibco, Bethesda, MD).
2. Dulbecco's Modified Eagle's Medium (DMEM; Gibco GlutaMAX High Glucose, Invitrogen).
3. Phosphate buffered saline (PBS): 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.7 mM KCl, 1.5 mM KH_2PO_4, 137.9 mM NaCl, and 8.1 mM Na_2HPO_4.
4. Tissue digestion solution: Mix 1,900 µL MEM with 38 µL of 30 U/mL papain (Worthington) and preincubate the solution for 10 min at 37°C. Weight out a tip of a spatula of L-cysteine and dissolve in an appropriate volume of MEM to obtain a 100× stock solution with a concentration of 24 mg/mL. After preincubation, add 20 µL DNase I and 20 µL L-cysteine to the Papain mixture. Sterilize the solution with a syringe filter.
5. Ovomocuid: Mix 1,950 µL DMEM with 20 µL of 1 mg/mL Trypsin inhibitor, 10 µL of 50 µg/mL Bovine Serum Albumine (BSA), and 20 µL DNase I.
6. 70% v/v Ethanol.

2.2. Neurosphere Culture

2.2.1. Proliferating Conditions

1. Neurospheres of human or mouse origin.
2. Proliferation medium: DMEM (Invitrogen) and Ham's F12 (Gibco), GlutaMAX (Invitrogen) 3:1 supplemented with 2% B27 (Invitrogen). EGF and recombinant human fibroblast growth factor (FGF) are dissolved at 10 µg/mL in sterile PBS containing 0.1% BSA and 1 mM DTT and stored at –20°C. Both are diluted in medium at a final concentration of 20 ng/mL. Add antibiotic solution (penicillin/streptomycin) to 1× final.
3. 10- and 6 cm culture dishes.
4. McIlwain tissue chopper.
5. Double-edged razor blade.

2.2.2. Differentiating Conditions

1. Neurospheres of human or mouse origin.
2. Differentiation medium: DMEM (Invitrogen) and Ham's F12 (Invitrogen) 3:1 supplemented with 1% N2 (Gibco, Invitrogen). Add antibiotic solution (penicillin/streptomycin) to 1× final.
3. Coverslips, multiwell plates, or multiwell chambers.
4. 0.1 mg/mL Poly-D-lysine (PDL).
5. 1 mg/mL Laminin.

2.3. Assessment of Cell Viability

2.3.1. Alamar Blue Assay (CellTiter-Blue Assay, Promega)

1. CellTiter-Blue Reagent (CTB, Promega, Mannheim, Germany).
2. 96-well plate compatible with fluorometer.
3. Proliferation or differentiation medium (depending on the experimental cell culture condition).
4. Fluorescence reader with the excitation 530–570 nm and emission 580–620 nm filter pair.

2.3.2. Lactate Dehydrogenase Assay (CytoTox-One, Promega)

1. CytoTox-One Reagent (Promega).
2. 96-well plate compatible with fluorometer.
3. Fluorescence reader with excitation 530–570 nm and emission 580–620 nm filter pair.

2.3.3. DAPI Exclusion Test

1. 1,000× stock 4′-6-Diamidin-2-phenylindole (DAPI) solution: Dissolve 10 mg/mL in H_2O. Prepare a 10 µg/mL working solution.
2. 1% Triton X-100 solution in PBS.

2.3.4. TdT-Mediated dUTP-Biotin Nick End Labeling Assay (In Situ Cell Death Detection Kit, Fluorescein, Roche)

1. 10× stock PBS: 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, and 100 mM NaCl. Adjust the pH to 7.5 with HCl and store at room temperature. Prepare a 2× working solution by the dilution of one part PBS with four parts water.
2. 12% (w/v) Paraformaldehyde (PFA) stock solution: Dissolve 12 g PFA in 100 mL PBS and add five drops of 1 N NaOH. Heat the solution carefully to 70–80°C (use a stirring hot plate in a fume hood) to dissolve the PFA and then cool to room temperature. Aliquots can be stored at –80°C until use and use a fresh aliquot for each experiment.
3. Ice-cold permeabilization solution (PBS-T): 0.1% (v/v) Triton X-100 in PBS.
4. DNase I.
5. 50% Glycerol in PBS.
6. TdT-Mediated dUTP-Biotin Nick End Labeling (TUNEL)-Mix for each slide chamber (20 µL): 13.55 µL PBS-T, 4 µL 5× Terminal Transferase Buffer (Roche Diagnostics), 2 µL 25 mM $CoCl_2$ (Roche), 0.1 µL Fluorescein-12-dUTP (Roche), 0.25 µL Terminal deoxynucleotidylexo-transferase (TdT, Roche), and 0.2 µL Hoechst 33258 (Stock solution: 10 mg/mL in H_2O). Mix and quick spin. Keep in ice.

2.3.5. Caspase Activity Assay (Apo-ONE Homogeneous Caspase-3/7 Assay, Promega)

1. Apo-ONE Homogeneous Caspase-3/7 Assay (Promega).
2. 96-well plate compatible with fluorometer.
3. Fluorescence reader with the excitation 485 ± 20 nm and emission 530 ± 25 nm filter pair.

2.4. Analyses of Neurosphere Proliferation

2.4.1. Diameter Determination and Metabolic Activity Measurement

1. 1× PBS (Subheading 2.3.4).
2. CTB (Promega).
3. Proliferation medium (see Subheading 2.2.1).
4. 6-cm culture dish.
5. Fluorescence reader with the excitation 530–570 nm and emission 580–620 nm filter pair.
6. 96-well plate U-bottom compatible with fluorometer.

2.4.2. Fluorescence-Activated Cell Sorting Cell Cycle Analysis

1. 1× PBS (Subheading 2.3.4).
2. 24-well plate.
3. Test tubes.
4. Proliferation medium (see Subheading 2.2.1).
5. 100% Accutase.
6. Centrifuge.
7. 4% PFA (Subheading 2.3.4).
8. Saponin solution: Dissolve 0.15 g Saponin in 100 mL ice-cold PBS and put in ice.
9. 1 mg/mL Ribonuclease A (RNAse) in H_2O.
10. 10 mg/mL Propidium iodide (PI) in H_2O.
11. Fluorescence-activated cell sorter (FACS) with compatible tubes.

2.5. Migration Analyses

1. PDL/laminin-coated 8-chamber slides (BD Bioscience).
2. Differentiation medium (see Subheading 2.2.2).

2.6. Immuncytochemistry

2.6.1. Immuncytochemistry of Differentiated Neurospheres

1. 1× PBS (Subheading 2.3.4).
2. 12% PFA (Subheading 2.3.4).
3. 0.1% (v/v) PBS-T: Triton X-100 in PBS.
4. Primary antibodies: rb IgG anti-GFAP (1:500, Sigma), ms IgG anti-β(III)tubulin (1:100, Sigma), ms IgM anti-O4 (1:50, Millipore; Billerica, MA), and ms IgG anti-Nestin (1:50, BD Bioscience).
5. Secondary antibodies: Alexa Fluor 488 anti-mouse IgG (1:500), Alexa Fluor 546 anti-rabbit IgG (1:100), and Rhodamine Red anti-mouse IgM (1:100, Dianova; Hamburg, Germany).
6. Nuclear stain: 10 µg/mL Hoechst 33258 (Stock solution: 1 mg/mL in H_2O).
7. Mounting medium: Vectashield Mounting Medium (Linaris; Wertheim, Germany).
8. Parafilm.
9. Goat serum.

2.6.2. Immuncytochemistry of Proliferating Neurospheres

1. Same material as in the Subheading 2.6.1.
2. Cryostat Microtome and compatible blades.
3. Tissue-freezing medium (Tissue-Tek OCT Compound (Sakura Finetek Europe, Zoeterwoude, the Netherlands)).
4. Microscope Slides for Cryosections (Menzel Glaeser Superfrost Plus, Braunschweig, Germany).
5. Cryomolds 15 mm × 15 mm × 5 mm (Sakura Finetek Europe, Zoeterwoude, the Netherlands).
6. 24- or 96-well plate (depending on the sphere number).
7. Saccharose solution 20% (w/v) in PBS.
8. Hydrophobic pen.
9. Dry ice.

3. Methods

3.1. Murine NPC Isolation

1. Fill one tissue culture dish with 1× PBS (sterile) and one with MEM.
2. Fill a 2-mL reaction tube with 1 mL MEM.
3. Euthanize the gravid mouse in the CO_2 chamber.
4. Sterilize the abdomen with EtOH (70%), sever fur, peritoneum, and musculature.
5. Remove the uterus from the abdomen and separate the embryos.
6. Transfer three embryos into the PBS-filled tissue culture dish.
7. Remove the uterus and placenta from the embryos, cut the umbilical cord, and determine the age of embryos according to the staging criteria of Theiler.
8. Separate the head from the thorax. Make two cuts with forceps: one over the eyes and one behind the brain, in such a way that a triangle is excised.
9. Remove the skin and cartilage with forceps to uncover the brain. Transfer the brain from the skull base to the tissue culture dish filled with MEM.
10. Remove the meninges from the brain and collect tissue in the reaction tube.
11. Add 1 mL tissue digestion solution to the tissue and incubate at 37°C, depending on the age, for 20–45 min.
12. Fill a 15-mL tube with 10 mL MEM.
13. Withdraw 1 mL cautiously from the supernatant of the tissue.

14. Triturate the tissue gently with a 1,000-µL tip.
15. Add 1 mL Ovomucoid to stop the digestion.
16. Transfer the single cell solution into the 15-mL tube.
17. Centrifuge for 5 min at $170 \times g$.
18. Discard supernatant and resuspend pellet in 1 mL proliferation media.
19. Determine the cell number with a Neubauer chamber.
20. Plate cells in a density of 1×10^5 cells/mL in a 10-cm cell culture dish with 20 mL proliferation media.

3.2. Neurosphere Culture

3.2.1. Proliferating Conditions: Thawing of Human NPCs

1. For one cryovial, distribute 50 mL of proliferation medium to five 10-cm culture dishes.
2. Add another 50 mL proliferation medium to a 50-mL tube.
3. Place the cryovial in a 37°C water bath very briefly, just until the cells have started to thaw (approximately 2 min).
4. Add the contents of the cryovial into the 50-mL tube and resuspend the cells gently.
5. Transfer the cell suspension to the 10-cm culture dishes, 10 mL each.
6. Put culture dishes in the cell culture incubator.
7. Feed culture dishes every 2 days by replacing half of the medium.
8. After 4 weeks of expansion, the human NPCs have built neurospheres of about 500 µm diameter and can be used for experiments.

3.2.2. Growing and Expanding Human Neurospheres by Mechanical Chopping

1. To increase the growth and survival of human neurospheres, cultures should be chopped every 2–4 weeks.
2. Therefore, put the McIlwain tissue chopper into a laminar-flow culture hood and clean with 70% ethanol.
3. Soak a double-edged razor blade in 100% acetone and sterilize the sliding table and the copping arm with 70% ethanol.
4. Carefully secure the blade onto the chopping arm. Make sure the blade is parallel to the chopping surface.
5. Check the chopper settings. The blade force should be set at 12:00 (straight up) and, for optimal growth, the chop distance should be set between 0.2 and 0.3 mm.
6. Prepare 10-cm culture dishes for the newly chopped neurospheres by filling them with 20 mL proliferation medium each (usually two to three new dishes for chopping the neurospheres of one old dish).
7. Transfer neurospheres with as little medium as possible from the old 10-cm dish to the middle of an inverted lid of a 5-cm culture dish.

8. Carefully remove the remaining medium with a pipet in order to prevent the neurospheres from moving during the chop.
9. Place the dish lid on the chopper and move the sliding table to the starting position.
10. Turn on the power and press "reset."
11. When all of the spheres on the lid have been chopped, stop and raise the chopping arm and reposition the table on the starting position.
12. Rotate the dish 90° and repeat the steps 10 and 11 one more time.
13. When the neurospheres have been chopped in the second direction, remove the dish from the chopper and add about 1 mL proliferation medium to the cells.
14. Resuspend them by gently pipetting them up and down and then evenly distribute the cell suspension to the new dishes.
15. Put the cells back in the cell culture incubator.
16. After chopping is complete, clean the chopper with 70% ethanol and eventually discard the razor blade (usually one blade can be used for three times each side).
17. Feed culture dishes every 2–3 days by replacing half of the medium (see Note 1).

3.2.3. Differentiating Conditions

1. Wash the sterile hollowware with 100% ethanol prior to use and wash the chambers one time with sterile-distilled water.
2. Coat with 1 mg/mL PDL solution in sterile-distilled water for at least 1 h at 37°C.
3. Wash the chambers one time with sterile-distilled water.
4. Coat with 10 µg/mL laminin solution in sterile-distilled water for at least 1 h at 37°C.
5. Wash again with sterile-distilled water, cover the surface with sterile PBS, and store for at most 1 week at 4°C.
6. Distribute 100 µL differentiation medium for each neurosphere in each chamber or well (normally 5 spheres per chamber).
7. Use a 100-µL tip to transfer the neurospheres into the prepared chambers or wells. Make sure that each neurosphere hold adequate space for migrating cells.
8. Feed the cultures every 2–3 days by replacing half of the medium.

3.3. Compound Exposure

1. We recommend to set up several controls for compound testing.

2. Untreated Cells Control: Set up wells or chambers with untreated cells to serve as a vehicle control. Add the same solvent used to deliver the test compounds to the vehicle control media.
3. Optional No-cell or Background Control: In case of fluorescence-based assays, set up wells or chambers without cells to serve as the negative control to determine background fluorescence.
4. Optional Test Compound Control: Especially for enzyme-based assays, set up wells or chambers without cells containing the vehicle and the test compound to test for possible interference with the assay chemistry.
5. Endpoint specific control: Expose your cells to a chemical compound that is known to influence the endpoint you are interested in. For example, for viability determination, set up wells or chambers containing cells treated with a compound known to be toxic to the cells (staurosporine, Triton-X100, H_2O_2) and for proliferation analysis, culture spheres in medium without the growth factors to inhibit the sphere growth.
6. We recommend a maximal solvent concentration of 0.25% for DMSO or other organic solvents and 1% for water or PBS in the cell culture media. Keep this in mind during the preparation of your compound stock solutions.
7. For setting an EC_{50} value, use at least six serial dilutions of your test compound with a dilution factor between 2 and 5 (three serial dilutions with a dilution factor of 10 for range-finding experiments).
8. We recommend a constant medium volume of 100 µL medium/sphere during the exposure period.
9. If you want to expose your cells for more than 72 h, you have to feed your cells by replacing half of the test media.

3.4. Viability

3.4.1. Alamar Blue Assay (CellTiter-Blue Assay, Promega)

1. Culture cells for the desired test exposure period.
2. Thaw CTB in a 37°C water bath. Protect the CellTiter-Blue Reagent from direct light.
3. Dilute CTB 1:3 with proliferation medium (for viability testing of proliferating spheres) or differentiation medium (for viability testing of differentiating spheres).
4. For spheres cultured in 100 µL medium (e.g., in the proliferation assay), add 30 µL of the respective CTB dilution and for spheres cultured in 500 µL medium (e.g., in the migration assay), add 150 µL of the respective CTB dilution.
5. Incubate using the standard cell culture conditions for 2 h.

6. Spheres cultured in a 96-well plate can be directly measured in the same plate. In case of cells cultured in chambers or other plate formats, transfer 100 µL of the supernatant media without cells to a 96-well plate.
7. Shake plate for 10 s and record the fluorescence ad 544/590 nm.
8. Because CTB is relatively non-destructive to cells during short-term exposure, it is possible to use the same experimental setup to measure the enzyme activity at more than one timepoint and multiplex it with other assays.

3.4.2. Lactate Dehydrogenase Assay (CytoTox-One, Promega)

1. Culture cells for the desired test exposure period.
2. Equilibrate Substrate Mix and Assay Buffer at room temperature.
3. Prepare CytoTox-One Reagent by adding 11 mL of Assay Buffer to each vial of Substrate Mix. Gently mix to dissolve the substrate. Protect the Reagent from direct light. Unused portions of the CytoTox-One Reagent could be stored tightly capped at –20°C for 6–8 weeks.
4. Place 100 µL of cell culture medium of your exposed cells in a 96-well plate and add an equal amount of CytoTox-One Reagent.
5. Incubate at 37°C for 4 h.
6. Shake the plate for 10 s and record fluorescence ad 544/590 nm.
7. Because you only need a small portion of the supernatant media, it is possible to use the same experimental setup to measure lactate dehydrogenase (LDH) activity at more than one timepoint and multiplex it with other assays.
8. The half-life of LDH that has been released from cells into the surrounding medium is approximately 9 h at 37°C. But the collected medium can be stored at –20°C for 2–4 weeks prior to adding the CytoTox-One Reagent without remarkable loss of LDH activity.

3.4.3. DAPI Exclusion Test

1. Culture cells for the desired test exposure period.
2. Add DAPI solution to obtain a final concentration of 0.2 µg/mL.
3. Incubate for 2 min at room temperature.
4. Take a photo with a fluorescent microscope to detect dead cells.
5. Add 200 µL Triton X-100 solution to lyse cells.
6. Incubate for 2 min at room temperature.
7. Take a photo with a fluorescent microscope to detect all cells.

3.4.4. TdT-Mediated dUTP-Biotin Nick End Labeling Assay (In Situ Cell Death Detection Kit, Fluorescein, Roche)

1. Thaw on ice: $CoCl_2$, Fluorescein-12-dUTP and 5× Terminal Transferase Buffer.
2. Dehydration: Replace 250 µL medium with 250 µL 2× PBS.
3. Incubate for 5 min.
4. To fix the cells, add 166 µL of 12% PFA solution to the 500 µL culture medium (to receive a final concentration of 4% PFA) and incubate for 15 min.
5. Wash twice for 5 min with 500 µL PBS.
6. Add the TUNEL-Mix to the cells.
7. Incubate for 1 h at 37°C in a moist and dark chamber.
8. Wash the slides three times with 500 µL PBS for 5 min.
9. Remove the PBS and add 10 µL Glycerol/PBS to each culture field and place a cover slip on the slide. Remove abundant Glycerol/PBS by a paper towel and gently pressure. Nail varnish should be used to seal the samples. The sample can be viewed immediately after the varnish is dry or be stored in the dark at 4°C for up to a month.

3.4.5. Caspase Activity Assay (Apo-ONE Homogeneous Caspase-3/7 Assay, Promega)

1. Culture neurospheres in 96-well plates with one sphere per 100 µL medium/well and freeze the plate after the desired test exposure period at –80°C.
2. After at least 2 h, thaw the plate at room temperature.
3. Thaw the 100× Caspase Substrate and the Caspase Buffer at room temperature. Mix by inversion or vortexing. Dilute the substrate 1:100 with the buffer to obtain the desired volume of Apo-ONE Reagent. Freshly prepare the Apo-One Reagent for every experiment. Do not freeze and store the Apo-ONE Reagent.
4. Add 100 µl of Apo-ONE Caspase-3/7 Reagent to each well of the 96-well plate.
5. Incubate at room temperature for at least 1 h, depending upon the expected level of apoptosis (and thus caspase-3/7 activity) in the spheres analyzed.
6. Shake the plate for 10 s and record fluorescence ad 485/530 nm.

3.5. Analyses of Neurosphere Proliferation

3.5.1. Diameter Determination and Metabolic Activity Measurement by CellTiter-Blue Assay

1. Chop the spheres, as described in the Subheading 3.2.1; growing and expanding human neurospheres by mechanical chopping.
2. On the next day, collect neurospheres of exactly the same diameter. You will need six spheres per concentration and additional spheres for the respective controls (see the Subheading 3.3). Leave blank the outer wells of the plate. These wells will be filled with distilled water to prevent the evaporation of test media during the exposure time.

3. Dilute CTB 1:3 with proliferation medium and equilibrate the solution for at least 15 min in the cell culture incubator.
4. Put every sphere in a single well of a 96-well plate filled with 100 µL proliferation medium.
5. Add 30 µL of the CTB dilution per well.
6. Incubate 96-well plate using the standard cell culture conditions for 2 h.
7. Shake the plate for 10 s and record fluorescence ad 544/590 nm.
8. Determine the diameter of every neurosphere.
9. Prepare a second 96-well plate with the test media (chemical test substance in the proliferation medium) and the control media. Use again 100 µL medium per well and fill outer wells of the plate with distilled water.
10. Transfer neurospheres from the CTB plate to the second 96-well plate filled with the test media. Prevent the contamination of the test media with CTB by washing neurospheres in a Petri dish with sterile PBS. Place again one sphere in a single well at the same position as in the first plate.
11. Put the plate in the cell culture incubator.
12. Feed the neurospheres by the replacement of half of the medium every 2–3 days.
13. Repeat the steps 3–8 after 14 days.
14. Determine the proliferation rate by subtracting the values for diameter and metabolic activities before the exposure from the values after the 14-day exposure (see Note 2).

3.5.2. Fluorescence-Activated Cell Sorting Cell Cycle Analysis

1. Chop spheres as described in the Subheading 3.2.1; growing and expanding human neurospheres by mechanical chopping.
2. On the next day, collect neurospheres of exactly the same diameter. You will need at least ten spheres per concentration step and additional spheres for the respective controls.
3. Prepare a 24-well plate with 1 mL proliferation medium per well with the chemical test substances or the respective control media.
4. Transfer neurospheres to the 24-well plate (up to 10 neurospheres/well).
5. Culture spheres for the desired test exposure period (usually 2–3 days) under standard conditions.
6. To obtain a single cell suspension, all neurospheres of the same exposure condition are washed once in PBS, transferred to a test tube, and incubated with 200 µL Accutase at 37°C

for 20 min. Singularize the cells by gently pipetting during and at the end of the Accutase treatment.

7. Add 400 µL of proliferation medium. From now on, work on ice unless otherwise noted.
8. Centrifuge the suspension at 4°C and 1,400×*g* for 5 min. Discard supernatant.
9. Resuspend the cell pellet in 100 µL of PBS.
10. Add 500 µL of 4% PFA and fix the cells for 30 min at 4°C.
11. Centrifuge at 4°C and 1,400×*g* for 5 min. Discard supernatant.
12. Resuspend in 200 µL of PBS containing 0.15% saponin and 10 µg/mL RNase, and incubate for 30 min at 37°C.
13. Transfer the cell suspension to FACS tubes.
14. Add 1 µL PI (1 mg/mL stock concentration) 5 min before the FACS analysis.

3.6. Migration Analysis

1. Prepare a dilution series of the test compound.
2. Fill each chamber of a PDL/laminin-coated 8-chamber slide with 500 µL.
3. Transfer five neurospheres to each chamber using a 100-µL tip. All neurospheres should be of the same size. Make sure that each neurosphere hold adequate space for migrating cells.
4. Incubate for 2–3 days at 37°C and 7.5% CO_2.
5. Use a phase-contrast microscope to measure the migration distance in four directions for each neurosphere from the edge of the sphere until the farthest migrated cell (see Note 3).

3.7. Immunocytochemistry

3.7.1. Immunocytochemistry of Differentiated Neurospheres

1. Culture cells for the desired test exposure period under differentiating conditions as described under the Subheading 3.2.2.
2. To fix the cells, add 100 µL of 12% PFA solution to the 500 µL culture medium (to receive a final concentration of 2% PFA) and incubate for 30 min at 37°C.
3. Remove the media from the cells and take off the gasket carefully from the slide.
4. Wash the slide three times for 5 min in a coplin jar in PBS.
5. Prepare the primary antibodies in an appropriate dilution in PBS (for surface epitopes) or PBS-T (for intracellular epitopes) with 10% goat serum. You will need 200 µL of antibody solution for each slide.
6. Place the slide on a piece of parafilm in a humidified chamber and add 200 µL antibody solution on the slide. Cover the

slide with another piece of parafilm and incubate for 30 min at 37°C.

7. Wash the slide three times for 5 min in a coplin jar in PBS.
8. Meanwhile, prepare the secondary antibodies in an appropriate dilution in PBS supplemented with 1% Hoechst.
9. Incubate the slide with the secondary antibody solution, as described previously, for 20 min at 37°C.
10. Wash the slide three times for 5 min in a coplin jar in PBS.
11. Dip the slide once in distilled water to avoid saline residuals.
12. Dry off the back of the slide and the rubber lining; the samples are then ready to be mounted.
13. Add a drop of mounting medium to each culture field and place a cover slip onto the slide. Remove abundant mounting medium with a paper towel and gently press down. Seal the slide with nail varnish. The immunohistochemical specimen can be examined immediately microscopically or be stored in the dark at 4°C for up to a month.

3.7.2. Immuncytochemistry of Proliferating Neurospheres

1. Culture cells for the desired test exposure period in 24- or 96-well plates (depending on the sphere number).
2. To fix the cells, transfer the spheres to a 24- or 96-well plate filled with 4% PFA (100 µL/sphere) and incubate for 30 min at 37°C.
3. Place the spheres in a new well filled with 20% saccharose solution (100 µL/sphere) and incubate over night at 4°C.
4. Fill a cryomold half-full with cryomedium and place up to ten neurospheres in the center. Prevent the transfer of saccharose solution to the cryomold. Fill up the cryomold with cryomold.
5. Freeze the filled cryomold on dry ice till the cryomedium is completely solid. Frozen neurospheres in cryomedium can be stored for short terms at –20°C; for long-term storage, use a –80°C freezer.
6. The correct cryostat microtome settings are dependent on the individual instrument used for the cryosections. We use a chamber temperature of –20°C and an object temperature of –18°C. For a cryosection with a single layer of cells, adjust the slice thickness to 10 µm.
7. The microscope slides with the cryosections can be stored at –20°C for several months.
8. To stain cryosections, take the slides out of the freezer and let them adjust to room temperature. Encircle cryosections with a hydrophobic pen to prevent leaking of the antibody solution in the next steps.

9. Wash the slide one time for 5 min in a coplin jar in PBS.
10. Prepare the primary antibodies in an appropriate dilution in PBS (for surface epitopes) or PBS-T (for intracellular epitopes) with 10% goat serum. You will need about 200 µL for each slide (depending on the number of cryosections per slide and the size of the encircled area).
11. Carefully pipet the antibody solution on the cryosections.
12. Place the slide in a humidified chamber and incubate for 1 h at 37°C.
13. Meanwhile, prepare the secondary antibodies in an appropriate dilution in PBS supplemented with 1% Hoechst.
14. Incubate the slide with the secondary antibody solution, as described previously, for 30 min at 37°C.
15. Wash the slide three times for 5 min in a coplin jar in PBS.
16. Dip the slide once in distilled water to avoid saline residuals.
17. Add a drop of the mounting medium to each culture field and place a cover slip onto the slide. Remove abundant mounting medium with a paper towel and gently press down. Seal the slide with nail varnish. The immunohistochemical specimen can be examined immediately microscopically or be stored in the dark at 4°C for up to a month (see Note 4).

4. Notes

1. If neurospheres settle on the culture dish bottom during the cultivation under proliferating condition, this might be due to a lack in growth factors. Either seed spheres more sparsely or add more medium.
2. Usually used proliferation assays rely on the detection of tritiated thymidine ^{3}H or BrdU uptake. They have both in common that you cannot use them to analyze the proliferative capacity directly in the neurosphere, but have to break the neurosphere into single cells. Therefore, we have established methods that indirectly measure the number of cells present within intact neurospheres.
3. If migration is irregular, coating might be patchy. Moreover, do not start a migration experiment the day after chopping.
4. If differentiation pattern changes, it might be due to the age of the spheres in culture. We routinely use our neurospheres for 3–4 months after thawing.

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Chapter 8

Acute Hippocampal Slice Preparation and Hippocampal Slice Cultures

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Abstract

A major advantage of hippocampal slice preparations is that the cytoarchitecture and synaptic circuits of the hippocampus are largely retained. In neurotoxicology research, organotypic hippocampal slices have mostly been used as acute ex vivo preparations for investigating the effects of neurotoxic chemicals on synaptic function. More recently, hippocampal slice cultures, which can be maintained for several weeks to several months in vitro, have been employed to study how neurotoxic chemicals influence the structural and functional plasticity in hippocampal neurons. This chapter provides protocols for preparing hippocampal slices to be used acutely for electrophysiological measurements using glass microelectrodes or microelectrode arrays or to be cultured for morphometric assessments of individual neurons labeled using biolistics.

Key words: Acute hippocampal slice, Biolistics, Electrophysiology, Hippocampal slice culture, Microelectrode arrays, Morphometry

1. Introduction

As an experimental model, hippocampal slice cultures provide distinct experimental advantages over other in vitro and in vivo preparations of the hippocampus. The most significant advantages are that, except for the absence of afferent input, hippocampal slice preparations retain the cytoarchitecture and synaptic circuits of the intact hippocampus (1), yet are readily accessible for optical imaging or electrophysiological studies. Hippocampal slices have proven to be a powerful experimental model for investigating the structural and functional features of synaptic connectivity at the molecular, cellular, and circuit levels. The most widely used hippocampal slice preparations are the acute hippocampal

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slices and the organotypic hippocampal slice cultures. Acute hippocampal slice preparations are most often obtained from the adult rodent brain, are intended to be used for experimentation the same day that they are prepared, and are typically used to study the electrophysiological properties of individual neurons or circuits. In contrast, hippocampal slice cultures are usually derived from neonatal rodent brains and can be maintained in vitro for weeks to months (2, 3). Molecular biology, imaging, electrophysiology, and immunohistochemical techniques are routinely used in slice cultures to investigate molecular and cellular processes of neurodevelopment, synaptic plasticity, and cell death in both physiologic and pathophysiologic contexts.

The advantages of acute slices relative to slice cultures are that they involve less work to prepare and maintain, and the pattern of synaptic connections within the slice is minimally altered relative to the in vivo patterns at the time of harvest. In contrast, hippocampal slice cultures require more effort, particularly if the goal is to maintain the cultures beyond several days, and the neuronal injury and disruption of afferent and efferent connections that occur during the preparation of hippocampal slices induce axonal and dendritic remodeling and reorganization of synapses among the remaining neurons (4). The tissue isolation and slicing procedure also induces glial cell activation, which can lead to the formation of an astrocytic scar that surrounds the healthier tissue in the center of the slice within several weeks after harvest. There are several advantages of the slice cultures relative to the acute slice preparation beyond the obvious advantages afforded by being able to track processes that occur over extended periods of time. First, the dead cells and tissue debris caused by the slicing procedure disappear after 1–2 weeks in vitro (5). Second, the slice has time to recover from the altered metabolic state caused by the enzymes and ions released during the cutting of the tissue (6).

This chapter describes the techniques for preparing acute hippocampal slices for electrophysiological measurements as well as culturing and transfecting hippocampal brain slices for morphometric analyses. The preparation of hippocampal slices involves two major steps: (1) preparation of equipment, substrates, and media and (2) dissection and slicing of the hippocampus. While there are many similarities, the details of these steps vary depending on whether the goal is to generate acute slices or slice cultures. Protocols for each preparation are, therefore, described separately. With regards to hippocampal slice cultures, several methods have been developed over the years, but currently, the two most frequently used are the roller tube technique, which was developed and characterized by Gahwiler (5), and the membrane interface technique introduced by Stoppini et al. (7). With the roller tube technique, slices are attached to glass coverslips and incubated in rotating tissue culture tubes for several weeks. The slow rotation results in periodic alteration of the gas–liquid

interface to which the cultures are exposed. This process leads to considerable thinning of the slice, eventually resulting in a monolayer of cells. With the membrane interface technique, slices are placed on porous, transparent membranes and maintained at the interface between air and culture medium. These membrane slice cultures do not thin to the same extent as seen with roller-tube cultures, but rather remain five to eight cell layers thick. The preparation and equipment needs are much simpler using the membrane interface technique, making it more accessible to a greater number of laboratories, and this is the technique described for preparing hippocampal slice cultures in this chapter.

Freshly prepared (acute) slices of rat or mouse hippocampus are widely used to study how drugs and/or targeted mutations influence synaptic excitability and changes in neuroplasticity. Traditionally, glass microelectrodes have been used to record electrically evoked extracellular potentials, including the excitatory and inhibitory postsynaptic potentials (EPSPs and IPSPs) and population spikes (PSs). Glass microelectrodes have several advantages, permitting the investigator to precisely place the recording and stimulating electrodes within the subregions of the hippocampus and to a depth beyond the surface of the slice, where cells are likely injured by the slicing protocol. The use of multiple or multibarreled electrodes permit the measurements of evoked potentials simultaneously with the measurements of local pH, oxygen, or neurotransmitter release. Glass microelectrodes also afford the measurements of excitatory and inhibitory currents from a single neuron using an electrode placed intracellularly or using the whole cell voltage clamp configurations. Application of single electrode methods to hippocampal slices has been invaluable in determining how xenobiotics influence the neuronal excitability and plasticity on an acute timescale and have contributed to our knowledge of underlying mechanisms. However, a major limitation of single electrode approaches for toxicological research is that they are not amenable to rapid throughput screening and do not provide information about the patterns of network activity across the entire hippocampus. Recent advances in multielectrode array (MEA) technology and fabrication have resulted in clear advantages over the traditional extracellular pulled-glass electrode approaches. Advances in printed MEA chips have permitted the fabrication of electrodes at high density (arrays of 64, 128, or more electrodes) spaced ≤50 μm, apart with limited interference from cross talk. Moreover, the spatial orientation of individual electrodes within an MEA chip can be customized to measure patterns of neural activity among hippocampal regions in response to stimuli delivered to one or more discrete locations within the slice. The major steps common to both the electrode and MEA methods include: (1) cutting and equilibration of hippocampal slices in ACSF; (2) determining whether the interface or submerged perfusion will be used; (3)

placement of recording and stimulating electrodes or positioning of slice on the electrode array; (4) defining the input/output (I/O) relationship for each slice; and (5) recording a stable baseline response to a constant electrical pulse protocol determined from the I/O relationship (stimulus giving ~50% of maximum response) prior to perfusion of the test substance.

Optical imaging of hippocampal slice cultures has proven to be a powerful experimental approach for elucidating cellular and molecular mechanisms contributing to both functional and structural plasticity in the developing hippocampus. Various labeling techniques have been successfully applied to image neuronal and glial cells in hippocampal slice cultures, including: (1) immunohistochemistry; (2) bath application, injection, or ballistic delivery (8) of fluorescent structural or physiological indicators; and (3) biolistic (9) or viral transfection (10) to express cDNAs or siRNAs in a subset of neuronal or glial cells. In this chapter, we describe a method for labeling the soma, dendritic arbors, and axon of a subset of neurons in slice cultures using the biolistic delivery of cDNA encoding a fluorescent protein. The major steps include: (1) preparation of DNA-coated gold microcarriers or DNA bullets; (2) coating of tubing with DNA bullets; and (3) transfection of organotypic slice cultures.

2. Materials

2.1. Preparation of Acute Hippocampal Slices

1. Tissue slicer to generate 300–400-μm thick slices of the hippocampus. Suitable slicers include the Vibratome (model VT1000S, Leica, Bannockburn, IL), McIlwain tissue chopper (Brinkman, Westbury, NY), and the Dosaka microslicer (Model DTK-1000, Kyoto, Japan).
2. Artificial cerebral spinal fluid (ACSF): 124 mM NaCl, 2.5 mM KCl, 1.25 mM KH_2PO_4, 26 mM $NaHCO_3$, 2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 10 mM D-glucose, and 4 mM D-sucrose bubbled with 95% O_2/5% CO_2 (*aka* carbogen) (see Notes 1 and 2).
3. Six- to twelve-week old rats (125–200 g) or mice (20–25 g) (see Note 3).
4. Vaporizer for the administration of volatile anesthetic.
5. Guillotine (WPI, Sarasota, FL).
6. Preincubation chamber (Medical Systems, Corp., New York, NY) (Fig. 1).

2.2. Preparation of Hippocampal Slice Cultures

1. Four- to five-day old rat pups (see Note 4).
2. Laminar flow tissue culture hood (see Note 5).

3. Tissue slicer to generate 400-μm thick slices of the hippocampus. Suitable slicers include Vibratome (model VT1000S, Leica, Bannockburn, IL), McIlwain tissue chopper (Brinkman, Westbury, NY), and the Dosaka microslicer (Model DTK-1000, Kyoto, Japan).
4. Dissecting microscope.
5. Glass bead sterilizer.
6. Sterile double-edged razor blades.
7. Large scissors, small round-ended spatula, scalpel handle and blade, small fine-tipped straight forceps, two pairs of #4 and #5 Dumont forceps, one pair of #7 curved Dumont forceps, and iridectomy scissors (or small spring scissors).
8. Sterile 6-cm Whatman filters.
9. Aklar squares are cut to fit the chopping platform of the tissue slicer.
10. Sterile the plastic transfer pipettes.
11. Sterile 10-cm diameter glass Petri dishes, 6-cm diameter tissue culture plastic dishes, and 6-well tissue culture plastic plates (NUNC 140685).
12. Culture the plate inserts: 0.4 μm Millicel membrane, 30-mm diameter (Millipore, PICM0RG50).
13. Dissection buffer: 1 mM $CaCl_2$, 5 mM $MgCl_2$, 4 mM KCl, 26 mM $NaHCO_3$, 8% sucrose, 0.5% phenol red, and

Fig. 1. Illustration of prechamber for holding brain slices before transfer to the recording chamber, such as the Haas chamber (Fig. 2). The prechamber contains a mesh bottom permitted exchange of ACSF once placed inside the outer chamber. The outer chamber possesses an inlet for bubbling carbogen from an external source via tubing.

filter-sterilized and bubbled with 95% O_2/5% CO_2 (*aka* carbogen) (see Note 1).

14. Slice culture medium: MEM with Earle's salts and L-glutamine (Gibco 11095) supplemented with 1 mM $CaCl_2$, 2 mM $MgSO_4$, 1 mg/L insulin, 1 mM $NaHCO_3$, 20% heat-inactivated horse serum, 0.5 mM L-ascorbate, 30 mM HEPES, 2.3 g/L D-glucose, and pH 7.3.

2.3. Measuring Electrical Excitability in Acute Slices Using Glass Microelectrodes

1. Nichrome wire (50-μm diameter).
2. Flaming/Brown type – Micropipette/Patch pipette puller – P-97 (Sutter Inst, Novato, CA).
3. Recording electrodes of approximately 1 MΩ resistance.
4. Amplifier KS-700 (WPI, New Haven, CT).
5. Digitizer (DigiData 1200, Axon Instruments, Molecular Devices, Sunnyvale, CA).
6. Data acquisition and analysis software (pClamp 9, Axon Instruments).
7. Interface or submerged recording chamber (Medical Systems, Corp., New York, NY) (Fig. 2).

2.4. Measuring Electrical Excitability in Acute Slices Using Microelectrode Arrays

1. MEA probes and amplifiers: These can be purchased from a number of vendors. The MED64 MEA from Tensor Biosciences (Irvine, CA) is an integrated system that supports 64-channel data acquisition and analysis. For rat hippocampal slices, a MED64 probe 8 × 8 array with 150-μm interelectrode spacing is the best, but other configurations are commercially available.
2. Microinjection pump (Harvard apparatus, Holliston, MA).
3. 0.1% polyethylenimine (PEI) in 25-mM borate buffer.

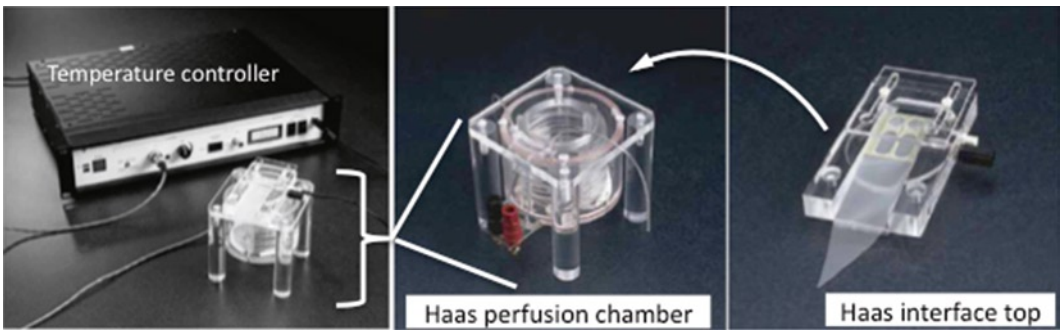

Fig. 2. Illustration of the perfusion (HAAS) chamber (*middle panel*) and the interface top (*right panel*) that holds slices on which stimulating and recording electrodes are placed. *Left panel* shows the interface chamber assembled and connected to an external temperature controller.

4. ACSF: 124 mM NaCl, 2.5 mM KCl, 1.25 mM KH_2PO_4, 26 mM $NaHCO_3$, 2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 10 mM d-glucose, and 4 mM d-sucrose bubbled with 95% O_2/5% CO_2 (carbogen).
5. Data acquisition and analysis software (Performer® software, Tensor Biosciences, Irvine, CA).

2.5. Labeling Hippocampal Slice Cultures for Morphometric Analyses

1. Helios Gene Gun system (BioRad, Hercules, CA), including Helios gene gun, tubing preparation station, and tubing cutter.
2. Clean bench or tissue culture hood.
3. Nitrogen gas cylinder and nitrogen regulator.
4. Helium gas cylinder and helium regulator.
5. Gold-Coat tubing (BioRad).
6. Transwell® permeable support (3.0-μm polyester membrane 24 mm, Costar).
7. Gold microcarriers, 1.6-μm diameter (BioRad) (see Note 6).
8. 0.5 M spermidine (Sigma S-0266).
9. Ethanol (100%).
10. 1 M $CaCl_2$.
11. 50 μg/mL polyvinylpyrrolidone (PVP, BioRad).
12. Purified cDNA: To visualize dendritic arbors in hippocampal slice cultures, we typically use pCAGGS expressing tomato fluorescent protein (see Note 7).

3. Methods

3.1. Preparation of Acute Hippocampal Slices

1. Anesthetize rat or mouse by applying isoflurane through a nose cone using a calibrated vaporizer set at 2–3%. Once a deep plane of anesthesia has been reached, euthanize the animal by decapitation, preferably using an appropriately sized guillotine.
2. Remove the brain quickly and place in chilled (5°C) ACSF (see Notes 8 and 9).
3. Dissect the hippocampus quickly on an ice-cold platform with plenty of ACSF.
4. Using a tissue slicer, section hippocampus (transverse plane) at 400-μm intervals beginning at approximately 10 mm from the rostral ends (see Note 10).
5. Transfer the slices to a preincubation chamber (Fig. 1) and hold them at 35.0 ± 0.5°C in carbogenated ACSF prior to transfer to the recording chamber (see Note 11).

6. Transverse slices 400 μm thick are cut from each hippocampus and placed, in turn, upon platforms within an interface incubation chamber (four to eight slices per platform) with ACSF that is continuously gassed with carbogen (see Note 12).

3.2. Preparation of Hippocampal Slice Cultures

1. Warm a 50-mL aliquot of slice culture medium to 37°C.
2. Place separate 50-mL aliquot of slice culture medium and 50-mL aliquot of dissection medium on ice. Bubble dissection medium with carbogen.
3. Sterilize the surgical equipment using the bead sterilizer.
4. Place the bottom of a 10-cm glass Petri dish on a pan of packed ice and fill with cold dissection medium (see Note 9). Spray a 11-cm Whatman filter with 70% ethanol and place it in overturned lid of the Petri dish on top of the ice. Cover this with cold dissection medium. Place a second 10-cm glass Petri dish on packed ice and place a 6-mm plastic tissue culture dish inside it. Add cold dissection medium to cover the bottom of the 6-mm plastic tissue culture dish.
5. Prepare the inserts for plating the slices. Place an insert into each well of a six-well plate. Add 800 μL of slice culture medium under each insert. Make sure that the bottom of each insert is completely wet and there are no air bubbles. Place the culture dishes with inserts into a cell culture incubator at 35°C at 5% CO_2 until the slices are ready to plate.
6. Prepare the tissue slicer (this protocol is based on the use of the McIlwain tissue slicer, but can be adapted for other slicers) and wipe the slicer down thoroughly with 70% ethanol. Sequentially sterilize the following with 70% ethanol, dry, and then stack on the stage: the two plastic-cutting platforms provided by the manufacturer, 6-cm Whatman filter, and Aklar square cut to fit the stage. Ensure that the edge of the Aklar extends just beyond the edge of the filter. Insert a sterile razor blade and align it to the chopping platform.
7. Wipe the head of the pup with 70% ethanol and then decapitate using large scissors. Cut the skin down the midline from the neck up to and between the eyes with a scalpel while gently holding the skull with small forceps. Insert the scissors into the foramen magnum and cut the skull along the midline up to and between the eyes. Make four small incisions perpendicular to the main cut at the ends of the main incision. Peel the skull back with straight forceps, remove the brain using the small spatula, and submerge it immediately in the 10-mm Petri dish with chilled dissection medium, using the transfer pipette to add more dissection medium as necessary.
8. Place the brain in the Petri dish lid containing the Whatman filter covered with cold dissection medium. Be sure the brain remains covered with dissection medium throughout the

remainder of dissection. Using a scalpel or iridectomy scissors, remove the cerebellum by making a coronal cut just behind the inferior colliculi. Make a second cut sagittally down the midline completely through the brain to separate the hemispheres. Place the hemibrain so that the medial surface is facing up. Separate the neocortex with the underlying hippocampus from the midbrain and brain stem, being sure to remove the meninges and blood vessels surrounding the hippocampus. The hippocampus should now be exposed. Using the iridectomy scissors or curved Dumont forceps, disrupt the connection of the hippocampus on the ventral side (fimbria) by gently rotating the hippocampus around its longitudinal axis. Use a wide-bore pipette (precoated with slice culture medium) to transfer the hippocampus to the second 10-mm dish containing chilled dissection medium. Repeat this process until all hippocampi have been removed (see Note 8).

9. Remove the hippocampi (four to six at a time) with a wide-bore pipette and place them on the chopping block. Position hippocampi, smooth side up, using a pipette filled with dissection medium to push hippocampi into the correct position. Place them so that the long axis of each hippocampus is parallel to the direction of the stage movement, but allow the septal end to curve away from the parallel axis. Remove the excess liquid (see Note 13). Repeat this until there are 10–11 hippocampi lined up on the stage.
10. Set the chopper to move the stage 400 μm. Position the stage to the edge of hippocampi and turn the chopper on. Transfer the slices from the Aklar square into the 6-mm dish with chilled dissection medium using a wide-bore pipette. Repeat until all the hippocampi are sliced.
11. Separate the slices under a dissecting microscope either by gentle agitation of the dish or by using Dumont forceps. Be careful to not poke or tear the slices.
12. Select the slices with an intact structure that display distinct CA1, CA3, and DG cell layers, are of even thickness, and do not have tears or holes.
13. Use a wide-bore pipette (precoated with slice culture medium) to transfer three to four of the good slices per Millicell insert. Keep the slices distant from each other, but relatively centered in the middle of the membrane. Gently remove the excess medium from the top of the insert, as medium on top of the insert prevents oxygen exchange. Place the cell culture dishes in a cell culture incubator at 35°C with 5% CO_2.
14. To maintain the cultures for several weeks to several months, it is necessary to change the medium the first day after plating and every 3 days thereafter. To change the medium, tilt the plates and aspirate the medium in each well. Slowly add

800 μL of warm slice culture medium to each well, placing the pipette tip along the edge of the well below the insert. Be sure the medium covers the bottom of the filter and there are no air bubbles. An alternative method which accounts for possible conditioning of the medium by the slice culture is to partially replace the medium (see Note 14).

3.3. Measuring Electrical Excitability in Acute Slices Using Glass Microelectrodes

1. Make recording electrodes of approximately 1 MΩ resistance using a Flaming/Brown type – Micropipette/Patch pipette puller.
2. Transfer several slices from the medial hippocampus from the preincubation chamber to the nylon netting of either an interface (11) or submerged (12) recording chamber (Fig. 2).
3. For interface preparations, make sure ACSF contacts the bottom of the slice that is in contact with the nylon mesh support (Fig. 1a). Perfuse carbogenated ACSF at 22°C underneath the slice for 30 min at a flow rate of 4 mL/min. After 30 min, perfuse the slices with carbogenated ACSF warmed to 35 ± 0.5°C for the duration of the experiment using a temperature controller (Medical System Corp.) (see Note 8). Measurements can also be made from a submerged slice preparation in which ACSF flows over the entire slice and carbogen flows over the bath solution. The slices are permitted to recover in perfused ACSF for 2 h prior to placing the electrodes for conventional recording with an extracellular microelectrode.
4. A bipolar stimulating electrode made from two lengths of nichrome wire, 50 μm in diameter, is directed through holes in the cover of the interface chamber and placed on the surface of the stratum radiatum (S. rad) of area CA1 to orthodromically stimulate Schaffer collateral/commissural fibers (Fig. 3).
5. Place the glass microelectrode filled with ACSF (1 MΩ resistance) 1–2 mm from the stimulating electrode near the stratum pyramidale to record field excitatory postsynaptic potentials (*f*EPSPs) or PSs (Fig. 3).
6. Responses are amplified (KS-700, WPI, New Haven, CT, USA) using a low-pass filter at 1 kHz, digitized with a DigiData 1200 (Axon Instruments, NY, USA) and stored on a personal computer for subsequent analysis with pClamp 9 (Axon Instruments).
7. During recording, a combination of stimulation intensity and micropipette placement is used to obtain maximal responses.
8. Stimuli consist of constant current rectangular pulses lasting 200 μs applied at 0.1 Hz, ranging from 10 to 170 μA. Once

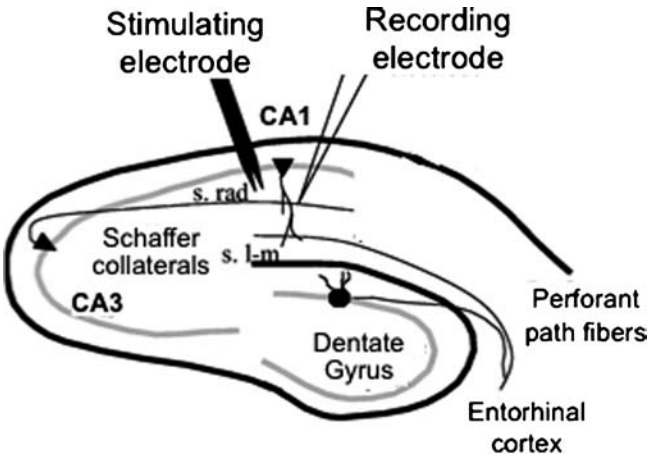

Fig. 3. Recording evoked potentials from the hippocampus CA1 using microelectrodes. Diagram showing the placement of stimulating and recording electrodes near the Schaffer collateral/commissural fibers and stratum radiatum (S. rad), respectively. The relative location of stratum lacunosum-moleculare (s. l-m) is also shown.

the stimulus–response curve is obtained at the beginning of each experiment, the stimulation intensity is fixed to obtain an fEPSP amplitude ranging from 50 to 70% of maximum (1.0–1.5 mV).

9. Begin baseline recording with constant perfusion of ACSF at 3–4 mL/min. Baseline synaptic responses are recorded for a period of 20–30 min to assure the slice is stable. Those slices not exhibiting stable responses should be discarded.
10. Record the magnitude of the *f*EPSP and PS (if present) as a function of stimulus intensity ranging from threshold of EPSP to that producing a maximal PS (control input/output curve).
11. The slices are then perfused at 3–4 mL/min with ACSF containing either vehicle or the test substance dissolved in vehicle for the duration of the experiment (typically 1 h).
12. After 30 min of perfusion with test substance, Input/Output data are acquired over the same stimulus intensity range.
13. A high-frequency protocol (tetanic pulse train) is then used to induce long-term potentiation (LTP) in the slice consisting of three trains of 100 Hz each 1 s in duration administered with 20 s intertrain intervals to induce LTP.
14. Responses are monitored continually for 20 min to access the stability of the LTP.
15. Record the magnitudes of fEPSP and PS responses at the same stimulus intensity range as the respective control (LTP data).
16. Digitized data are analyzed with pCLAMP software (Axon Instruments, version 9.2).

17. The initial slope of the population fEPSP is measured by a linear fit to a 1 ms window immediately before the onset of the PS.
18. PS amplitudes are measured from extrapolated fEPSP baseline to the peak of the PS. Input/output curves are generated by normalizing the PS amplitudes and EPSP slopes to their respective control maximum (13).

3.4. Measuring Electrical Excitability in Acute Slices Using Microelectrode Arrays

1. MED probes are relatively hydrophobic and need to be initially pretreated with PEI, 0.1% in 25-mM borate buffer for 12 h to facilitate tissue adhesion. The coating process is done once to new probes, since subsequent exposures to ACSF and tissue slices enhance slice adhesion.
2. Transfer the slice to the MED probe chamber, containing ACSF with sera (10% fetal bovine serum + 10% horse serum) using a small paintbrush to position the slice on the electrode array.
3. Use an inverted microscope equipped with a 4× phase lens to align the slice such that electrodes make contact with Schaffer-commissural fibers and the recording sites contact the pyramidal cell layer as indicated in Fig. 4.

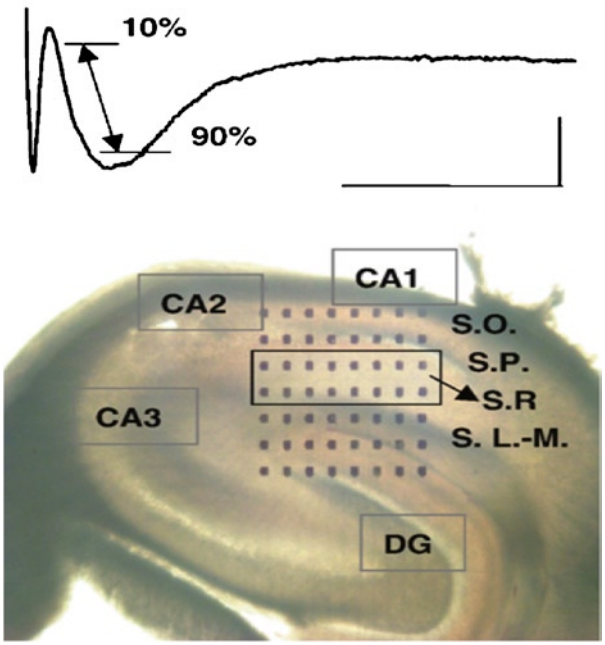

Fig. 4. Rat hippocampal slice placed on multielectrode array (MEA) showing the position of 64 microelectrodes relative to the hippocampal slice architecture. Trace depicts a typical waveform indicating the location where the fEPSP slope is measured. Abbreviations: *CA* Cornu Ammon, *DG* Dentate Gyrus, *S.O.* stratum oriens; *S.T.* stratum pyramidale, *S.R.* stratum radiatum (*the region indicated by the box*), and *S. L.-M.* stratum lucidum–moleculare.

4. After positioning the slice on the electrode array, completely remove the culture medium with the sera used for incubation and replace with 250 μL of fresh culture medium with sera.
5. During the adhesion process, it is critical that the slice receives sufficient oxygen. Prepare an airtight container with a secure lid. Flow oxygen into the container, optionally through a Teflon plate with very fine orifices.
6. Pour a small amount of water into the bottom of the container to ensure sufficient humidity.
7. Place the MED probe in the container and close the lid tightly.
8. Place the container securely in a water bath at 38°C with 95% oxygen and 5% carbon dioxide flow for 1 h. Make sure the airflow pressure is positive, typically 20 mmHg.
9. The incubation period should not exceed 1 h since the culture medium contains 20% sera, which may cause damage to the slices.
10. Apply a weight at the margins of the slice with several twists of fine wire (100–200 mg). Avoid applying pressure directly on the electrodes.
11. Take a photomicrograph (using a 4× objective) of the position of the slice on the probe.
12. Import the photomicrograph to the MED conductor software and confirm the relative placement of the slice on the electrode array.
13. Place the MED probe on the MED connector, replace the connector cover, and finally place the probe cover on the probe.
14. Referring to the photomicrograph, select the channels for stimulation. Adjust the stimulation magnitude with the MED amplifier. MED Conductor sends the stimulation signal while simultaneously recording and storing evoked responses from all 64 channels.
15. During the electrophysiological recording, the slice is perfused with oxygenated 35°C ACSF at a rate of 2–3 mL/min using a microinjection pump.
16. The stimulation electrode is chosen within the MEA based on its proximity to the Schaffer-Collateral/Commissural pathway (Fig. 4).
17. Apply field pulses, biphasic stimuli (10–80 mA, 0.1 ms) delivered to Schaffer-Collateral/Commissural pathway at 0.05 Hz (i.e., once every 20 s).
18. The recording electrode is chosen based on its proximity to the S. rad within the CA1 region of hippocampus (Fig. 4).

19. The evoked fEPSPs are sampled at 20 kHz using an MED 64 multichannel amplifier, digitized and graphically displayed using Performer® software.
20. Baseline fEPSPs are experimentally set between 50 and 60% of maximum amplitude for each slice, and the slope of each fEPSP is calculated and displayed by the Performer® software.
21. Slices that exceed more than 20% fluctuation during the stabilization period (at least 30 min) are discarded.
22. Once the slice is stabilized, fEPSPs are collected for an additional 10 min period in the absence of test compound to establish a baseline control (i.e., 100% baseline).
23. Test compounds or solvent controls are added directly into the ACSF after the recording of baseline fEPSP. The volumes of vehicle or text solutions added to the ACSF should not exceed 0.05% (v/v).
24. Perfusion with vehicle or test compound dissolved in ACSF is initiated at the completion of baseline period, and the effects on fEPSP slope are recorded for an additional 30–40 min minimally depending on the experimental design.
25. For each experimental condition, we typically record from 5 to 15 slices per experimental paradigm. The mean change in fEPSP slope (normalized to each slice's baseline period) is calculated. After a 10 min recording to establish a baseline period, the slice is perfused for 30 min (test period) with vehicle (DMSO, ≤0.025% v/v) or test compound dissolved in ACSF. After an additional 30 min of recording during the test period, LTP can be induced using the protocol described above.

3.5. Morphometric Analyses of Hippocampal Slice Cultures

1. Make up the PVP solution: Weigh out 20 mg PVP in a 1.5-mL sterile microfuge tube. Add 1 mL of 100% ethanol and mix until dissolved. Dilute the PVP to 50 μg/mL in 100% ethanol.
2. Weigh out 12.5 mg of gold microcarriers and add to 100 mL of 0.05 M spermidine. Sonicate (in a bath sonicator) for several seconds to resuspend the gold microcarriers.
3. Add 50 μg of total DNA (typically, we use 16.6 μg fluorescent marker, e.g., tomato fluorescent protein, and 33.3 μg of test plasmid or carrier DNA) and bath sonicate briefly to mix and resuspend the mixture.
4. While vortexing the DNA mixture at a moderate to slow rate, add 100 μL of 1 M $CaCl_2$ dropwise.
5. Allow the gold and DNA to precipitate at room temperature for 10 min. Mix gently by flicking the tube periodically during precipitation.

6. Spin for 15 s at maximal rpm in a microcentrifuge. Remove and discard the supernatant. Wash 3× in 1 ml 100% ethanol; spin for 5 s at maximal rpm between each wash.
7. Resuspend the DNA-coated gold microcarriers in 500 μL of PVP (50 μg/mL) and transfer to a 15-mL centrifuge tube. Add PVP to a final volume of 3 mL (see Note 15).
8. Place a 30-in. length of Gold-Coat tubing into the tubing prep station as described by the manufacturer. Turn on the N_2 gas and adjust the flow rate to 0.35 L/min and maintain for 30 min to completely dry the tubing. Remove the tubing and attach a syringe to one end using adaptor tubing.
9. Bath sonicate the DNA-coated gold microcarrier suspension for 5–10 s, invert the suspension several times, and then immediately place the free end of tubing into the suspension and pull the suspension into the tubing using the syringe. Remove the tubing from the 15-mL tube of DNA-coated gold microcarrier suspension, but continue to apply suction with the syringe to leave a 2-in. air gap on the free end.
10. Holding the syringe and attached tubing horizontally, insert the loaded tubing into the prep station until the end is 1 in. from the O-ring.
11. Allow the gold microcarriers to settle for 5 min.
12. Slowly and steadily suck out the fluid from the tubing without disturbing the gold microcarriers. This should take approximately 30 s.
13. Immediately insert the tubing into the O-ring and turn the tubing 180° using the switch on the tubing prep station, pause for 5 s, and then turn on the switch to rotate the tubing for 30 s. This is to ensure an even spread of gold in the tubing (see Note 16).
14. Open the valve on the flowmeter of the N_2 tank at 0.35 L/min for 5 min to dry the gold to the tubing.
15. Remove the tubing from the prep station and insert it into the tubing cutter to create cartridges (see Note 17).
16. Set up the gene gun in a clean bench or tissue culture hood, wiping it down thoroughly with 70% ethanol. Load cartridges into the chamber of the gene gun per the manufacturer's directions. Attach the gene gun to a tank of helium gas using the high-pressure hose supplied by the manufacturer. Adjust the helium pressure to 200 psi.
17. Remove the organotypic slice cultures from the incubator and place inside the sterile hood. Remove the lid of the tissue culture plate and set it aside.
18. Place a sterile Transwell® permeable support (3.0-μm polyester membrane 24 mm, Costar) over the barrel of the gun.

19. Place the end of the gene gun barrel with the transwell support about 1 cm above the slice cultures and fire the gun.
20. Replace the lid to the tissue culture plate and return cultures to the incubator (see Note 18).

4. Notes

1. Unless stated otherwise, all solutions should be prepared in water that has a resistivity of 18.2 MΩ-cm and total organic content of less than five parts per billion. This standard is referred to as "water" in the text.
2. Glucose concentrations between 1 and 10 mM optimize several electrophysiological properties of the in vitro hippocampal slice preparation, such as the amplitude of population spikes (14, 15). Concentrations in excess of 5-mM exert neuroprotection (16–18). Cerebrospinal fluid (CSF) contains numerous amino acids that are omitted from ACSF. Of these, glutamine, which is a major precursor of glutamate and GABA, is the most abundant in CSF (0.4–0.8 mM). However, systematic analysis of varying concentrations of glutamine added to slices perfused in ACSF containing 10 mM glucose indicated that the inclusion of glutamine in ACSF did not enhance the population spike amplitude and higher concentrations resulted in spreading depression (14).
3. The majority of electrophysiological studies performed on acute hippocampal slices use slices prepared from adult male rats or mice. Males are typically used because this limits the influence of variable hormonal status inherent in adult females. However, for questions that address how the alteration of hormonal status in vivo impacts hippocampal neuroplasticity ex vivo or to identify gender differences in electrical excitability when hormones are exogenously applied in vitro, hippocampal slices derived from male and female animals can be incorporated in the experimental design. For example, intact and ovariectomized mice have been used to address specific questions of how estrogen influences electrophysiological properties of the hippocampus (19). Similarly, the age of the animal used as the source of hippocampal slices can be an important aspect of the experimental design. For example, electrical recordings from hippocampal slices obtained from juvenile mice (3–4 weeks of age) have been used to probe how neuromodulators differentially affect plasticity at developing versus adult stages (20). Acute hippocampal slices have also been prepared from neonatal (PND 2–4) mice to study

the ionic and synaptic basis of periodic population discharges known as interictal bursts, as well as the pacemaker mechanisms underlying interictal rhythmicity (21).

4. Hippocampal slice cultures fare best when they are derived from early postnatal rats or mice (postnatal days 4–10). The typical experience has been that as the age of the donor increases beyond postnatal day 7, long-term culture success decreases; however, there are reports of healthy organotypic slice cultures being obtained from adult animals (22–24).
5. All steps in this procedure should be performed in a laminar flow tissue culture hood using sterile equipment and good aseptic technique.
6. Gold particles come in a variety of sizes, and the size needs to be optimized for each particular instrument and biological system under investigation.
7. It has been the empirical observation of our colleague, Dr. Gary Wayman (Department of Veterinary and Comparative Anatomy, Pharmacology and Physiology, Washington State University, Pullman), that cDNA constructs which are driven by CMV promoters in hippocampal neurons show dramatic upregulation following stimuli, such as increased synaptic activity (bicuculline or KCl stimulation) or neurotrophic stimulation (BDNF) as well as other stimuli that elevate intracellular Ca^{2+} in hippocampal neurons. This can greatly complicate the interpretation of experiments in which these stimuli are used. In his experience, the pCAGGS vector gives the most consistent results with very slight to no modulation of the transgene expression under any culture or stimulation condition. The pCAGGS vector consists of the CMV immediate early enhancer and chicken β-actin promoter (25). When expressed in hippocampal or cortical neurons, stimuli, such as bicuculline or BDNF, do not significantly affect the expression of cDNAs contained with the pCAGGS vector. This vector has the added benefit of restricted expression. Within a mixed culture of neurons and glia, this expression vector only detectably expresses cDNA in neurons and not glia. A potential disadvantage of this vector may be that in neurons, it may not express cDNA inserts to the same high level in the first 12–48 h after transfection as seen when using a CMV driving expression system. Tomato fluorescent protein is preferred over other more traditionally used green fluorescent proteins because of its brighter expression which facilitates the imaging of individual neurons by confocal microscopy.
8. It is imperative that the dissections should be completed quickly to minimize the length of time between euthanasia of

the animal and separation and exposure of cut tissue slices to culture medium. Prolonged delays result in deterioration of the tissue and reduced quality of the slices. A reasonable goal is to have cut slices separated in less than 5–10 min after the animal has been euthanized.

9. Keeping all dissection solutions cold and performing the entire procedure over ice help delay the deterioration of the tissue during dissection.
10. Depending on the tissue slicer available for your use and your specific application, slices ranging from 250 to 500 μm have been successfully used for electrical recordings. In some applications, the CA1 region can be separated from the CA3 area to minimize the spontaneous action potentials in the Schaffer collaterals in the CA1 area.
11. Slices can be stored in the prechamber (Fig. 1) for 4–6 h before their integrity is compromised resulting in unstable electrical recordings.
12. The interface chamber maintains the slice at the interface between the perfused ACSF fluid and the oxygenated atmosphere. With the interface configuration, oxygen is supplied to the slice by the warmed, humidified carbogen flowing immediately over the slice. By contrast, slices completely submerged in ACSF (submerged recording chamber configuration) are oxygenated by the perfused ACSF (26). Field potential recordings have been widely published using both slice configurations to study pharmacological responses and changes in neuroplasticity. However, some notable differences were revealed in systematic comparisons of how anoxia alters ion transport when hippocampal slices are maintained at a gas–liquid interface versus being completely immersed. Some of the factors underlying this difference include heterogeneous rates of glucose diffusion into the tissue slices and washout of accumulating substances, such as extracellular K^+ during perfusion (14, 26). It is important to note that the responses typically measured with hippocampal slices maintained in either interface or submerged chambers can be differentially influenced by O_2 deprivation and may be of pathophysiologic interest. For example, hippocampal slices maintained in the interface chamber were shown to recover their responses to othodromically evoked stimuli after they were subjected to anoxia, whereas submerged slices did not (26).
13. If the tissue is too wet, hippocampi tend to move each time the blade is lifted; however, if the tissue is too dry, hippocampi tend to stick to the blade.

14. Healthy hippocampal slices should have smooth edges and their surface should not appear sandy or grainy. The neuronal cell body layers should remain tight and transparent. If cultures are overfed and become flooded, they will become opaque. After 1 week in vitro, cultures initially cut at 400 μm normally thin down to about 150–200-μm thick. Underfed cultures thin out much sooner and become nearly invisible.
15. This solution can be stored at –20°C for up to 2 months if capped with Parafilm wrapped around the cap. Let it warm to room temperature before removing the Parafilm and opening the cap.
16. The gold microcarriers must be evenly distributed within the tubing for efficient transfection. Uneven labeling of the gold suspension inside the tube may be due to the PVP solution, which should be replaced every 6–8 weeks.
17. Cartridges can be kept for up to 1 year at 4°C if stored in a tightly capped vial wrapped with Parafilm.
18. The time and level of expression vary depending on the expression vector used, as discussed above in Note 7. With the pCAGGS vector, we typically see the expression of transfected proteins within 12 h with maximal expression at about 48 h. In a typical experiment, we obtain 20–100 transfected neurons per slice.

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Chapter 9

Glia-Neuron Sandwich Cocultures: An In Vitro Approach to Evaluate Cell-to-Cell Communication in Neuroinflammation and Neurotoxicity

Mariaserena Boraso and Barbara Viviani

Abstract

Glia-neuron sandwich cocultures are "in vitro" cell systems suitable to evaluate cell-to-cell interactions relaying on the release of soluble factors, such as proinflammatory cytokines. This chapter presents a protocol to obtain a sandwich coculture from primary rat glial cells and hippocampal neurons. Furthermore, it provides details to evaluate the release of tumor necrosis factor-α by a biological assay and neuronal cell survival, as well as examples of practical application in the investigation of neuroinflammation and its impact on neuronal function.

Key words: Primary hippocampal neurons, Primary glial cells, Sandwich coculture, TNF-α, Biological assay, MTT-test

1. Introduction

The concepts about the development and progression of neurodegenerative diseases and neurotoxic insults have been completely revised, mainly because of the recognition that most neurological disorders are the consequence of a complex relationship between glia and neurons. Following an insult to the CNS, glia becomes activated and releases new molecules not normally detectable in quiescent cells that can affect neuronal functions (1, 2). As well, damaged neurons may induce glial response through the release of neurospecific proteins (3–5). This interplay may result in a vicious cycle of damage progression.

Cytokines are among the molecules that have been implicated both in the modulation of glial activation and neuronal dysfunction and death. In the CNS, a large number of cytokines and

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cytokine receptors are expressed in astrocytes, microglia, neurons, and oligodendrocytes, either constitutively or by induction following brain damage (6). Initially, attention has been mainly devoted to the ability of cytokines in activating astrocytes and microglia (the "immune cells" resident within the CNS). Presently, it has been realized that these molecules may also exert a direct impact on neuronal function, both in health and disease (7). Thus, cytokines are now regarded as novel neuromodulators, providing new perspective in the current view of brain behavior and new frontiers in therapeutical intervention.

Cytokines recruit different responses in glial cells and neurons through cell type-specific mediated signaling (6, 8). Sandwich cocultures of primary neuron and glia are particularly feasible to study such a cross talk, discriminating the cell type-specific molecular mechanisms involved. A sandwich coculture is an in vitro cell system formed by two different cell populations growing on different surfaces, usually a coverslip and a Petri dish (Fig. 1). These surfaces are separated by small paraffin dots at the edges of the coverslip, on which one of the cell populations is seeded. In this way, the two cell populations face each other without touching. However, soluble substances can diffuse between them. The great advantage of this cell system is the possibility of separating the two cell populations at any time (e.g., prior to or after a treatment) while retaining their integrity and organization. This allows the investigator to (1) manipulate the cell types differently before

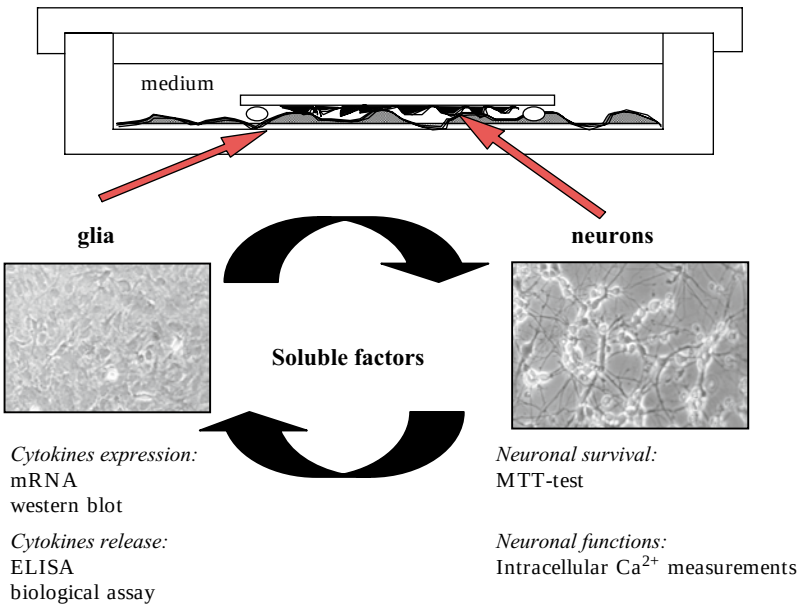

Fig. 1. *Glia-neuron sandwich coculture.* Glial cells are seeded at the bottom of Petri dishes while neurons on the top of glass coverslips. Coverslips are inverted so that neurons face the glia monolayer. Paraffin dots create a narrow gap to separate neurons from glial cells. The two cell populations can be exposed together to tested compounds dissolved in the common culture medium and then separated at the end of the treatment to perform different evaluation (examples are described under the pictures).

they are treated together, thus providing information on the involvement of specific mediators or biochemical pathways (9), (2) perform different biochemical measurement on the two cell populations separately at the end of treatment (e.g., quantification of cytokines), and (3) evaluate the viability and activity of highly differentiated neurons in the presence or absence of glial layer (e.g., MTT viability assay) (10, 11) (Fig. 1).

In this chapter, we describe the methodological approach to sandwich cocultures providing some examples of practical application in the investigation of neuroinflammation (i.e., the method for TNF-α measurement) and its impact on neuronal function (i.e., methods for cell death evaluation).

2. Materials

2.1. Cell Cultures

1. Dulbecco's Phosphate-Buffered saline (PBS) without calcium chloride and magnesium chloride, sterile (Sigma).
2. Ethanol 95%, needed to keep dissecting tools sterile while in use.
3. Hanks' Balanced Salts Solution (HBSS) (pH 7.4): Dissolve Hanks' Balanced Salts powder (Sigma without $CaCl_2$, $MgSO_4$, $NaHCO_3$ – use at 9.5 g/L) in 850 mL of distilled water, add 10 mL of 10 mM HEPES (pH 7.3), 10 mL of 10,000 U/mL penicillin, and 10 mg/mL streptomycin. Adjust the volume to 1 L with distilled water. Sterilize in a vacuum-driven disposable filtration system (0.22-μm pore size, cellulose acetate). Store up to 1 month at 4°C.
4. 10 mg/mL Deoxyribonuclease I (DNase I) solution: Dissolve 100 mg of 536 Kunitz units/mg DNase I in 10 mL of HBSS. Sterilize with disposable syringe filter (0.22-μm pore size). Store up to 6 months in 1.5-mL aliquots at –20°C.
5. Dissecting tools, sterile: Stainless steel scissors with ~4- and ~2-cm blades, curved forceps (2 pairs), Dumont forceps, no. 3c and no. 5, and bistouries.
6. Dissecting microscope (e.g., Zeiss Stemi DV 4).
7. Inverted light microscope.
8. Laminar flow hood equipped with UV lamp to prepare cell cultures.
9. 37°C, 95% air/5% CO_2, and 95% relative humidity incubator to grow cell cultures.
10. 100-, 60-, and 35-mm Petri dishes, plates, and flasks to be used during dissection and to plate and grow cell cultures.
11. Equipment for the determination of cell number and viability: Cell counter, hemacytometer or Burker chamber, squared

glass coverslips (22×22), and 0.04% (w/v) Trypan blue solution prepared in 0.81% sodium chloride and 0.06% potassium phosphate, dibasic (Sigma) (see Note 1).

12. Pipettes and pipette tips (P20, P200, P1000).

2.2. Primary Hippocampal Cultures

1. Pregnant female rats (Sprague-Dawley) at gestational day 18 (E18).
2. Poly-L-ornithine hydrobromide: Prepare a stock solution by dissolving 10 mg of poly-L-ornithine in 6.67 mL of distilled water. Sterilize with disposable syringe filter (0.22-μm pore size). Store up to 6 months in 1-mL aliquots at –20°C. Dilute 1:100 with sterile-distilled water just before use.
3. Trypsin (1×) solution: 0.5 g/L porcine trypsin in HBSS.
4. Ethylenediamine tetraacetic acid (EDTA) solution: 0.2 g/L EDTA in HBSS.
5. Neurobasal complete medium: To 500-mL Neurobasal (Invitrogen), add 5 mL of 10,000 U/mL penicillin and 10 mg/mL streptomycin, 1.25 mL of 200 mM L-glutamine, and 1% B-27 (Invitrogen). Sterilize in a vacuum-driven disposable filtration system (0.22-μm pore size, cellulose acetate). Store up to 1 month at 4°C.
6. 2 mM Cytosine-1-β-*D*-arabino-furanoside (CyARA) stock solution in distilled water. Store up to 1 year, protected from light, at –20°C.
7. Paraffin wax is melted in a baker using a heating plate (150°C) just before use.
8. 5–10-mL sterile syringe with 0.95×40-mm needle can be used more than once.
9. Sterile 12-mm glass coverslips.
10. Sterile 1.5-mL eppendorf tubes.
11. Sterile 1-mL and 200-μL pipette tips.
12. Tissue culture plates or dishes.
13. Heating magnetic stirrer.
14. Microwave oven, a useful and fast approach to sterilize glass coverslip or, if needed, tips.

2.3. Primary Glial Cultures

1. Rat pups 1- to 2-days-old (Sprague-Dawley) (see Note 2).
2. Trypsin (10×) solution cell culture tested.
3. Minimal Essential Medium (MEM) complete medium: Add to 500 mL of MEM Earle's salts (MEM, Sigma) 3 g of D(+)-glucose, 5 mL of 10,000 U/mL penicillin, 10 mg/mL streptomycin, and 5 mL of 200 mM L-glutamine. Shake vigorously to dissolve glucose, and then add 20% or 10% fetal bovine

serum (FBS). To avoid excessive foam formation, do not add FBS before shaking. Sterilize in a vacuum-driven disposable filtration system (0.22-μm pore size, cellulose acetate). Store for up to 1 month at 4°C.

4. 100-μm nylon cell strainer (Falcon).
5. 50-mL conical polystyrene centrifuge tubes.
6. Shaking water bath.

2.4. Sandwich Cocultures

1. Confluent cortical glial monolayer in 24-well plates.
2. Culture of hippocampal neurons on coated glass coverslips.
3. Neurobasal complete medium for primary hippocampal cells.
4. 2 mM Cytosine arabinoside solution.
5. Sterile sharpened forceps.

2.5. Biological Assay for TNF-α

1. L-929 cells, adherent murine fibroblast line (available from American Type Culture Collection) (see Note 3).
2. Roswell Park Memorial Institute (RPMI)-1640 complete medium: Add to 500 mL of RPMI-1640 medium (Sigma), 5 mL of 10,000 U/mL penicillin, 10 mg/mL streptomycin, 5 mL of 200 mM L-glutamine, and 10% FBS. Store up to 1 month at 4°C.
3. Dulbecco's PBS.
4. Trypsin (1×) solution.
5. EDTA solution.
6. 10 mg/mL Actinomycin D solution: Dissolve 10 mg of Actnomycin D in 1 mL of Dimethyl sulfoxide. Store up to 6 months in the dark at 4°C. Actinomycin D increases sensitivity of L929 for TNF-α.
7. 1,000 U/mL Recombinant Mouse TNF-α: Dilute 5 μL of 400,000 U/mL (10 μg/mL) Recombinant Mouse TNF-α in 2 mL of cultured medium, store up to 6 months in single-use aliquots at −80°C. Use recombinant Mouse TNF-α for standard curve.
8. 0.2% Crystal Violet solution: Dissolve 2 g of Crystal Violet in 980 mL of water and add 20 mL of ethanol. Filter using a 0.22-μm cellulose-acetate disposable vacuum filtration system and store for up to 6 months at room temperature. This solution is used to fix and stain L929 monolayer at the end of the treatment.
9. 1% Sodium dodecyl sulfate (SDS) solution: Dissolve 10 g of SDS in 1 L of distilled water. Store up to 6 months at room temperature.

10. 15- and 50-mL conical polystyrene centrifuge tubes.
11. Microtiter plates 96-well flat bottomed.
12. 5–50 µL and 50–200 µL of multichannel pipettes.
13. Multiscan reader (e.g., Molecular Devices, Emax precision microplate reader).

2.6. Viability Assay: MTT-Test

1. 7.5 mg/mL MTT solution: Prepare MTT (3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide) solution as a 7.5 mg/mL stock in PBS. Before use, filter through a 0.22-µm cellulose-acetate disposable syringe filter to remove any crystal of formazan. Store for no more than 2 days in the dark at 4°C.
2. 1 N HCl/isopropanol (1:24).
3. Microtiter plates 96-well flat bottomed.
4. Pipettes and micropipet tips.
5. Multiscan reader (e.g., Molecular Devices, Emax precision microplate reader).

3. Methods

3.1. Primary Glial Cultures

1. Arrange all the sterile-dissecting tools on a sterile surface (e.g., sterile lid of a Petri dish).
2. Anesthetize and sacrifice the animal by an officially approved procedure.
3. Place one head in a 35-mm Petri dish with 2 mL of HBSS. Secure the head by holding down the snout with a curved forceps and cut the skin and skull along the midline from the base of the skull to the snout with sharpened scissors. To ease the removal of the brain, cut the left and right side of the skull in the center perpendicularly to the midline.

   Place the head in the Petri dish with neck down and skull-cap toward the operator. Place the anterior end of the head toward the topside of a Petri dish and posterior end toward the bottom. This disposition greatly facilitates the dissection.

   To avoid damaging the underlying brain tissue, place one scissors tip just under the skull bone and lift upward with the scissor blade while cutting.
4. Still keeping the head secured, remove the skull with a curved-tip forceps and expose the brain. Sever the olfactory bulbs at the anterior end of the brain and the spinal cord at the posterior end with Dumont forceps.
5. Remove the brain with a curved-tip forceps and collect it in a 60-mm Petri dish with 5 mL of HBSS. Keep on ice. Place the

curved tips of the forceps under the brain and gently lift it upward.

6. Repeat for each head.
7. To separate the cortices from the rest of the brain, move one brain to a 35-mm Petri dish with 2 mL of HBSS. Working under the dissecting microscope, place the two tips of Dumont no. 5 forceps along the brain midline under the cortices, one at the anterior and the other at the posterior end. Use a single cut to isolate the cortices from the brain stem.
8. Repeat this step for each brain and store all the cortices on ice in a 60-mm Petri dish with 5 mL of HBSS.
9. Place one cortex in a 35-mm Petri dish (2 mL of HBSS) and, still working under the dissecting microscope, gently remove any extraneous tissue as well as the meningeal coverings on the cortical surface by pulling them off gently with Dumont forceps. Take care not to destroy the tissue by pressing too hard with the forceps (see Note 4).
10. Repeat this step for each cortex.
11. Place all the cortices obtained in a 35-mm Petri dish on ice without any medium and chop up with a bistouries. Accumulate cortices all together in the center of the dish, and to obtain a homogenate, cut the tissues several times in different directions.
12. Add to the dish 2 mL of HBSS and resuspend the minced cortices with a 2-mL disposable plastic pipette (sterile).
13. Remove the suspension and transfer to a 50-mL centrifuge tube.
14. Repeat the washing of the Petri dish with 2 mL of HBSS till all minced cortices are removed.

    Usually, three washing for a total of 6 mL of HBSS are enough.
15. Add 750 µL of 10× trypsin and 750 µL of 10 mg/mL DNase to the suspension (6 mL of HBSS).
16. Seal the tube cap with parafilm. Vigorously agitate in a water bath at 37°C for 15 min to favor enzymatic digestion of the tissue.
17. Prepare another 50-mL centrifuge tube containing 12 mL of MEM complete medium 10% FBS.
18. After agitation, remove the tube from the shaking water bath and allow the undissociated tissue to collect at its bottom.
19. Collect 5-mL dissociated cells with a disposable sterile plastic pipette and transfer to the 50-mL centrifuge tube containing MEM complete medium 10% FBS. Take care not to include undissociated tissue pieces.

20. Add other 6 mL of HBSS, 750 µL of 10× trypsin, and 750 µL of 10 mg/mL DNase to the 50-mL tube containing the remaining undissociated tissues.
21. Agitate again as in 18 and add these dissociated cells to 50-mL tube containing the cells from the first digestion.
22. Filter dissociated cells through a 100-µm nylon cell strainer and collect them in a fresh 50-mL centrifuge tube.
23. Centrifuge the pooled cells for 5 min at 200–300 × *g* at room temperature, in a swinging bucket rotor to pellet the cells. Carefully remove the medium from the pelleted cells.
24. Resuspend the pellet in ~5 mL of MEM complete medium 10% FBS. To disaggregate the pellet, first add 2 mL of MEM 10% FBS, then gently pipette with a 2-mL disposable plastic pipette until the solution becomes homogeneous, and add an additional 3 mL of MEM 10% FBS.
25. Gently mix 100 µL of cell suspension to 200 µL of 0.04% (w/v) trypan blue solution. Determine the total cell number and cell viability with a hemacytometer and an inverted phase-contrast microscope (see Note 5).
26. Prepare the desired number of flasks or plates. To obtain (a) 24-well plates (i.e., for sandwich cocultures), seed 50,000 cells/1 mL of medium/well; (b) 6-well plates, seed 140,000 cells/2 mL of medium/well; and (c) 75 cm^2 flasks, seed 5×10^6 cells/10 mL. Thus, dilute the cell suspension to obtain the desired number of cells/mL with MEM 20% FBS and then pipette the proper volume of cell suspension into each well, dish, or flask.
27. Place the plates/flasks in a 37°C, 5% CO_2 95% relative-humidity incubator.
28. 24 h after plating, to eliminate unattached cells, remove the medium, and add fresh MEM supplemented with 20% FBS for each well (see Note 6).
29. Five days later, remove the MEM complete medium supplemented with 20% FBS, replace the medium with fresh MEM 10% FBS, and continue to grow to confluence, changing the medium twice a week. Confluence will be reached in ~10 days.

3.2. Primary Hippocampal Cultures

1. Arrange all the sterile-dissecting tools on a sterile surface (e.g., sterile the lid of a Petri dish).
2. Fill the Petri dishes required for the preparation with appropriate amount of HBSS (10 mL for 100-mm dishes, no. needed 2; 5 mL for 60-mm dishes no. needed 1; and 2 mL for 35-mm dishes, no. needed 6).
3. Set up the dissection microscope.

4. Sacrifice the animal by an officially approved procedure after anesthesia.
5. Set the anesthetized animal on a dissecting table, ventral side up, and sterilize the abdomen by pouring 95% ethanol over it.
6. Rinse forceps and scissors in ethanol, grasp the abdominal skin with forceps and cut the abdomen completely open from the vagina to the thoracic cavity. Cut the diaphragm.
7. Gently grasp uterine horns at one of the constrictions and lift up. Remove the horns by cutting the attachments to the abdominal cavity and place them in a 100-mm Petri dish filled with 10 mL of cold HBSS. Keep on ice (see Note 7).
8. Remove the fetuses from the uterine horns and place them in a 100-mm Petri dish with cold HBSS. Keep on ice. To remove the fetuses, grasp at the upper constriction of each yolk sac and cut along one side; the fetuses will slip out of it.
9. Decapitate the fetuses with scissors and place the heads in a 60-mm Petri dish with cold HBSS. Keep on ice.
10. Place one head in a 35-mm Petri dish filled with 2 mL of HBSS, with the neck down and the skullcap toward the operator. Under the dissection microscope, gently grasp the cut edges of the skin and skull with both the Dumont forceps and pull in opposite directions to expose the brain.
11. Place the anterior end of the head toward the topside of the Petri dish and posterior end toward the bottom. This disposition greatly facilitates the dissection.
12. Sever the cerebral hemispheres from the cerebellum and the spinal cord with Dumont forceps.
13. Remove the brain by lifting upward out of the skull with curved-tip forceps and place it in a 60-mm Petri dish with 5 mL of HBSS. Keep on ice. Place the curved tips of the forceps under the brain and gently lift it upward.
14. Repeat for each head.
15. Place the brain in a 35-mm Petri dish filled with 2 mL of HBSS, with the dorsal side up and the posterior end toward the operator. Working under the dissecting microscope, place the two tips of Dumont forceps along the brain midline under the cortices, one at the anterior and the other at the posterior end. Use a single cut to isolate the cortices from the brain stem. Repeat for the other hemisphere.
16. Discard the brainstem and orient one of the hemispheres with the medial surface upward (see Note 8).
17. Gently remove the meninges by pulling them off with Dumont forceps.

18. To obtain the hippocampus, make a curving incision with Dumont forceps along the dorsal hippocampal fissure. Release the hippocampus cutting at the end of the curved incision.
19. Transfer the hippocampus to a 35-mm Petri dish with 1 mL of HBSS and keep on ice.
20. Repeat for each brain.
21. Collect the hippocampi isolated from rat embryos in a sterile 1.5-mL microcentrifuge tube.
22. Centrifuge 4 min at 100–150 × *g* room temperature. Carefully remove the supernatant with a pipette. To avoid aspirating the hippocampi as well, do not use a vacuum system.
23. Add 400 µL of 1× trypsin/EDTA and 80 µL of 10 mg/mL DNase I. Shake gently (do not vortex) and place in a 37°C incubator for 5 min (see Note 9).
24. Prepare three 1.5-mL eppendorf tubes with 400 µL of MEM plus 10% FBS each, and one with neurobasal medium.
25. Gently aspirate off the trypsin/DNAse solution with a sterile 1-mL pipette (avoid vacuum), add 400 µL of MEM complete medium 10% FBS, and gently shake until the hippocampi are floating in the medium (see Note 10).
26. Allow the hippocampi to pellet at the bottom of the Eppendorf tube.
27. Gently collect the pelleted hippocampi with a 1-mL pipette in the smallest possible volume of medium and transfer to the first of the four tubes with 400 µL of MEM complete medium 10% FBS.
28. Gently shake and allow the hippocampi to pellet at the bottom of the tube.
29. Repeat the steps 25 and 26 another three times in the remaining tubes in succession; these repetitive passage through tubes with fresh medium wash the hippocampi free from trypsin and DNAse I.
30. In the last tube, the one containing neurobasal medium, dissociate the hippocampi by gently pipetting (with sterile tips) through a 1-mL pipette first and then through a 200-µL pipette which gradually yields a single cell suspension. Adjust the volume of the cell suspension to 1 mL with neurobasal medium.
31. Gently mix 10 µL of cell suspension with 10 µL of 0.04% trypan blue solution. Determine the total cell number and cell viability with a hemocytometer and an inverted phase-contrast microscope.
32. Prepare the desired number of plates. To obtain (a) 24-well plates, seed 50,000 cells/1 mL of medium/well and (b) 6-well plates, seed 250,000 cells/2 mL of medium/well.

Thus, dilute the cell suspension to obtain the desired number of cells/mL with neurobasal medium and then pipette the proper volume of cell suspension into each well or dish. If you desire to prepare a sandwich coculture, place before seeding the cells a glass coverslip properly treated in each well of a 24-well plate (see the Subheading 3.3).

33. To grow the primary hippocampal medium, change the medium once a week by replacing only one-third of the total medium (see Note 11).

3.3. Glass Coverslips for Sandwich Coculture

1. Place one 12-mm glass coverslip in each well of a 24-well tissue culture plate. Prepare the exact number of coverslips to be used.
2. Sterilize the coverslips by microwaving 10 min at the highest power setting. Set on max heat for 10 min in the presence of a beaker full of water to avoid plastic distortion.
3. Heat paraffin wax to ~100°C. Take an aliquot (~2 mL) in a 5- or 10-mL syringe and apply three small drops near the outer edge of each coverslip at roughly equal distances from each other (see Note 12).
4. Resterilize the coverslips by UV irradiation for 30 min with a germicide lamp (see Note 13).
5. In a laminar flow hood, add 1 mL of 1× poly-L-ornithine solution in each well containing the coverslips (see Note 14). Check that the coverslips do not float in the well but are completely covered by polyornithine. To prevent floating, be sure to eliminate any air bubble under the coverslip by gently pressing over it. Incubate the plates 2 days at room temperature *or* for 2 h at 37°C. When the plates are out of the laminar flow hood, store them sealed with parafilm in the dark.
6. Immediately before isolating the cells, remove the polyornithine and rinse twice with PBS.
7. After the final rinsing, add 1 mL of neurobasal medium and incubate at 37°C, 5% CO_2, to equilibrate before cell seeding.

3.4. Sandwich Cocultures

1. On the day hippocampal neurons are to be prepared, replace the medium in confluent cortical glial monolayer with 1 mL of neurobasal complete medium and return them to the incubator overnight (see Note 15).
2. Prepare the primary hippocampal neurons and seed on glass coverslips (see the Subheading 3.2 and 3.3).
3. The day after, use one tip of a pair of sharpened forceps to lift the edge of a coated glass coverslips plated with neurons. Seize the coverslip with the forceps and transfer and turn it over glial monolayer. Neurons must face glial monolayer. Repeat for each well to be used (see Note 16).

4. Add to each well containing neurons and glia 1 μM CytARA to reduce the proliferation of glial cells (see Note 17).
5. Maintain the cocultures in a humidified 5% CO_2 incubator at 37°C.
6. Routinely feed cultures once a week by replacing one-third of the medium with fresh neurobasal.

3.5. Biological Assay for TNF-α (See Note 18)

1. L-929 cells are grown until confluence in RPMI medium in 75-cm^2 flasks. At confluence, you will obtain roughly 15×10^6 cells/flask. Replace culture medium three times a week.
2. The day prior to assay, samples for TNF-α. L-929 are harvested from confluent cultures. Thus, remove the culture medium from L-929 cell flasks and wash with 5 mL of PBS.
3. Add 2 mL of 1× trypsin/EDTA solution per 75-cm^2 flask and incubate no longer than 5 min at 37°C.
4. Collect trypsinized cells with a disposable sterile plastic pipette and transfer to a centrifuge tube containing 2 mL of RPMI-1640 complete medium 10% FBS. Tryspinized cells from different flasks can be collected in the same tube; in this case, add an equal amount of RPMI-1640 medium plus 10% FBS to deactivate trypsin.
5. Centrifuge for 5 min, 200–300 × *g* at room temperature, in a swinging bucket rotor and then carefully discard supernatant.
6. To dissociate the pellet, add 2 mL of RPMI-1640 10% FBS and then gently pipette until the solution becomes homogeneous; add an additional 4 mL of RPMI 10% FBS and resuspend.
7. Gently mix 100 μL of cell suspension to 200 μL of 0.04% (w/v) trypan blue solution. Determine the total cell number and cell viability with a hemocytometer and an inverted phase-contrast microscope.
8. Adjust the cell number to 250,000 cells/mL and plate 100 μL into each well of flat-bottomed 96-well microtiter plate (25,000 cells/well) (see Note 19).
9. Place microtiter plates back into a CO_2 incubator at 37°C overnight to allow L-929 to attach.
10. The day after, dilute the stock solution of actinomycin D (10 mg/mL) to 6 μg/mL in L-929 tissue culture medium. Prepare 8 mL for each 96-well microtiter plate.
11. Vigorously tap the 96-well microtiter plate in the sink to eliminate the medium in each well. Replace with 100 μL of 6 μg/mL actinomycin D by using a multichannel pipette (see Note 20).

12. Incubate plates at 37°C for at least 1 h.
13. In the meanwhile, prepare the standard curve. Make serial dilutions of recombinant mouse TNF-α (1,000 U/mL) in RPMI 10% FBS, from 2 U/mL (25 pg/mL) to 0.04 U/mL (0.5 pg/mL), at least 1 mL final volume for each point of the curve. Prepare a standard curve for each 96-well plate.
14. Add 100 µL of sample to the actinomycin D treated L-929 cells, establishing a final volume of 200 µL in each well, in duplicate along the rows B and C from column 2 to 10 so that sample no. 1 will be added in B2 and its duplicate in C2, sample no. 2 will be added in B3 and its duplicate in C3, and so on. Keep the last two columns of the 96-well plate (11–12) free from sample; these are dedicated to the standard curve (Fig. 2).
15. Make serial dilutions of the samples. Add 25 µL of sample to the actinomycin D treated L-929 cells placed along the lanes D and E, establishing a final volume of 125 µL in each well (Fig. 2).
16. With a multichannel pipette, mix the medium along the lane D (D2 to D10) and then transfer 25 µL from lane D to lane F (F2 to F10) (Fig. 2).
17. Mix the medium along the lane F and discharge 25 µL (Fig. 2).
18. Repeat the steps 15 and 16 for lane E to lane G.
19. Add 100 µL of standard to the actinomycin D-treated L-929 cells in the two end columns (11 and 12, from B to G). Make a duplicate for each point of the curve.

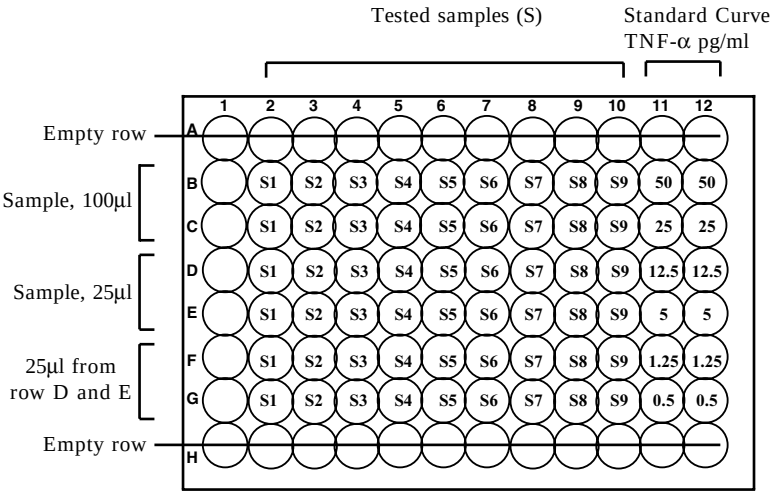

Fig. 2. *96-well plate setting for TNF-α measurement.* Distribution of samples to be tested (S) and standard curve points with suggested concentrations (pg/ml). For details see the text.

20. Add 100 µL of RPMI 10% FBS medium in each well included between the row D-G and column 2–10, establishing a final volume of 200 µL in each well. You will thus obtain a 1:10 dilution of the samples in the row D-E and 1:50 dilution in row F-G.
21. Place the plates in a 37°C incubator with 5% CO_2 for 18–24 h.
22. At the end of incubation, discard the medium by vigorously tapping the plates over the sink. Stain the residue cells by adding to each well 100 µL of 0.2% crystal violet solution in 2% ethanol. Wait for 10 min at room temperature.
23. To remove staining, vigorously tap the plates over the sink and abundantly wash with water. Let the plates dry and then dissolve the stained cells into 100 µL volumes of 1% SDS.
24. Shake the plates for 1 h and then read the absorbance of each well using a plate reader at 595 nm.
25. Calculate TNF-α concentrations for each sample against the standard curve.

3.6. Viability Assay: MTT-Test (See Note 21)

1. Dilute MTT 7.5 mg/mL solution in the culture serum-free medium to a final concentration of 0.75 mg/mL (see Note 22).
2. Remove the medium from treated cells and add 500 µL of MTT 0.75 mg/mL for each 24 well.
3. Incubate the cells in a humidified 5% CO_2 incubator at 37°C for 3 h.
4. At the end of incubation, remove the medium and add 100 µL of HCl (1 N)/isopropanol (1:24) to each well to dissolve the dark blue crystals.
5. Transfer 80 µL of the obtained solution in flat-bottomed 96-well microtiter plate.
6. Read the absorbance of each well using plate reader at 595 nm. Cell viability is expressed as % of the control.

4. Notes

1. Trypan blue is used to distinguish viable from dead cells. Viable cells exclude trypan blue while dead or damaged cells are stained dark blue.
2. Mixed glial cells are obtained by mechanical and enzymatic dissociation of cerebral tissues from 1- to 2-day-old rat pups. The use of such young rats ensures the absence of viable neurons in the cell suspension.

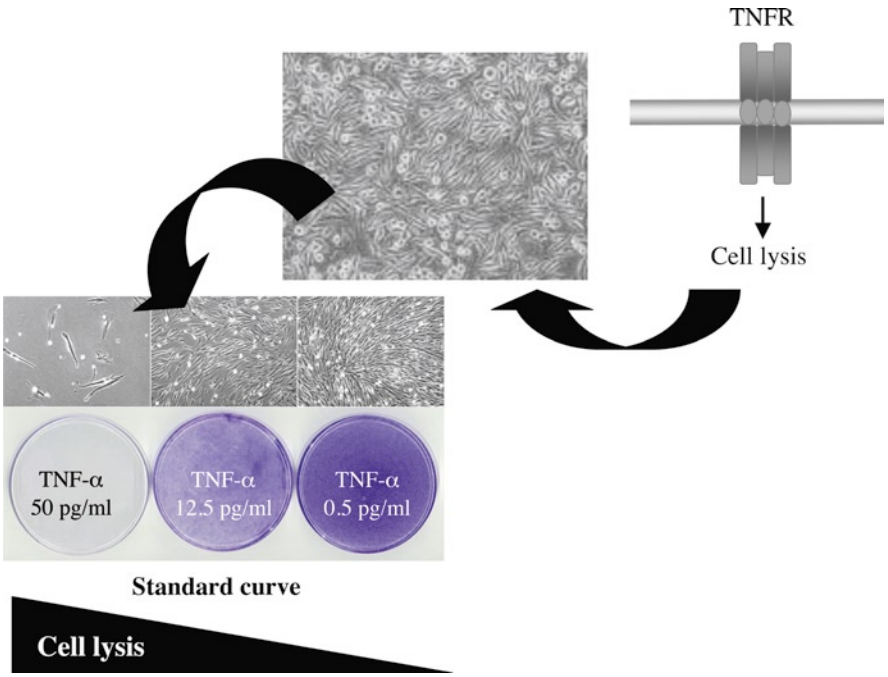

Fig. 3. *Principle of TNF-α biological assay.* Murine fibroblasts L-929 are particularly sensitive to TNF-α, which, through the specific receptor TNFR, induce their lysis. L-929 cell lysis is proportional to the amount of TNF-α present in the samples to be tested. Cell death is revealed at the end of the procedure by eliminating culture medium and then staining the residue cells alive with crystal violet. A standard curve allows calculating the concentrations of TNF-α (pg/ml) present in the samples.

3. These cells are very sensitive to the killing action of TNF-α and adhere to microtiter plates, which provide an excellent target for the cytolytic bioassay described below (Fig. 3). Another cell line with similar characteristics is WEHI 164 cells, also available from American Type Culture Collection. Since TNF-α bioassay relies on the ability of this cytokine to produce cell death in certain target cell lines, one potential source of error is the presence of factors that are toxic to L-929 in the samples to be tested (i.e., the substance used to treat glial cells, still present in the medium together with the released TNF-α). The toxicity of such substances on L-929 cells has to be determined and excluded before performing TNF-α biological assay. In case of toxicity due to the tested substances, perform an ELISA test.
4. A semitransparent tissue, the meninges, surrounds the surface of the cortex. They are recognizable for their slightly pink color due to the presence of blood vessels. When the meninges are completely removed, the surface of the cortex appears completely white.
5. From one 2-day-old rat pup, the authors usually obtain ~5×10^6 cells.

6. Remember, the addition of 20% FBS during the first 5 days of culture is mandatory to obtain a proper growth and confluence within 10 days.
7. Keeping the embryos, brains, and later the hippocampi cold reduces the metabolic activity and associated cellular damage.
8. The cerebral hemisphere is defined by a dorsal side much more rounded than the ventral side and usually by the olfactory bulb that projects anteriorly. The hippocampus is located in the medial posterior part of the cerebral cortex on the back of the olfactory bulb. When the medial cortex is perfectly clean, the hippocampus is clearly visible as a C-shaped structure, whose dorsal margin is separated from the adjoining cortex by a fissure while the ventral margin is free.
9. DNase is added to prevent the DNA released by damaged cells from making the dissociation medium too viscous during digestion.
10. The serum in the MEM inhibits residual trypsin and prevents overdigestion of the cells.
11. During the feeding, it is important not to change the culture medium completely, since neurons depend on conditioning of the medium for long survival. Replace about one-third of the medium each time. Under such conditions, neurons survive for several weeks and become richly innervated. These cells reach a high degree of maturation (developed neuronal network, functional glutamatergic system, and the development of postsynaptic density almost complete) after about 7–9 days of culture.
12. The temperature of the paraffin is very important; if it is too hot, it spreads too thin and wide while if it is too cool, the dots do not adhere to the coverslip and will subsequently detach.
13. The germicide lamp of a flow hood is sufficient. Avoid exposing the coverslips to UV after they have been coated with polyornithine.
14. Hippocampal neurons do not readily adhere to glass. Thus, to promote their attachment, maturation, and survival, coverslips are coated using poly-L-ornithine. In addition, three dots of paraffin are placed on each coverslip to create a narrow gap between the two cultivated cell populations. The coverslips are prepared at least a day before the hippocampal neurons.
15. The addition of neurobasal to glia prior to adding glass coverslips with hippocampal neurons allows conditioning of the medium to favor early phases of neural maturation.
16. Neurons can be cocultured with glia even later than the day after preparation. In this case, glial medium must be replaced with the neurobasal medium in which neurons have been grown and differentiated (conditioned neurobasal).

17. Addition of cytosine arabinoside, toxic to dividing cells, is important to block astrocytes proliferation in the neuronal culture. Addition of cytosine arabinoside immediately after neuronal cells are attached allows us to obtain a 98% pure neuronal culture on the glass coverslip, as assessed by immunocytochemistry of microtubule-associated protein 2 (a marker for neurons) and glial fibrillary acidic protein (a marker for astrocytes). Under these experimental conditions, cytosine arabinoside is not toxic to neurons.
18. The methodology employed to quantitate TNF-α in biofluids has remained fairly stable. To date, two techniques are generally employed, bioassay and immunoassay (e.g., ELISA). Immunoassay will measure the total immunoreactive TNF-α, which may not necessarily be bioactive. The molecule may have become partially denatured and hence inactive, but the key epitope recognized in the immunoassay might remain intact. Immunoassay would then result in an overestimate of the amount of bioactive TNF-α present. Bioassay detects only the cytokines that have been processed to be biologically active.
19. 2×10^6 cells/8 mL are needed to seed one 96-well plate. Do not plate L-929 cells in rows A and H (Fig. 2).
20. Actinomyin D greatly increases the sensitivity of L-929 cells to the lytic action of TNF-α. Actinomyin D is toxic; pay attention and use gloves when manipulating the substance.
21. The cleavage of the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide) into a blue-colored product (formazan) by the mitochondrial reductase enzyme is potentially useful for assaying cell survival and proliferation. The conversion takes place only in living cells, and the amount of formazan produced is proportional to the number of alive cells. Another method to assess cell viability is the release of the cytoplasmic enzyme, lactate dehydrogenase (LDH), in the culture medium. This method has proved useless for primary hippocampal neurons probably for the low amount of this enzyme within these types of cells.
22. High protein levels (serum, albumine, etc.) in the cultured medium may form a precipitate when MTT solution is added.

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Chapter 10

In Vitro Models to Study the Blood Brain Barrier

Hilary Vernon, Katherine Clark, and Joseph P. Bressler

Abstract

The blood brain barrier regulates the transport of chemicals from entering and leaving the brain. Brain capillaries establish the barrier and restrict transport into the brain by providing a physical and chemical barrier. The physical barrier is due to tight membrane junctions separating the capillary endothelial cells resulting in limited paracellular transport. The chemical barrier is due to the expression of multidrug transporters that mediate the efflux of a broad range of hydrophobic chemicals. Because of the unusual nutrient demands of the brain, this limited permeability is compensated by the expression of a large number of transporters that are responsive to the metabolic demands of the brain. Consequently, the blood brain barrier indirectly regulates brain function by directly controlling the uptake of nutrients. Two widely used methods for studying the blood brain are a cell culture model using rat, pig, or cow brain endothelial cells and isolated microvessels. The cell culture model is more popular likely because it is easier to use and less costly compared to isolated microvessels. In some laboratories, brain endothelial cells are cocultured with astrocyte- or astroglial-conditioned media. The endothelial cells express many of the transporters displayed in vivo but not all. Although cell culture models vary, none express the tight barrier observed in vivo. Because microvessels are isolated directly from the brain, they express all of the transporters displayed in vivo. Their disadvantage is that the preparation is laborious, requires animals, and has a shorter lifespan in vitro. We present an approach in which transport is first verified in isolated microvessels, and then the mechanism is studied in cell culture.

Key words: Transport, Microvessels, Endothelial cells, Brain, RBE4 cell line

1. Introduction

The blood brain barrier is largely impermeable to the transfer of most solutes. The tightness of the blood brain barrier is attributable to tight junctions expressed by the endothelial cells forming the capillary (1). Other tight barriers, kidney tubules and bile ducts, are due to tight junctions expressed by epithelial cells. A useful indicator of tight barrier permeability is the oil water coefficient of the chemical. The higher the solubility in oil, the

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more likely the chemical will be permeable. Not surprisingly, the tightness of the barrier would attenuate the transport of many polar nutrients. Because of the unique nutrient demands of the brain, endothelial cells express a broad range of transport mechanisms for nutrients and hormones. Interestingly, the transporters are regulated by metabolic demand. For example, brain regions with increased neuronal activity display increased glucose uptake mediated by the increased expression of glucose transporter 1 in endothelial cells (2). The interactions between neurons and endothelial cells are likely mediated by astrocytes. The endfoot of the astrocyte neighbors the endothelial cell, whereas astrocyte processes participate in synaptic activity. Several investigators suggest that intense synaptic activity promotes signals derived from astrocytes to endothelial cells resulting in the increased expression of transporters for glucose and other nutrients (3).

However, the blood brain barrier also expresses a metabolic barrier. Brain endothelial cells express the multidrug transporters p-glycoprotein and breast cancer resistance protein at the luminal surface that prevent a broad range of hydrophobic chemicals from entering the brain (4). Consequently, the permeability of chemicals with similar oil water partition coefficients across the blood brain barrier might be very different. The pharmaceutical industry is profoundly interested in drug transporters in the blood brain barrier because of their potential to impede transport of drugs for treating neurological and mental illnesses. There are other enzymes expressed by the blood brain barrier that could be argued which function to protect the brain (5). Butyrylcholinesterase possibly protects against the acetylcholine from entering the brain or exposure to chemicals that inhibit acetylcholinesterase (6). Similarly, monoamine oxidase might prevent the transport of catecholamines. Finally, gamma-glutamyl transpeptidase could prevent the uptake of glutathione conjugates across the blood brain barrier (7).

In vivo, techniques for studying the blood brain barrier include the brain uptake index, microdialysis, and positron emission spectroscopy in rodents (8). The distinct disadvantages of in vivo assays include the cost and use of anesthetics. Consequently, attention has focused on in vitro assays. Several models have been developed in the past 50 years, but two continue to be used; cell culture and isolated microvessels. A valid model must retain the tightness and transport (blood to brain and brain to blood) mechanisms observed in vivo. Cell culture models have received the most attention because cell culture techniques are commonly performed in most laboratories. The source of cells for the model include, the rat clonal cell line RBE4 cells (9) primary cultures of rat, bovine or porcine brain endothelial cells, and cell lines derived from primary cultures (10–12). Cell culture models retain some features of the blood brain barrier though not all. Cell lines express enzymes that are enriched in the brain capillary, such as

butyrylcholinesterase and alkaline phosphates but lose the expression of gamma-glutamyl transpeptidase and glucose transporter 1 (13). They have been successfully used to study unidirectional transport mediated by the multidrug transporters and monocarboxylate transporter (luminal to/from abluminal) (9, 14). In addition to the loss of properties, another concern is the expression of new transporters in cell culture models. Indeed, rat brain endothelial cells were found to acquire the expression of aquaporin 1, which is not found in the blood brain barrier (15). Likely the more distinct problem with cell culture models is their tightness, which is best assessed by measuring electrical resistance though some groups have measured transport of sucrose and mannitol. The electrical resistance value that best represents the blood brain barrier is an estimate because it cannot be measured directly in brain capillaries in vivo. In blood vessels within the pia mater on the surface of the brain, which displays a tight barrier, a resistance between 1,200 and 1,900 Ω-cm (2, 16). In brain capillaries in the parenchyma, estimates of electrical resistance have been as high as 8,000 Ω-cm^2 on the basis of radiometric fluxes of ions (17). Although this value might be too high, the 1,000-fold greater permeability to potassium displayed by peripheral capillaries compared to brain capillaries provides us with an appreciation of the tightness of the blood brain barrier (18).

To increase the expression of the properties of the blood brain barrier, several laboratories have cocultured endothelial cells with astrocytes or use astroglial-conditioned media (19). The suggestion to coculture is based on early studies that demonstrated that abdominal vessels grafted to neural tissue take on properties more similar to brain vessels, such as the formation of tight junctions, and express enzymes that are highly enriched in brain capillaries, such as alkaline phosphatase and butyrylcholinesterase (20). Several studies have shown increased expression of markers of the blood brain barrier, for example tight junctions and gamma-glutamyl transpeptidase, in endothelial cell cocultured with astrocytes compared to endothelial cells alone (21–23). In addition to cocultures, addition of hydrocortisone, increased cAMP levels, and removing serum have also been shown to increase barrier properties (22, 24–27). A range of resistance values between 200 and 800 Ω-cm^2 have been reported in different culture models. Because these values are much lower than even those found in blood vessels in the pia matter on the surface, culture models must be used with caution. Very likely the culture conditions needed to sustain the same differentiation program in vivo have not been defined.

A model that expresses all of the transporters is brain microvessels (capillaries, small venules, and arterioles) isolated directly from the brain (28, 29). Isolated microvessels have been used to study transport of a number of solutes including amino acids (30) and drugs (31) and they have also been used to study gene expression

(32, 33). Conceptually, isolated microvessels are similar to brain slices. They can be isolated from the adult brain and have been shown to express the same transporters and other proteins expressed by capillaries in vivo. Microvessels consist of astrocyte endfeet and pericytes in addition to endothelial cells (34, 35). Their size ranges from 1 to 10 microns. Microvessels are metabolically active but they have shortened lifespan, which is a distinct disadvantage compared to cultures. In this chapter, methods are described for preparing and using brain endothelial cell cultures and isolated microvessels to study the blood brain barrier in vitro.

2. Materials

2.1. Microvessel Preparation

1. Hanks' Balanced Salt Solutions (HBSS) supplemented with 1% bovine serum albumin (BSA) (see Note 1).
2. HBSS supplemented with 30% dextran (see Note 2).
3. 118 and 53 micron nylon mesh (Tetko, Elmsford, NY).
4. Dounce homogenizer with a Teflon pestle (0.25-mm clearance, machine shop alters the pestle; see Note 3).
5. Motorized homogenizer.
6. Kim wipes.
7. Buchner funnel fitted with the 118 micron mesh and placed onto a vacuum flask. The mesh is secured with a rubber band and dented in the middle forming a trough.
8. 100-mm bacteriologic Petri dishes.
9. Glass rods.
10. 25-mm glass beads.
11. 3-mL syringes.

2.2. Cell Culture

1. RBE4 cells: They are not in the ATCC collection, but are available from different laboratories.
2. RBE4 medium: Alpha minimum essential medium (αMEM) and Ham's F-10 medium in a 1:1 ratio supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM glutamine, 300 μg/mL G418, 1-ng/mL human recombinant basic fibroblast growth factor (bFGF).
3. 0.1-mg/mL bFGF in 10 mM Tris, pH 7.6, stored at –20°C. For long term storage, aliquot into buffer containing 0.1% BSA and store in polypropylene vials at –20°C. Refilter through a 0.22-μM filter before using.
4. 1× Phosphate buffered solution (PBS).

5. Rat tail Collagen I (5 mg/mL, Sigma), stored at 4°C in an opaque container to avoid light exposure.
6. 0.02 M acetic acid.
7. Culture dishes: Must be collagen coated.
8. 0.25% trypsin (0.25%).
9. 1 mM ethylenediamine tetraacetic acid (EDTA).

2.3. Transport Studies

1. HBSS with 20 mM HEPES, pH 7.4.
2. The pore size of transwell membrane inserts range from 0.4 to 1.0 μM, and are either nitrocellulose or polyethylene terephthalate. They are coated with collagen similar to the method used to coat tissue culture plastic.
3. 10 μg/mL sodium fluoroscein (final concentration).
4. World Precision Instruments EVOM with chopstick electrodes. Newer instruments are available.

2.4. Staining Collagen-Coated Porous Membrane Insert for Histological Examination

1. 2.5% glutaraldehyde.
2. 0.5% triton X.
3. Gills hematoxylin No.1, filtered through a 0.2-μM membrane.
4. 0.4% hydrochloric acid in 70% ethanol.
5. 0.04% ammonium hydroxide.
6. Pyrex dish for rinsing inserts.

3. Methods

3.1. Isolating Brain Microvessels

The method takes advantage of the relative strength of the brain microvessel to mechanical force. The homogenizing pestle is shaved so that the clearance between the inner glass wall of the homogenizer and the pestle is 0.25 mm. The clearance allows the microvessels to remain intact but the smaller cells, glia and neurons, are disrupted. A density centrifugation and a series of filtrations are conducted to separate the microvessels from the other constituents (cell nuclei and debris). The filtration procedure does not distinguish capillaries from arterioles and venules. Thus, the procedure is for preparing microvessels (which includes capillaries). Because arterioles and venules also express tight junctions, the isolated microvessels are used to study the blood brain barrier. When first establishing the method in the laboratory, monitor purity microscopically and compare the specific activity of gamma-glutamyl transpeptidase in brain homogenates and the isolated microvessels. The specific activity should be 20-fold greater in microvessels.

3.1.1. Dissecting and Homogenizing the Brain

1. The preferred method of euthanizing rats or mice is with a guillotine so that the least amount of blood collects in the head. Carbon dioxide asphyxiation is acceptable. Isolate the brain from one rat before going on to the next. Each brain is excised quickly and immersed in a beaker of media that is prechilled and kept on an ice bath. Microvessels are very prone for losing ATP. Once the brain is removed, the yield of viable microvessels improves by conducting all procedures on ice (see Notes 4 and 5).
2. Remove meninges and blood by gently rubbing the brain with a Kim wipes. Remove the brain stem because it is mostly white matter. Take up to four sharp scissors in one hand and mince the brain in the beaker. Pieces should be no more than 1 or 2 mm. Pour the pieces in a centrifuge tube and repeat for each brain in a beaker with fresh media.
3. Homogenizing must be done carefully. The procedure essentially destroys the cellular integrity of glia and neurons but keeps the microvessels intact. Each homogenate consists of 1 volume tissue for 9 volumes HBSS/BSA. Pour the minced tissue into the homogenizing tube and apply 20 strokes, each stroke for 2 min at a rotor speed of 40 rpm. Because homogenizing generates heat, cool the tube in an ice bath after every three to four strokes. Pour the homogenate into a centrifuge tube and homogenize the next batch. Combine homogenates.
4. Add an equal amount of the 27% dextran and invert the tube several times for 60 s. The dextran and homogenates must be well mixed but not shaken too vigorously. An alternative is to centrifuge the homogenate at 1,000 × *g* for 30 min and resuspend the pellet directly in 13% dextran.
5. Centrifuge for 30 min at 4,400 × *g*.
6. The myelin is at the top and the grey matter containing the microvessels forms the pellet. Pour the supernatant out and keeping the tube inverted, swipe the myelin off the sides of the tubes with Kim wipes and drain excess liquid onto a paper towel. Place the tube on ice and add 1 volume (volume of the pellet) of ice-cold HBSS/BSA to the pellet. Tap the bottom of the tube vigorously or vortex slowly to resuspend the pellet. You should observe neither particles nor foaming. Add 9 volumes of HBSS/BSA to the suspension.

3.1.2. Filtering

1. Pipette the suspension slowly onto 118 micron nylon filter covering the Buchner funnel. The larger blood vessels are trapped by the nylon and the smaller ones flow through along with the nuclei and debris. You can use a glass rod and gently rub the filter to assure a continual flow of fluid. After all of the suspension is added, wash the filter several times with a generous amount of HBSS/BSA.

Fig. 1. Micrograph of purified brain microvessels. After elution from the glass bead column, a drop of the suspension of microvessels was placed on a microscope slide, air dried, and stained with Giemsa. Please note the abundance of microvessels and the absence of debris and nuclei.

2. Centrifuge the filtrate at 1,000 × *g* for 20 min.
3. Resuspend the pellet again as before with 9 volumes of HBSS/BSA and pipette to the glass bead column (see Notes 6 and 7). The nuclei and other debris pass through the column, but the microvessels cling to the glass beads. After allowing the suspension to pass through, wash the column with 5–10 volumes of media. Pour the beads into a beaker, pipette HBSS/BSA to cover the beads, and swirl several times to dislodge the microvessels from the beads. Allow the beads to settle and transfer the HBSS/BSA into a centrifuge tube. Repeat twice.
4. Combine the washings and centrifuge 1,000 × *g* for 10 min. Resuspend the pellet in a small volume of HBSS/BSA and place a drop of the suspension onto a microscope slide (see Fig. 1). Under phase contrast microscopy, microvessels with little or no cell nuclei should be observed.

3.2. Cell Culture

There are a number of brain endothelial cell lines available from cow, pig, rat, and human (11). It is also possible to prepare primary cultures of brain endothelial cells. Another choice is the RBE4 cell line that was derived by immortalizing primary

cultures of rat brain microvascular endothelial cells with the plasmid pE1A-neo containing the E1A region of adenovirus 2 and a gene that conveys neomycin resistance (9). No model has yet been shown to be better than the other. The choice is left to the investigator. The advantage of RBE4 cells is consistency but laboratories might have individual success by developing their own cell lines. An additional caveat is to augment expression of the blood brain barrier by incubating the endothelial cells with astrocyte-conditioned media or culturing rat brain astrocytes on the opposite side of the filter. Again, the benefit of adding an astroglial component varies. We describe the transport assay being conducted in our laboratory that uses the RBE4 cell line.

3.2.1. Cell Line Maintenance

1. RBE4 cells grow on collagen-coated plates (see Notes 8 and 9). Tissue culture Petri dishes are coated with 5 µg-collagen per cm^2 surface area with collagen at 0.125 mg/mL in 0.02 M acetic acid for 60 min at room temperature. The acetic acid/collagen is aspirated and the plate is washed three times with PBS. The coated dishes can be stored at 4°C or used immediately.
2. To pass cells, the monolayer is first washed with PBS and then incubated with 0.25% trypsin in EDTA at 37°C. As soon as the cells begin rounding up, media is added and the cells are dislodged with vigorous pipetting. The cell suspension is centrifuged at $250 \times g$ for 5 min and the pellet is resuspended in media and plated at 4,000 cells per cm^2. When the cells reach confluency, in approximately 72 h, the cells should be passed.

3.2.2. Staining Filters to Monitor Cell Integrity

Filters can be purchased for cell examination but phase contrast microscopy might be insufficient or difficult. Our laboratory stains one filter prior to conducting a transport assay.

1. Carefully (not to disturb either side of the filter) aspirate the media from both chambers and rinse both sides of the filter with PBS.
2. Carefully add 2.5% glutaraldehyde to cover the apical surface of the membrane and incubate at room temperature for 15 min.
3. Aspirate the fixative, add 0.5% triton X-100 and incubate at room temperature for 3 min. Aspirate.
4. Add Gill's hematoxylin No. 1, incubate at room temperature for 15 min, aspirate.
5. Remove the collagen-coated insert (still in its plastic holder) from the culture dish and rinse it in distilled water in a Pyrex dish four times, using fresh distilled water with each rinse.
6. Add 0.4% hydrochloric acid in 70% ethanol and incubate at room temperature for 3 min, aspirate and rinse four times.

7. Add 0.04% ammonium hydroxide, incubate at room temperature for 3 min, aspirate, rinse four times.
8. The inserts are air dried.
9. The inserts can now be examined with light microscopy for basic histology, as well as any obvious breaches or holes in the monolayer.

3.3. Transport Assays in RBE4 Cell Line

3.3.1. Specific Transport

The frequently used cell culture model comprises plating brain-derived endothelial cells on a collagen-coated porous membrane insert that is then placed into a multiwall plate. The media above the cells is considered the apical chamber and below the filter the basolateral chamber. The only pathway for a solute to pass from one chamber to the other is across the cell monolayer.

1. Cells are plated at 4,000 cells per cm^2 on collagen-coated inserts. Media is added to both the apical and basolateral chambers. Inserts are collagen coated with the same procedure as described above for Petri dishes. Also, inserts coated with collagen can be purchased. Inserts that fit into 24-, 12-, and 6-well dishes are available (see Note 10).
2. Cells reach confluency in approximately 7 days. The media is changed every 72 h, with care not to disturb the cell monolayer.
3. On the day of the assay, inserts are removed to a new plate and the apical and basolateral sides are washed three times with transport buffer. Our laboratory uses HBSS buffered with 20 mM HEPES, pH 7.4. There is calcium and magnesium to maintain the integrity of the cell monolayer, and glucose is the energy source. HBSS can be purchased without phenol red for fluorescent assays. The volume of the buffer in the basolateral chamber should be sufficient to bath the filter. The volume in the apical chamber varies depending on the size of the well.
4. The solute is added to either the apical (blood to brain) or basolateral (brain to blood) chamber. After the solute is added, the plate is returned to the incubator (assuming transport is measured at 37°C).
5. At different lengths of time, a sample is removed from the opposite chamber. When samples are taken from the basolateral chamber, the insert must be lifted with forceps. Some investigators find that lifting the inserts to be difficult under the time constraints of a transport assay. Alternatively, the insert can be transferred to a new plate with basolateral transport buffer.
6. After the transport assay is completed, the amount of solute remaining in the cells can be determined by cutting the filter

free from the insert. Radioactive solutes are measured by placing the filter in a scintillation vial with scintillation fluid. Fluorescent assays are measured by scraping the cells off the filter. The solute could also be directly extracted from filter for chemical analysis (e.g., mass spectroscopy).

7. Permeability coefficients are calculated from the following equation.

$$P_c = \frac{V_A}{A\left(C_D - C_D\right)} \frac{dC_A}{dt}$$

where $\frac{dC_A}{dt}$ (mg/s/mL) is the increase of solute concentration in the acceptor chamber during the time considered, A is the surface area exposed to the compound, V_A the solvent volume in the acceptable chamber (mL), and C_D the initial concentration of solute in the donor chamber (mg/mL). Transport kinetics can also be measured to determine K_m and V_{max} (see Note 11).

3.3.2. Transelectrical Resistance Measurements

Transelectrical resistance (TER) measurements across confluent cells (grown on filter inserts) can be used to assess the formation of tight junctions and integrity of the monolayer. This can be important both at the beginning of an assay, to verify that the cells are fully differentiated and not damaged, as well as following an experiment to assess the effect of test compounds on the monolayer integrity. There are several devices that can be used to measure TEER: We discuss the use of chopstick electrodes, which are among the less expensive and more widely available instruments. Additionally, measurements can be made in HBSS or media (or similar); however, we have had best results with HBSS, which we describe here.

1. In advance: Prewarm HBSS and media.
2. Aliquot prewarmed (37°C) HBSS into wells of a plate (same size as those used for filter inserts). Prepare as many wells as there are inserts, plus one extra. Use a fixed amount of HBSS in each well.
3. For each insert: Remove insert from plate using forceps, decant apical media, and place in the prepared HBSS plate. Add a fixed amount of the prewarmed HBSS to the apical side of each insert.
4. Place one blank insert (no cells, but with collagen or other treatment used) into HBSS plate.
5. Place plate in incubator for 20 min to give cells the opportunity to equilibrate.
6. Meanwhile, place the chopstick electrodes in 70% ethanol for 10 or 15 min to sterilize. Remove from ethanol in a sterile environment and let dry briefly, then place in a 15-mL

centrifuge tube filled with enough prewarmed HBSS to bathe the electrode tips.

7. Turn on the meter, make sure that it is set to resistance (or ohms) and connect the electrodes.
8. Test the meter according to instructions, usually by pressing the 'Test' button or similar.
9. To measure resistance: Place chopsticks into plate so that longer chopstick rests on the bottom of the well plate and the shorter chopstick rests in the apical section of the insert.
10. Press the measurement button on the meter and record the resistance. Continue with all wells, and repeat each well at least twice so that three measurements are made per well (see notes on resistance fluctuations).
11. Return cells to media plate and replace apical HBSS with media or treatment of interest.
12. Repeat at the end of experiment.
13. Resistance for each experiment well is calculated by subtracting the average resistance in the blank insert from the average of the experimental insert. This is multiplied by the surface area (in cm^2) of the insert and reported as ohms-cm^2. Additionally, changes in resistance following a treatment can be calculated by comparing changes in experimental wells (before and after exposure) to changes in untreated wells over the same period of time (see Note 12).

3.3.3. Paracellular Transport/Permeability

1. Cells are cultured and plated on transwell filters as described in Subheading 3.4 steps 1 and 2.
2. We set up enough wells to have two samples for every desired time point to be measured.
3. The apical and basolateral chambers are each washed three times in HBSS (phenol red free) taking care not to disturb the monolayer.
4. Add enough HBSS to the basolateral chamber such that it is in contact with the transwell membrane and to the apical chamber. Initiate transport by adding sodium fluoroscein to 10 μg/mL. Paracellular transport should be similar in both directions.
5. Remove the HBSS from the apical and basolateral chambers into separate containers at each desired time point. Be sure to store the samples in foil to protect them from quenching by light exposure and at –20°C if they are not to be analyzed immediately.
6. The samples are analyzed with a spectrophotometer at 490 nm or a spectrofluorometer at an excitation wavelength in the range of 440–480 nm and an emission wavelength of 517 nm. Transport is measured by the equations stated above.

3.4. Transport in Microvessels

In general, transport assays conducted in the cell culture and microvessels models are similar. The few notable differences are discussed here.

1. Microvessels are washed and resuspended in the transport buffer. All centrifugations and washings should be at 4°C at $500 \times g$ for 5 min. The type of buffer depends on the requirements of the transporter/receptor and was likely published in earlier studies. Buffers used in receptor assays on preparations of plasma membrane might not be osmotic. Because microvessels are intact cells, they burst at hyposomotic buffers and shrink in hyperosmotic buffers. If sodium or chloride interferes with the assay, osmolarity can be achieved with sucrose. Very often, however, transporters require a monovalent metal.
2. There are two choices for separating the solute taken up by the microvessels from the free solute and these are centrifugation and filtration. Each technique has advantages and disadvantages. The advantage of filtration is that the filter traps the microvessels and all washings are done on the filter. Filters can be washed multiple times until the solute is no longer detected in the washings. This achieves the best separation of free from bound. In contrast, more washings result in more loss when centrifugation is used. The disadvantage of filtration, however, is measuring the probe. The filter physically quenches the signal emitted from the probe. When centrifugation is used, microvessels are solubilized directly and physical is less important.
3. Data is reported as uptake normalized to time and amount of protein.

4. Notes

1. Oxygenating HBSS could help increase viability.
2. To make HBSS with 27% dextran, first dissolve dextran (sizes 60,000–120,000 kDa) in boiling water to make a 32% solution. Add 1 mL of 10× HBSS to every 8.5 mL of dextran and 0.5 mL of HEPES (1 M, pH 7.4).
3. A machine shop will shave the pestle to a 0.25 mm clearance. Each homogenizing tube and pestle will be matched and used only for microvessel preparation.
4. Microvessels can also be prepared from other larger mammals, such as pigs and cows. Special concern is to chill the brain immediately after slaughter and begin isolation as quickly as possible. Kosher slaughterhouses are preferred

because less blood collects in the brain. Isolating microvessels from grey matter will only increase yield.

5. Yield is 4–6 mg of microvessels from 20 g (wet weight) cerebral cortex. An adult rat brain is approximately 2 g.
6. The glass bead column is made by cutting both ends of a 3-mL syringe so that each end of the remaining cylinder has approximately the same size opening. One end is closed with a piece of 53 micron filter that is secured with a rubber band. Glass beads (25 mm) are poured into the column extending to 1.5 cm from the filter. The columns are supported by a test tube rack or ring stand. The beads have a rather large capacity.
7. An alternative to the glass bead column is filtering through a 53 micron mesh filter that is fitted around a glass beaker with a rubber band. The microvessels are trapped by the filter and the cell nuclei flow through. Cut the filter along the edges of the beaker and place in a beaker with a generous amount of HBSS/BSA. Swirl the filter several times to dislodge the microvessels. This step can be repeated to assure that the microvessels are dislodged. Centrifuge the microvessels at $1{,}000 \times g$ and examine for purity.
8. RBE4 cells were selected for their ability to form tight junction and express the transporters displayed by brain endothelial cells in vivo. In maintaining the RBE4 cell line, caution must be exercised so that these properties are retained. Most cell lines lose their expression of differentiated properties with repeated passage. In the RBE4 media, the addition of neomycin maintains the selective pressure for brain endothelial cells to survive. Nonetheless, there is also selective pressure favoring cells that proliferate quickly, are more resistant to trypsin, and to other adaptive responses in the tissue culture environment at the expense of differentiation. Consequently, there is the potential that, after many passages, RBE4 cell line loses its ability to mimic the blood brain barrier. Large batches of the cell line are frozen to provide a source of early passage cells. The specific passage number varies in different laboratories. Consequently, the laboratory must record the passage number so that a new batch of cells can be thawed to replace the older cells that have lost the ability to differentiate. To properly record passage number, plate the same number of cells after each passage and wait the same numbers of days before the cells are passed. The cell line provides more consistent data with greater consistency in maintenance.
9. Three main sources of contamination are bacteria, mycoplasm, and fungi. Heavy bacterial contamination can be spotted

by an opaque growth media but should always be confirmed by microscopic observation. Even when the growth media is clear, microscopic observation is absolutely required before feeding and passing. Contaminated cultures are destroyed by adding bleach to the flask outside of the tissue culture. Surprisingly, the best method of preventing contamination is not to use antibiotics. Contamination is due to a breakdown in aseptic technique and very often caused by human error. Consequently, all sources of contamination are introduced but bacterial growth is seen first. When antibiotics are used, the breakdown in aseptic technique is masked but is observed much later by the growth of mycoplasm and fungi. Mycoplasm contamination is not detected microscopically but by detecting mRNA or directly in a mycoplasma selective growth media. Fungi often can be observed under phase contrast microscope, but their sporadic growth might be difficult to detect. When mycoplasm and fungi are finally detected, the contamination has likely spread to other cell lines. To prevent a larger problem, it is recommended to detect a breakdown in aseptic technique early by not using antibiotics.

10. Many of the concerns in setting up transport assays generally do not depend on the model being studied. The concerns specific to the models described here are discussed. When getting started, the first objective is to determine whether the solute undergoes specific transport. To accomplish this objective, the investigator needs to decide the quantity of cells/microvessels, the amount of solute to add, and the length of time cells are incubated with the solute. Increasing the amount of microvessel or higher numbers of cells increases the likelihood of detecting the solute. It might be more difficult in the microvessel model because the starting material comes from animals. In the cell culture model, the higher the ratio of membrane surface area to chamber volume, the better chance of detecting transport. Although assays conducted for longer lengths of time will likely increase the probability of detecting transport, there are other concerns that must be considered. For example, the investigator must consider physiological relevance and also cell viability might decrease (see below).
11. Similar to other transport assays, nonspecific transport must be defined. When working with a solute that is labeled (e.g., radioactive or fluorescent), nonspecific transport is determined by measuring transport in the presence of excess (100–1,000-fold) nonlabeled solute. Specific transport is defined as total transport (only labeled solute) minus nonspecific transport. When a labeled solute is not available, various approaches have been taken for determining nonspecificity. Our laboratory defines it as transport at 4°C.

12. The loss of viability could increase paracellular transport and increase nonspecific transport in the cell culture model. One concern for viability is the length of time. Paracellular transport will likely increase in cells incubated for extended lengths of time in HBSS. Although cell culture media could be substituted, media also introduces more chemical constituents that potentially could influence transport kinetics. The length of time for the transport assays in the cell culture model should be no greater than a few hours. In the microvessel model, 1 h incubation should be sufficient. A second concern is whether the solute, or other chemicals added to the incubation buffer, affects cell viability. In addition to cytoxicity, changes in tight junction assembly increase paracellular transport, which mask a transporter mediated event. Accordingly, it is important to measure paracellular transport under the same conditions transport is measured.

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Part II

Cell Death

Chapter 11

Measurements of Cell Death in Neuronal and Glial Cells

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Abstract

During brain development, cell death is a physiological process which allows the elimination of cells produced in excess. During adulthood, when there is no or little physiologic cell death, an increase in cell loss is usually caused by neurologic disorders or by exposure to neurotoxic chemicals. Measurements of cell death are often used a first line of investigation on chemicals. Cell death in neuronal or glial cultures in vitro can be quantified with a variety of assays based on different properties of live and dead cells. Thus, healthy cells exclude dye (e.g., trypan blue, propidium iodide) or possess metabolic activity to cause a compound's conversion to a colored or fluorescent one (e.g., MTT, calcein AM) while dead cells do not. Conversely, dying cells release enzymes in the medium (e.g., LDH) whose quantification is proportional to the number of dead cells.

This chapter describes several relatively rapid, inexpensive and reliable methods for measuring cell death and in neurons and astrocytes in primary cultures or in neuronal and glial cell lines.

Key words: Cell death, Cell viability, Trypan blue assay, MTT assay, Lactate dehydrogenase

1. Introduction

In neurologic disorders, cell death may occur as an early event during the disease process or may occur secondarily in a late stage of the process (1). Cell death can occur via two processes, which are fundamentally different in their nature and in biological significance: necrosis and apoptosis. Typically, necrosis involves irreversible changes, such as the loss of cytoplasmic structure, dysfunction of various organelles and finally, cytolysis, as a result of high-amplitude swelling. In general, necrosis is considered to be a passive process that is usually caused by extreme trauma or injury, such as stroke, trauma, or infection (2, 3). The release of the dying cell's contents into the extracellular space can cause

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further injury or even death of neighboring cells, and may result in inflammation or infiltration of pro-inflammatory cells into the lesion, leading to further tissue damage. In contrast, when a neurodegenerative disorder, such as amyotrophic lateral sclerosis, Parkinson's disease, or Huntington disease develops, cell death seems to be an early and primary feature of the process, and the apoptosis is considered to be the probable mechanism (4, 5).

A variety of neurotoxic chemicals are known to cause death of neurons and/or glial cells by necrosis or apoptosis, often depending on their concentration (11). As such, methods to rapidly and inexpensively assess cell death are of great interest and relevance in neurotoxicology.

Cell death in neuronal and glial cells in culture can be quantified with a variety of assays based on different properties. Widely used methods are based on the uptake or exclusion of vital dyes. Dead and viable cells are discriminated by differential staining, and counted using a light or fluorescence microscope, these assay are those utilizing Trypan blue or Propidium Iodide. Though easy and cheap, these methods are usually time-consuming and do not allow the processing of a large number of samples. Furthermore, they do not account for the portion of dead cells which may have been lysed completely during the exposure time, thus, the actual rate of cell death in long-term cultures may be underestimated by these assays.

A second type of assay is based on the measurement of cytoplasmic enzyme activity (lactate dehydrogenase, LDH) released by damaged cells. The amount of enzyme activity detected in the culture supernatant usually correlates with the amount of lysed cells.

A third type of assay is designed to utilize the cell's metabolic activity to promote a dye's conversion to a visible or fluorescent state which can be quantified (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay).

A final type of common assays is based on the measure of fluorescence dyes (e.g., calcein acetoxymethylester (calcein AM)) from prelabeled cells. The disadvantage of these assays is that a spontaneous release of the probe from the pre-labeled target cells may occur, thereby reducing the sensitivity of the assay.

2. Materials

2.1. Trypan Blue Assay

2.1.1. Trypan Blue Assay Based on Microscopy

1. Phosphate-Buffered Saline (PBS) solution (pH 7.2): Can be purchased from Invitrogen as a 10× stock solution and diluted using double distilled water.
2. 0.4% (w/v) trypan blue stock solution.

3. 0.25% trypsin.
4. Complete medium: Neurobasal containing 2% B27 (primary neurons), DMEM containing 10% FBS (primary astrocytes, C6 rat glioma cells, human astrocytoma 132-1N1, PC12 rat pheocromocytoma cells, SH-SY5Y human neuroblastoma cells).
5. Hemocytometer.
6. Inverted microscope.

2.1.2. Trypan Blue Assay by Sprectrophotometry

1. 10% sodium dodecyl sulfate (SDS) stock solution.
2. Lysis buffer: 25 Tris–HCl (pH 7.4), 120 mM NaCl, 1% SDS.
3. Locke's buffer: 10 mM HEPES, 5.5 mM KCl, 2.3 mM $CaCl_2$, 10 mM glucose, 5 mM $NaHCO_3$, 2 mM glutamax, 100 μg/ml gentamicine, 1.2 mM $MgCl_2$, 130 mM NaCl.
4. Microplate Reader.

2.2. Propidium Iodide Method

1. 1 mg/ml propidium iodide (PI).
2. Fluorescence microplate reader with a detector capable of reading plates from underneath; 530 ± 25 nm excitation filter, and 610 ± 40 nm emission filter.
3. In alternative, a fluorescence microscope.

2.3. Lactate Dehydrogenase Assay

1. LDH cytotoxicity assay kit (Cayman), including: 100× nicotinamide adenine dinucleotide solution (NAD^+), 100× lactic acid, Assay buffer, diaphorase, L-lactic dehydrogenase standard, 100× p-iodonitrotetrazoliumviolet (INT).
2. LDH reaction solution: Add 100 μl of 100× lactic acid, 100× NAD^+ and 100× INT, 150 μl of diaphorase to 9.6 ml of assay buffer.
3. 96-well microtiter plate.
4. 98% Triton X-100.
5. Microplate reader (e.g., Molecular Devices).

2.4. MTT Assay

1. 5 mg/ml MTT stock solution: Mix 5 mg of MTT in 1 ml of PBS.
2. 99.9% dimethyl sulfoxide (DMSO).

2.5. Calcein AM Method

1. 1 mM calcein AM stock solution. Dilute to final concentration of 1 μM.
2. 1 mg/ml PI stock. Dilute to a final concentration of 2.5 μg/ml.
3. Fluorescence microscope.

3. Methods

3.1. Methods Based on Trypan Blue

We describe two methods to measure the neuronal cell death utilizing Trypan blue (6). The first method is based on visual counting of death cells, using a contrast-phase microscope. The second method is a spectrophotometric assay, which can be performed in a 96-well plate. The latter method allows a fast and reliable measure of the cell death, and avoids the tedious and time-consuming cell counting, which can be often a source of errors.

3.1.1. Quantitation of Trypan Blue Based on Microscopy

1. Plate primary astrocytes or neurons, or neuronal and glial cell lines (e.g., human astrocytoma 132-1N1, human neuroblastoma SH-SY5Y, C6 rat glioma cells, PC12 rat pheocromocytoma) in a 24-well plate.
2. After treatments wash cell three times with PBS.
3. Incubate cells in 250 µl of 0.25% trypsin for 3 min at 37°C in a 5% CO_2-containing incubator (see Note 1).
4. Add 250 µl of complete medium to inactivate the trypsin.
5. Detach cells from the plate using a pipette.
6. Mix 50 µl of cell suspension and 50 µl of trypan blue stock solution and resuspend manually using a pipette.
7. Incubate mix for 3 min at 37°C in a 5% CO_2-containing incubator.
8. Using a hemocytometer, count the number of live cells and the number of dead cells under an inverted microscope. Dead cells stain dark blue while live cells remain bright.
9. Quantify cell death by dividing the number of dead cells by the number of total cells.

3.1.2. Quantitation of Trypan Blue by Spectrophotometry

1. Plate cells in a 48-well plate (e.g., primary neurons at a density of 5×10^5/ml).
2. Perform treatment at 37°C in a 5% CO_2-containing incubator.
3. After treatment, wash medium twice with Locke's buffer.
4. Incubate cells in Locke's buffer containing trypan blue (0.05% final concentration).
5. Place the plates in the incubator for 15 min.
6. Wash cells twice with Locke's buffer. A slow, steady wash is necessary to prevent the loss of injured cells which may result from mechanical handling.
7. Add 200 µl of lysis buffer and gently collect cells.
8. Resuspend cells gently avoiding to introduce air bubbles.
9. Read absorbance using a Microplate Reader System setting the absorbance at 562.

3.2. Propidium Iodide Assay

The propidium iodide assay relies on the cell membrane-permeable dye, which becomes brightly fluorescent upon binding to DNA in dead cells. Propidium iodide is added to the culture medium and the number of dead cells can be determined by either measuring the fluorescence with a fluorescence microplate reader systems (7) or by directly counting cells using a standard fluorescence microscope.

1. Plate cells in a 48-well plate.
2. Perform treatment at 37°C in a 5% CO_2-containing incubator.
3. Add propidium iodide to culture medium to a final concentration of 5 µg/ml (see Note 1).
4. Return plates to incubator for 30 min.
5. Using a fluorometric multiwell plate reader equipped with a 530 ± 25 nm excitation filter and a 645 ± 40 nm emission filter, determine the absorbance of each well at desired time points. Alternatively, count stained cells manually on a fluorescence microscope.
6. Express results using arbitrary unit values of the emission at 610 nm.
7. If measurements are done with a fluorescence microscope, quantify dead cells by counting the total number of cells per well.

3.3. Lactate Dehydrogenase Assay

LDH is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant upon damage of the plasma membrane. LDH activity can easily be measured in culture supernatants (8). In the first step of this assay, the LDH present in the medium of the culture catalyzes the energetically unfavorable reduction of NAD^+ to NADH and H^+ by oxidation of lactate to pyruvate. In the second step, the diaphorase utilizes NADH and H^+ to reduce the tetrazolium to the dark blue-colored formazan (9, 10). The amount of formazan accumulated in the medium is proportional to the amount of LDH released into the culture medium. The use of a microplate reader guarantees the easy processing of a large number of samples (see Note 2). This assay was originally used to measure neuronal cell death occurring via necrosis, but more recently it has been shown to measure "late" neuronal apoptosis in primary neuronal cultures (11). Before performing the LDH assay, a variety interfering factors such as the presence of serum (see Note 3) or the pH-sensitive dye in the culture media should be considered. The linear range of the LDH molar absorbance is also limited.

1. Plate neuronal cell lines in a 48-well plate at a density of 7×10^4 cells/500 µl/well. Primary neurons are plated at a density of 20×10^5 cells/500 µl/well.
2. Incubate cell lines for 48 h to allow cells to attach and reach 60–80% confluency. Primary neurons are kept in culture for 10 days before treatments.
3. Treat cells with test compounds, using different concentrations or different time-time points. Include a vehicle control. As a positive control, treat cells with 10% Triton.
4. Collect medium from the plates containing treated cells.
5. Add 50 µl of each sample to a 96-well plate. For low density cell cultures (such as primary neurons), the sample volume can be increased to 100 µl.
6. Add 50 or 100 µl of LDH standards (see Note 4).
7. Add 100 of Reaction Solution to each well.
8. Incubate plate on an orbital shaker for 30 min at RT (see Note 5).
9. Set the microplate reader for kinetic measurement at 490 nm (every 5 s for 2 min).
10. Using the LDH standards, convert absorbance values into LDH activity (U/ml).

3.4. MTT Assay

The MTT assay, first described by Mosmann in 1983 (12), is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT to form dark blue formazan crystals, which are largely impermeable to cell membranes, thus resulting in their accumulation within healthy cells. Solubilization of the cells by the addition of a detergent or of DMSO results in the liberation of the crystals which are solubilized. The number of surviving cells is directly proportional to the level of the formazan product formed. The color can be quantified using a simple colorimetric assay. The results can be read on a multiwell ELISA reader.

1. Plate neuronal cell lines in a 48-well plate at a density of 7×10^4 cells/500 µl/well. Primary neurons are plated at a density of 20×10^5 cells/500 µl/well.
2. Incubate cell lines for 48 h to allow cells to attach and reach 60–80% confluency. Primary neurons are kept in culture for 10 days before treatments.
3. At the end of incubation, add 50 µl of MTT stock reagent to each well for 30 min.
4. Incubate (37°C, 5% CO_2) for 30 min to allow the MTT to be metabolized.
5. Remove the medium (dry plate on paper towels to remove residue).

6. Add 250 µl of DMSO and let the plate shake for 5 min to thoroughly mix the samples.
7. Transfer 160 µl of suspension to a 96-well plate.
8. Read the plates at 562 nm in a microplate reader.

3.5. Calcein AM

Calcein AM is a nonfluorescent, hydrophobic compound that easily permeates intact, live cells. The hydrolysis of calcein AM by intracellular esterases produces calcein, a hydrophilic, strongly fluorescent compound that is well-retained in the cell cytoplasm. By using propidium iodide, we can stain both live and dead cells, and the total number of cells can be counted, eliminating the possibility of an underestimation of cell death due to cell detachment. This double staining allows to easily distinguish between live cells (green) and dead cells labeled with propidium iodide (red). Other molecules that intercalate into and stain DNA may be used to stain dead cells while other fluorescent azoxymethyl or acetyl derivatives cleaved by intracellular esterases allow morphological discrimination of live cells.

1. Primary neurons are plated at a density of 20×10^5 cells/500 µl/well.
2. At the end of the treatment, add calcein AM and PI mixtures directly to the cultures making sure to use the proper final dilutions.
3. Leave plates for 15 min in the incubator.
4. Perform the assay within 1 h from the beginning of the staining.
5. Examine stained cells with a standard fluorescence microscope equipped with Nuance Software.
6. Score green cells as live and cells with red nuclei as dead (see Notes 6 and 7).

4. Notes

1. The optimal dye concentration and incubation time for the trypan blue assay and the propidium iodide assay may depend on culture conditions. This should be determined experimentally in each culture system.
2. LDH activity or levels can also be measured by HPLC or mass spectrometry, or by an immunological method (ELISA or electrophoresis).
3. The animal sera contain LDH in variable amounts which can interfere with the assay. Usually, the greater the serum contents of the medium, the greater the background LDH activity.

4. The conditions indicated for the LDH assay protocol were determined empirically for primary neurons grown in 48-well culture plates and 500 µl bathing volume.
5. It is critical to avoid foam formation at this or any other step; foam interferes with measurement of the plate reader. Foam bubbles can be disrupted by touching them with the tip of a sharp needle.
6. In this protocol, a total-kill condition is not required; however, wash-treated shams should still be used to determine baseline cell death. To determine mean cell death specifically due to each condition, subtract the mean baseline cell death for each condition.
7. The dye concentrations used in this protocol were determined empirically for neurons grown in glass-bottom 35-mm dishes and a 1-ml bathing volume. Since the optimal dye concentration can vary with the cell culture, the lowest concentration of propidium iodide that gives sufficient staining of dead cells should be used with the highest concentration of calcein AM that does not stain dead cells.

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Chapter 12

Measurements of Neuronal Apoptosis

Gennaro Giordano and Lucio G. Costa

Abstract

Apoptosis is a natural process occurring during the development of central nervous system resulting in the elimination of neurons that may have formed faulty synapses. Apoptosis can also be triggered by deprivation of growth factors or by exposure to a variety of endogenous or exogenous compounds. Several methods exist to assess apoptosis in neuronal cells. The choice of a particular method for apoptosis detection is dependent on the cell system, the nature of the toxin or toxicant, the type of information being sought, and, finally, on technical limitations. In this chapter, we describe techniques to evaluate apoptosis in primary neuronal cell cultures based on the evaluation of three criteria: cell morphology, biochemical changes, and DNA degradation. To draw correct conclusions regarding the mode of cell death, a combination of some of the methods mentioned above should be used.

Key words: Apoptosis, Hoechst assay, DNA laddering, Caspases

1. Introduction

The development of the CNS requires the generation of new cells (glia and neurons) and at the same time the death of those cell which are no longer necessary (or produced in excess) to control the overall cell number. This programmed cell death is called apoptosis, a term (set in the early 1970s) derived from Greek roots and meaning "dropping of leaves off a tree". Apoptosis is the results of a well-regulated and complex chain of events and shows different morphological features from necrosis which is associated with a rapid explosion of cells following a rapid degradation and increase in the permeability of the cell membrane (1). The failure of this well-regulated chain of events during the development of the brain can have serious effects in the adulthood. Congenital brain malformations often represent the product of a

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less efficient and incomplete apoptotic process. Brain disease processes may be accompanied by either necrosis or apoptosis or often both. This may depend on the intensity and extent of injurious stimuli. Ischemia, neurotoxicants, or ionizing radiation may result either in apoptosis (at low doses) or in necrosis (at higher doses) (2).

In brain, apoptosis can also occur as the result of oxidative stress that may increase with age or because of accumulation or excessive release of excitatory neurotransmitters. In chronic degenerative disorders, the nature of the cell loss is less clear, although a number of factors seem to point at the involvement of apoptosis. For example, examination of spinal cord in amyotrophic lateral sclerosis has shown evidence for apoptosis by TUNEL stain (3); similar observations have also been made in striatum in Huntington's disease (4). Evidences of apoptotic markers have been found in brain specimens from autopsies of Alzheimer's disease patients (5), and studies examining the substantia nigra pars compacta (SNpc) in brains of humans with Parkinson's disease have in some cases shown signs of neuronal apoptosis (6).

The key apoptotic morphological features, observable under a microscope, include shrinkage and blebbing of the cytoplasm, preservation of the structure of cellular organelle, including the mitochondria, and condensation of chromatin (7). However, it is interesting to note that not all these phenomena are seen in all cell types. Primary cell brain cultures, especially cerebellar granule neurons (8), have been established almost 30 years ago and have become, since then, very popular in vitro models to study not only aspects of developmental, functional, and pathological neurobiology but also to understand mechanisms related to neuronal apoptosis during developmental stages and in response to stress and toxicity. Physiological and toxic conditions determining apoptotic cell death in vivo brain can be easily mimicked changing the culture conditions.

In this chapter, we describe some methods to detect apoptotic cell death in brain cells in culture. The first protocol described (the Hoechst assay) represents an easy way to detect apoptotic morphological features (cell shrinkage and nuclear condensation) in neuronal primary cultures and primary astrocytes. Apoptosis is also characterized by the presence of changes which ultimately result in chromatin and DNA degradation. The TUNEL assay is a method to detect chromatin cleavage and allows the detection of apoptosis in individual cells. The DNA laddering is a classical biochemical method for demonstrating apoptosis based on the presence of oligonucleosomic-sized fragments of DNA. Protease activation is one of the most important events, which characterizes the process of apoptosis. Among proteases, caspases have been identified as the enzymes responsible for the proteolysis of

numerous nuclear and cytoplasmic proteins that are cleaved during apoptosis. Caspase activity can be assessed by detecting proteolytic cleavage of fluorescent substrates or by immunodetection.

A combination of these methods allows detection and quantification of apoptosis, and to distinguish between apoptotic changes (cell shrinkage, high cytoplasm density, membrane blebbing, compaction of chromatin, and formation of apoptotic bodies) and those indicative of necrosis (chromatin clumping, gross swelling of organelles, early membrane breakdown, and cell disintegration) measurable with different techniques. Usually necrotic damage predominates in response to acute, intense excitotoxic or free radical-related insults (i.e., high concentration of NMDA or OONO–). In contrast, neuronal cell death with apoptotic features developed over many hours after less severe insults (9). During the initial phases of apoptosis the plasma membrane remains intact, and most functions of the membrane are preserved. During this phase, vital dyes such as trypan blue are excluded: an effect of the apoptotic process is to spare surrounding cells from exposure to products which are being released from dying cells (10).In contrast, loss of membrane structural integrity is an early event during the necrosis (11) and results in the release of LDH in the medium and uptake of vital dyes such as trypan blue.

In addition to the methods described in this chapter, additional techniques can be used to detect apoptosis. Visual inspection by light microscopy represents a fast and inexpensive way to detect cell death. This can be achieved on living samples using phase contrast mode, to monitor the conditions of cultured cells. However, light microscope fails to recognize completely disintegrated cells, whose fragments are too small to be seen, especially when the fraction of dead cells is low. Electron microscopy allows detection of the first phases of chromatin condensation through the visualization of fine ultrastructural details, which became visible by light microscopy only during later stages (12).

2. Materials

2.1. Hoechst Assay

1. Locke's buffer: 10 mM HEPES, 25 mM KCl, 2.3 mM $CaCl_2$, 10 mM glucose, 5 mM $NaHCO_3$, 2 mM glutamax, 100 µg/ml gentamicine, 1.2 mM $MgCl_2$, and 130 mM NaCl.
2. Hank's balanced salt solution can be purchased from Invitrogen or prepared as a 10× stock solution as follows: 1.4 g/l $CaCl_2$, 4.0 g/l KCl, 0.6 g/l KH_2PO_4, 1.0 g/l $MgCl_2$ $6H_2O$, 1.0 g/l $MgSO_4 \cdot 7H_2O$, 80 g/l NaCl, $NaHCO_3$, 0.9 g/l $Na_2HPO_4 \cdot 7H_2O$, 10 g/l glucose, and 0.1 g/l phenol red.
3. 4% (w/v) Paraformaldehyde (PFA) solution: add 4 g PFA to 80 ml HBSS in a beaker and begin stirring while heating.

PFA should dissolve upon heating and stirring for a while (do not let the PFA solution boil). Add 100 µl 1 M NaOH to clear the solution. Once PFA is in solution, allow it to cool to room temperature. Bring the volume up to 100 ml using HBSS. Adjust the pH of the solution to ~7.4. Prepare a fresh solution before each experiment.

4. Phosphate-buffered saline (PBS) solution (pH 7.2) can be purchased from Invitrogen as a 10× stock solution and diluted using double-distilled water.
5. 10% Triton X-100 (TX) solution: add 1 ml of TX stock solution (minimum 98%) to 9 ml PBS.
6. 0.1% Triton X-100 solution: add 100 µl of 10% Triton X-100 solution to 9.9 ml of PBS.
7. 10 mg/ml Bisbenzimide trihydrochloride (Hoechst 33342) stock solution: add 10 mg of Hoechst 33342 to 1 ml of double-distilled water.
8. 5 µg/ml Bisbenzimide trihydrochloride (Hoechst 33342) solution: add 5 µl of Hoechst 33342 stock solution to 10 ml of double-distilled water.
9. Sterile round plastic or glass coverslips.
10. Antifade mounting medium.
11. Fluorescence Microscope (e.g., Nikon).
12. Poly-D-lysine hydrobromide (PLYS) stock solution: dissolve 10 mg of PLYS in 1 ml of high-quality distilled water. Dilute to 200 µg/ml for primary neurons and to 50µg/ml for primary astrocytes with water and sterilize by filtration through a 0.2-µm filter. Divide stock in aliquots and store at –20°C.

2.2. DNA Laddering

1. RNase A solution: add 100 mg RNase A to 10 ml of 15 mM NaCl and 10 ml 100 mM Tris–HCl (pH 7.4). Boil for 15 min, slowly cool to room temperature, and store in aliquots at –20°C for 6 months.
2. Tris buffer: 10 mM Tris–HCl (pH 8.0) and 10 mM EDTA. Store at room temperature indefinitely.
3. TAE electrophoresis buffer (50×, pH 8.5): 242 g Tris base, 57.1 ml glacial acetic acid, 37.2 g EDTA, in 800 ml double-distilled water. Adjust the volume to 1 l by adding double-distilled H_2O. Store at room temperature indefinitely.
4. Gel agarose: dissolve 1.8 g SeaKem agarose in 100 ml 1× TAE buffer. Microwave until dissolved, and then cool down. Once the solution is cool, add 10 µl ethidium bromide stock solution and pour into mold.
5. 10 mg/ml Ethidium bromide stock solution (Invitrogen).

6. Proteinase K solution: add 10 mg proteinase K to 1 ml of water.
7. Gel loading buffer (4×): 40% (w/v) sucrose, 0.25% (w/v) bromophenol blue in TAE buffer. Store up to 2 months at 4°C.
8. Load DNA marker VI (Roche).

2.3. TUNEL Assay

1. In situ cell death detection kit, fluorescein (from Roche-Boehringer Mannheim), including: TdT enzyme, FITC-labeled 11-deoxyuridine triphosphate (dUTP-FITC), and Td reaction buffer containing nucleotide mixture.
2. PBS/BSA solution: dissolve 0.5 g of serum albumine bovine (BSA) in 100 ml of PBS.
3. PBS/4′,6-diamino-2-phenylindole (DAPI) solution: dissolve DAPI in PBS to a final concentration of 0.5 μg/ml for immunocytochemistry.

2.4. Detection of Caspase 3 and Cleaved Caspase-3 by Immunoblotting

1. Lysis buffer: 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) deoxycholate, 0.1% (v/v) SDS. This buffer can be stored at room temperature, and protease inhibitors can be added immediately prior to use. Phosphatase inhibitors are optional, depending on whether the experiment is aimed at assessing phosphorylation of caspases.
2. Lysis buffer with protease inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 μM aprotinin, 50 μM leupeptin, 1 mM benzamidine, 1 μg/ml pepstatin, in lysis buffer.
3. Lysis buffer with phosphatase inhibitors: 10 mM sodium 3-glycerophosphate (pH 7.4), 1 mM sodium orthovanadate, 5 mM NaF, 2 mM pyrophosphate, in lysis buffer.
4. 200 mM Orthovanadate stock solution: add 366 mg of sodium orthovanadate to 10 ml of double-distilled water. Adjust pH to 10.0 with 1 M NaOH or 1 M HCl (the starting pH varies, depending on the lot). At pH 10.0, the solution is yellow. Boil the solution until it turns colorless (about 10 min). Allow solution to cool to room temperature. Readjust pH to 10.0. If the solution turns yellow again, boil until it turns colorless and readjust the pH. Repeat these steps until the solution remains colorless and the pH stabilizes at 10.0. Store in aliquots at –20°C (see Note 1).
5. Primary antibodies: rabbit polyclonal antisera generated against peptides corresponding to sequences of the following proteins: procaspase-3 and cleaved caspase-3 (Cell Signaling).
6. 4× Laemmli buffer (Invitrogen).
7. 10% Polyacrylamide premade gels (Invitrogen).
8. Polyvinylidene fluoride (PVDF) nylon membranes.

9. 1 M DTT solution (Sigma). Dilute to 50 mM with double-distilled water (see Note 2).
10. Tris–glycine buffer: 25 mM Tris–HCl (pH 8.3), 192 mM glycine, 10% (v/v) methanol in double-distilled water.
11. Tris-buffered saline (TBS): 100 ml 1.5 M NaCl solution, 100 ml 1.0 M Tris solution, and 800 ml distilled water.
12. Tris-buffered saline–Tween (TBS–T): Add 0.5 ml Tween 20 stock solution to 1 l of TBS.
13. 100% Polyoxyethylene-sorbitan-monolaurate (Tween 20) stock solution.
14. X-ray film (Eastman-Kodak).
15. 4–12% (w/v) gradient SDS–polyacrylamide gel (Invitrogen).

2.5. Assay of Caspase Activity

1. Buffer 1: 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 1 mM EGTA in double-distilled water. Immediately before use add 1 mM (final concentration) PMSF, 0.5 mM dithiothreitol (DTT), 10 μg/ml aprotinin, 10 μg/ml leupeptin in double-distilled water.
2. 0.4% (w/v) Trypan blue solution in PBS.
3. Buffer 2: 25 mM HEPES (pH 7.5), 0.15 (w/v) 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS), 10 mM DTT, 10 μg/ml aprotinin, 0.5 mM PMSF in double-distilled water.

3. Methods

3.1. Hoechst Assay

Hoechst 33342 is a cell-permeant dye which binds DNA. Hoechst 33342 fluoresces bright blue upon its binding to DNA allowing a morphological evaluation of the nucleus. Hoechst 33342 is water soluble and relatively nontoxic and it can be excited with a UV lamp. Cells are scored as apoptotic if they have chromatin condensation (Fig. 1). This method can also be applied to cells growing attached to coverslips or to chambered culture slides (see Note 3).

1. Plate primary neurons or astrocytes on sterile round plastic (or glass) coverslips. Coverslips are previously coated with Poly-D-lysine (200 μg/ml for primary neurons and 50 μg/ml for primary astrocytes).
2. At the end of the treatment, wash cells twice with pre-warmed Locke's buffer.
3. Incubate coverslips (cells) in pre-warmed PFA for 20 min at 37°C in the incubator.

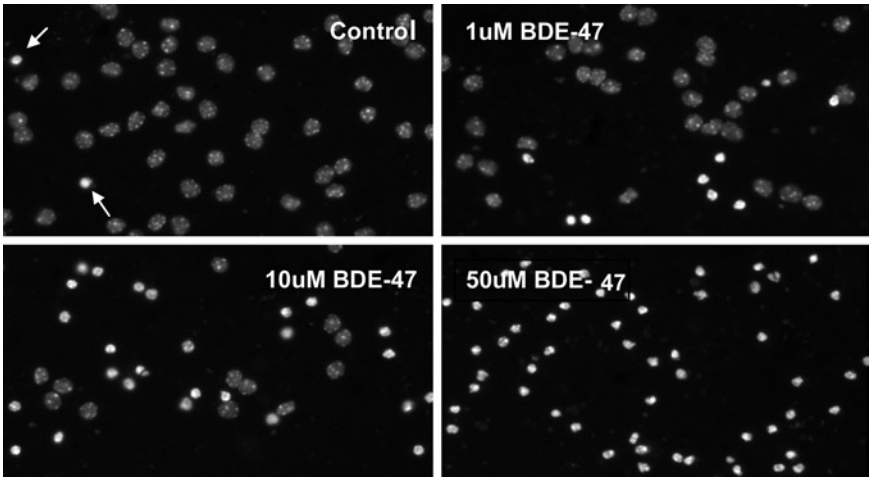

Fig. 1. Morphometric detection of apoptotis by Hoechst staining. Cerebellar granule cells were treated with indicated concentration of pentadiphenylether-47 (BDE-47) for 24 h. Apoptotic cells (indicated with *arrows* in the control image), detected as described in this chapter, are characterized by smaller size, nuclear fragmentation, and chromatin condensation (Magnification ×20).

4. Wash cells three times in PBS at room temperature using an orbital shaker (5 min for each wash).
5. Incubate coverslips in 1 ml/well PBS/TX solution for 10 min. Incubate plates on a shaker and shake gently.
6. Wash cells three times with PBS (5 min each, at room temperature, on a shaker).
7. Stain cells with the Hoechst solution in the dark for 10 min (wrap the plate in aluminum foil).
8. Wash cells three times with PBS (5 min each, at room temperature, on a shaker).
9. Remove the coverslips from the plates using a needle and a small forceps (in the dark).
10. Drain coverslips using a filter paper (do not touch the middle of the coverslips).
11. Mount coverslips on slides for a fluorescence microscope and cover using antifade mounting medium.
12. Cover slides with coverglass and seal using polish. A maximum of three coverslips can be used on each slide.
13. View by epifluorescence microscopy.

3.2. Apoptosis Assay using DNA Laddering

Apoptosis is associated with the fragmentation of chromosomal DNA into multiples of the 180 bp unit known as DNA laddering (13). In the DNA laddering assay, fragments of oligonucleosomal DNA are isolated, separated by electrophoresis and visualized using ethidium bromide (Fig. 2). The method we describe was

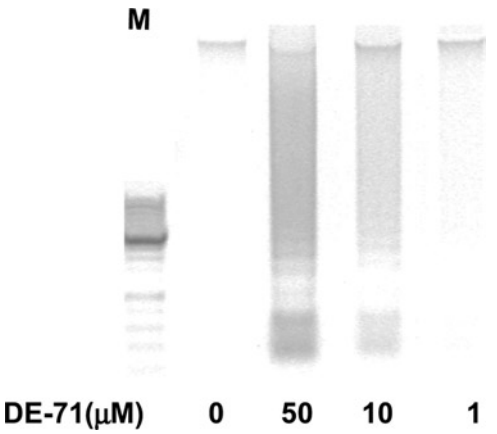

Fig. 2. DE-71-induced apoptosis in CGNs assessed by DNA laddering. Cerebellar granule cells were treated for 24 h with different concentrations of the pentadiphenylether (BDE) mixture DE-71. DNA laddering was performed as described in this chapter.

developed by Sorenson et al. (14) and does not require DNA purification.

1. Remove the medium from the plates. Use approximately 1×10^6 cells for each treatment.
2. Wash cells twice with pre-warmed Locke's buffer.
3. Collect cells in 300 μl of Tris buffer.
4. Incubate the cell suspension on ice for 30 min.
5. Gently swirl the tube to resuspend the cells.
6. Set aside 50 μl of this cell lysate. This sample can be saved for further analysis such as protein assay or immunoblot.
7. Centrifuge the remaining lysate at 15,000×*g* for 15 min at 4°C.
8. Transfer the supernatant to a fresh microcentrifuge tube. Add 0.25 ml cold ethanol (99%) and 10 μl 5 M NaCl. Mix and place in −20°C freezer overnight.
9. Centrifuge sample for 15 min at 15,000×*g*, 4°C. Remove and discard supernatant.
10. Place precipitate in speed vacuum lyophilizer for 20–25 min.
11. Add 20–30 μl TE buffer and 1 μl RNase stock solution. Incubate 1 h at 37°C (see Note 4).
12. Add 1 μl 20 mg/ml proteinase K solution and incubate an additional 1 h at 37°C.
13. Add Gel Loading Buffer (see Note 5).
14. Mount the gel in the electrophoresis tank and add just enough 1× TAE buffer to cover the gel.

15. Load DNA marker VI in the first well, and the samples in the other wells.
16. Run the gel at 70 mA until the bromophenol blue front is 1 or 2 cm from the end of the gel.
17. Photograph under UV light (see Note 6).

3.3. TUNEL Assay

DNA fragmentation is considered to be a key event in apoptosis and can be detected as a typical DNA ladder on agarose gels (note, however, that this method does not provide information regarding apoptosis in individual cells). DNA laddering can be assessed by enzymatic in situ labeling of 3′-OH ends of fragmented DNA permitting incorporation of labeled nucleotides into DNA strand breaks, using a terminal deoxynucleotidyl transferase (TdT) (15). This method is known as the TUNEL (TdT-mediated conjugated dUTP nick end-labeling) assay (see Note 7).

1. Plate cells on glass coverslips previously coated with 100 µg/ml Poly-D-lysine. Let neurons grow in culture for 11–13 days.
2. At the end of treatments, wash cells twice with HBSS.
3. Fix cells in cold 99.8% methanol (–20°C) for 5 min.
4. Rinse twice (5 min each) with PBS on an orbital shaker.
5. Prepare the TUNEL reaction mix according to the manufacturer's instructions.
6. Incubate cells with the TUNEL reaction mix for 60 min at 37°C in a humidified atmosphere in the dark. During this incubation, FITC-dUTP is incorporated to 3′-OH DNA fragments by the enzyme TdT.
7. Rinse twice (5 min each) with PBS/BSA on an orbital shaker.
8. Incubate with a fluorochrome (non-FITC)-conjugated secondary antibody, to visualize the antibody of interest for 60 min at room temperature in the dark.
9. Rinse twice with PBS/BSA.
10. Add 0.5 µg/ml DAPI to the cells.
11. Mount coverslips on slide for fluorescence microscope cover using antifade mounting medium (Vectashield Vector).
12. Analyze using a fluorescence microscope (see Note 8).

3.4. Detection of Caspase-3 and Cleaved Caspase-3 by Immunoblotting

Protease activation is one of the most important events that characterizes the process of apoptosis (16). Among the proteases implicated in apoptosis, caspases (cysteine-dependent, aspartate-specific protease) have been identified as the enzymes responsible for the proteolysis of numerous nuclear and cytoplasmic proteins

that are cleaved during the apoptotic process. The important role of caspases in apoptosis is supported by several lines of evidence. For example, low molecular weight peptide caspase inhibitors, as well as overexpression of endogenous caspase inhibitors, protect against apoptosis. Mice lacking the genes for caspase-3 or caspase-9 have severe defects in brain development, presumably due to the lack of extensive apoptosis that normally occurs during brain development. To date, 13 mammalian members of this family of enzymes have been described. All caspases are expressed as pro-enzymes (30–55 kDa) that contain three domains: an NH_2-terminal domain, a large subunit (17–35 kDa), and a small subunit (10–12 kDa). Activation involves proteolytic cleavage of domains, followed by the association of the large and small subunits to form a heterodimer. Detection of large or small subunits by western blotting can be used as a marker of caspase activation (Fig. 3). Among all caspases, the activation of caspase-3 has been shown most often to occur (17). A reasonable approach is thus to initially investigate the activation of caspase-3, and then examine activation of other up- and downstream caspases.

1. Aspirate the culture medium completely from the culture dish.
2. Add Locke's buffer and shake it gently to wash cells, then completely remove the Locke's buffer.
3. Wash cells once again with Locke's buffer.
4. Add 200–300 μl lysis buffer with proteases inhibitors to the cells, and scrape them off with the aid of a rubber policeman (do not trypsinize primary neurons!).
5. After measuring the protein levels, prepare samples loading equal amount of protein for each sample (see Note 9).
6. Add 4× sample buffer to each sample.

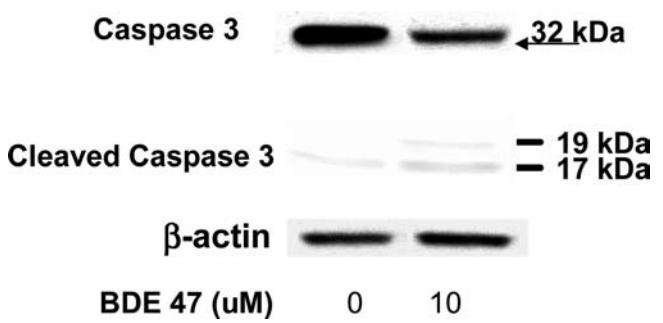

Fig. 3. Procaspase-3 and cleaved caspase-3 induced by PBE-47 treatment in mouse cerebellar homogenates. Cerebella were removed from pups 24 h after oral in vivo treatment with 10 mg/kg on postnatal day 10 (PND 10). Immunoblot was performed as described in this chapter.

7. Load samples into wells of a 4–12% (w/v) premade gradient SDS–polyacrylamide gel (see Note 10). Run the gel setting the current at 30 mA for 30 min then increase to 60 mA for about 90 min.
8. Transfer protein to a PVDF membrane using an apparatus for electroblot. Perform the transfer overnight at 4°C at 210 mA.
9. Block nonspecific binding sites by incubating blots in blocking solution for 1 h at room temperature on a shaker. To lower background and increase the sensitivity, PVDF membrane can be air dried before this step.
10. Dilute the primary antibody in blocking solution at a required dilutions (1:500 for anti-procaspase 3 antibody and 1:250 for anti-cleaved caspase-3 antibody). Flip the membrane over (see Note 11).
11. Incubate the membrane overnight at 4°C.
12. Remove the primary antibody and store it at 4°C if needed after short time. For long-term storage, leave the antibody dilution at –20°C. Flip the membrane over so that the side carrying the protein will return up.
13. Rinse the blot briefly three times in double-distilled water; wash the membrane in TBS-T (three times for 10 min). If a high background is present on the film after the first immunoblotting, it may be helpful to use a longer washing time or to use a different buffer, such as PBS-T.
14. Rinse the blot briefly three times in double-distillated water; wash it three times (10 min each) with TBS-T.
15. Incubate the blot for 1 h in secondary antibody solution containing secondary antibody HRP-conjugated.
16. Collect the secondary antibody solution, and save it at –20°C. (it can be reused for three or four times).
17. Rinse the blot briefly three times in double-distillated water and then wash it three times (10 min each) with TBS-T.
18. After a short wash in water, eliminate excess fluid from the membrane prior using a paper tissue (Kimwipe). Do not keep the paper tissue on the blot too long.
19. Place the blot in a plastic tray and add just enough ECL reagent to ensure complete coverage of the blot surface; incubate for 1 min.
20. Eliminate excess fluid from the membrane prior using the Kimwipe wiper. Do not keep the paper tissue on the blot too long.
21. Seal blot in a plastic bag and expose it to a X-ray film in the dark. Exposure times vary widely, from a few seconds to 10 min.

We usually develop the first exposure after 5 s and then adjust the exposure time based on this result (see Note 12).

3.5. Assay of Caspase Activity

Blotting with anti-caspase antibodies can be utilized to establish that caspase zymogens are cleaved during apoptosis. Unfortunately, this approach does not establish whether the resulting caspase fragments have enzymatic activity. One way to address this question is to determine whether caspase substrates have been cleaved. The demonstration that caspase substrates (polypeptides, including pro-caspases) have been cleaved provides evidence that caspases might have been activated. Release of a fluorogenic group from the peptide substrates is dependent on caspase activity and is proportional to the percentage of apoptotic cells in a cell population. In measuring caspase activation in cell lysis, it is critical that cell lysis does not cause caspase activation itself. To avoid release of lysosomal and other granule proteases, the use of detergent should be avoided as much as possible. A lysis procedure that avoids detergents is strongly recommended.

1. Wash cells twice in Locke's buffer. All further steps are performed at 4°C unless otherwise indicated.
2. Collect cells in a small volume of buffer 1. Typically, 0.5 ml buffer 1 are added to 1×10^7 cells. This usually yields a cytosolic extract with a protein concentration ranging from 0.4 to 0.7 mg/ml.
3. Incubate the sample for 20 min on ice.
4. Lyse the cells with 20–30 strokes in a tight-fitting Dounce homogenizer. Mix 3–20 µl homogenate with an equal volume of 0.4% (w/v) trypan blue and examine under a microscope.
5. Remove the nuclei by sedimentation ($800 \times g$ for 10 min). Supplement the supernatant with EDTA to a final concentration of 5 mM. Prepare cytosol from the post-nuclear supernatant by centrifugation at $100{,}000 \times g$ for 30 min in a Beckman ultracentrifuge using a Ti.1 rotor.
6. Freeze the cytosol in aliquots at –80°C.
7. Determine the protein concentration of the cytosol or other subcellular fraction using the method of Bradford (or other methods).
8. Thaw aliquots of cytosolic or subcellular fractions.
9. Load 30 µg cytosolic protein (about 50–60 µl) in a Eppendorf tube.
10. Add 200 µl of freshly prepared buffer 2, containing fluorescent substrate to the samples (see Note 13).
11. Incubate at 37°C for 30 min in a microplate reader.

12. Continuous monitoring of fluorochrome release can be utilized to examine the kinetics of product release.
13. Measure the fluorescence in a fluorometer using an excitation wavelength of 360 nm and an emission wavelength of 475 nm.

4. Notes

1. Activation depolymerizes the orthovanadate, converting it to a more potent inhibitor of protein tyrosine phosphatases.
2. Dithiothreitol (DTT) is used to maintain sulfhydryl (–SH) groups in the reduced state. By reducing the disulfide bonds, DTT preserves enzymatic activity. DTT is soluble in water and has less odor, and lower toxicity, than other thiol compounds (e.g., 2-mercaptoethanol).
3. In the Hoechst assay, a minimum of 300 cells for each treatment should be evaluated.
4. The presence of RNA fragments can generate an unusual band in the middle of the gel in all lanes. In this case, incubate gel longer with RNase.
5. Samples can become viscous and this can make it difficult to load them on the gel. To avoid this, dilute samples using the 1× DNA loading buffer.
6. If bands are difficult to visualize because of a high background level, wash gel abundantly with water.
7. Apoptosis can occur also without DNA degradation. In this case, an independent assay, along with the TUNEL assay, should be used to confirm whether cells have undergone apoptosis. Costaining cells with Hoechst is a very useful method to discriminate between apoptosis and necrosis in samples found positive in the TUNEL assay.
8. The presence of background in TUNEL-treated samples can be caused by the presence of DNase-contaminated test tubes, plates, or buffers.
9. Using the right amount of protein is very important since overloading the gel will result in band distortion during the separation process. In contrast, underloading the gel will result in weak bands, too faint to be recorded and quantified. In addition, the correlation between amount of target protein and intensity of the band is linear within a limited range. To ensure that the amount of proteins fall within that range (before starting to work on a new protein), we load crescent amount of protein and plot them against the relative band intensities.

10. Polyacrylamide gels work as porous matrix where two protein of different size but identical charge densities can be separated when subjected to an electric field. Gels with low concentration of polyacrylamide are necessary to separate protein with higher molecular weight, while higher concentrations work better for low molecular weight protein. For the detection of caspases and their products, we use 4–12% (w/v) premade gradient SDS–polyacrylamide gels, which allow separation of proteins with molecular weights between 14,000 and 280,000. In alternative, a 10% SDS–polyacrylamide gel can be used.
11. It is possible to incubate the membrane in primary antibody for shorter periods of time (e.g., 1 h), but this requires a higher concentration of antibody without any improvement in the signal-to-noise ratio. Flip the membrane over during the primary antibody incubation so that the face of the membrane carrying protein will be face down. Make sure that no bubbles are trapped between the blot and the bottom of the tray. This way, a thin layer of antibody solution will be trapped between the membrane and the bottom of the plastic tray ensuring a very efficient antibody-protein binding; furthermore, very little amount of primary antibody is necessary.
12. The presence of many bands on the film indicates that the concentration of primary and/or secondary antibodies are too high. In this case, decrease the concentration of antibodies or increase the washing time with TBS-T. If no bands are detected on the film, increase the concentration of primary antibodies first, and then the concentration of the secondary antibody. If white spots are present, bubbles may have formed during the protein transfer. In this case, the whole procedure must be repeated.
13. A number of caspase substrates conjugated to fluorogenic groups are now commercially available. These include AFC (7-amino-4-trifluoromethyl coumarin), MNA (4-methoxy-2-naphthylamine), and pNA (*p*-nitroaniline).

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Chapter 13

Measurement of Isoprostanes as Markers of Oxidative Stress

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Abstract

Oxidative stress results from an imbalance between production of reactive oxygen and nitrogen species (ROS and RNS, respectively) and endogenous antioxidant defense mechanisms. Increased generation of ROS/RNS is implicated in the pathogenesis of a variety of human diseases, including neurodegenerative disease, atherosclerosis, cancer, and aging. However, measuring oxidative stress in biological systems is complex and requires accurate quantification of either free radicals or damaged biomolecules. One method to quantify oxidative injury is to measure lipid peroxidation. Lipids are readily attacked by free radicals, resulting in the formation of a number of peroxidation products. F_2-isoprostanes (F_2-IsoPs) are one group of these compounds and they are derived by the free radical peroxidation of arachidonic acid (AA). The F_2-IsoPs, prostaglandine F_2-like compounds, provide an accurate measure of oxidative stress both in vitro and in vivo. This protocol details current methodology used to quantify these molecules using gas chromatography-mass spectrometry (GC/MS).

Key words: F_2-isoprostanes, Oxidative damage, Lipid peroxidation, Reactive oxygen species

1. Introduction

Oxidative stress is a prominent feature of many acute and chronic diseases including cancer, cardiovascular disease, neurodegenerative disease, lung disease, and even the normal aging process (1–3). When excess formation of free radicals overwhelms the capacity of endogenous cellular antioxidant defense mechanisms, these reactive species may cause cell and organ damage by distressing the normal physiology and even activate and/or accelerate disease processes. Free radicals can be generated endogenously from various sources (for example, mitochondria and oxidative burst during phagocyte activation) or derived from exogenous sources, such as environmental toxins and cigarette smoke (4).

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Reactive radicals readily attack a variety of critical biological molecules, including lipids, DNA, and essential cellular proteins. Hence, the high content of unsaturated lipids in the brain leads to pronounced lipid peroxidation, the central feature of oxidant injury in neuronal and glial cells.

Lipid peroxidation is the mechanism by which lipids are attacked by chemical species that have sufficient reactivity to abstract a hydrogen atom from a methylene carbon in their chain. Lipid peroxidation, through a free radical pathway, requires a polyunsaturated fatty acid (PUFA) and a reactant oxidant inducer that together form a free-radical intermediate. The free-radical intermediate subsequently reacts with oxygen to generate a peroxyl radical, which, in turn, with unpaired electrons may additionally abstract a hydrogen atom from another PUFA, thus initiating a propagation reaction that spreads as a brushfire. Hence, greater number of double bonds in the molecule and higher instability of hydrogen atom adjacent to the double bond explains why unsaturated lipids are particularly susceptible to peroxidation (5, 6). Such reactions have been long recognized, but the biological importance of lipid peroxidation has been explored only in the last three decades (7).

There are a number of analytical approaches, which permit the quantification of lipid peroxidation, or free radical-catalyzed damage to DNA or proteins (8). However, many of these techniques suffer from lack of sensitivity and specificity. In a recent multi-investigator study, termed the Biomarkers of Oxidative Stress Study (BOSS), sponsored by the National Institutes of Health, it was found that the quantification of F_2-IsoPs represents the most accurate method to assess oxidative stress status in vivo (9). F_2-IsoPs are prostaglandin-like compounds which are produced by a noncyclooxygenase free radical-catalyzed mechanism involving the peroxidation of the PUFA, arachidonic acid (AA, C20:4, ω-6). Formation of these compounds initially involves the generation of four positional peroxyl radical isomers of arachidonate, which undergo endocyclization to PGG_2-like compounds. These intermediates are reduced to form four F_2-IsoP regioisomers, each of which can consist of eight racemic diastereomers (10). In contrast to cyclooxygenase (COX)-derived prostaglandins (PGs), nonenzymatic generation of F_2-IsoPs favors the formation of compounds in which the stereochemistry of the side chains has a *cis* orientation in relation to the prostane ring. A second important difference between F_2-IsoPs and PGs is that F_2-IsoPs are formed primarily in situ, esterified to phospholipids, and subsequently released by a phospholipases (11, 12), whereas PGs are generated only from free AA (13).

Several methods have been developed to quantify the F_2-IsoPs from biological materials (3). Our laboratory uses a gas

chromatography/mass spectrometry (GC/MS) to quantify the F_2-IsoPs, methodology which was originally established at our University (by the pioneering work of Dr. Roberts and Dr. Morrow, Vanderbilt University Medical School) (10, 13). More specifically, after isolation and derivatization of the F_2-IsoPs, we take advantage of stable isotope dilution, negative ion chemical ionization (NICI) GC/MS with select ion monitoring (SIM) for quantification. This methodology allows the lower limit of detection of the F_2-IsoPs to be in the low picogram range. These properties, along with the assay's high sensitivity and specificity, allow the F_2-IsoPs to be an excellent biomarkers of and the most robust and sensitive measure of oxidative stress. Accordingly, we highlight this method and address purification and derivatization of the compounds for analysis by GC/MS.

2. Materials

1. Tissue or cell samples, fresh or frozen. It is important to process the samples immediately after isolation or assure their immediate storage at −80°C for later quantification.
2. Blade homogenizer and sonicator for tissue processing.
3. Folch solution: 2:1 (v/v) chloroform/methanol, ice-cold, containing 0.005% (w/v) butylated hydroxytoluene (BHT). A free radical scavenging agent such as BHT is added to the organic solvent during extraction of phospholipids to prevent oxidation and formation of F_2-IsoPs.
4. 0.9% (w/v) NaCl solution. Store at room temperature.
5. Organic solvents: ethyl acetate, heptane, chloroform, ethanol, acetonitrile, and methanol, with and without 0.005% (v/v) BHT.
6. 15% (w/v) KOH solution (to release esterified isoprostanes).
7. 1 M HCl and pH 3 water, adjusted by adding 1N HCl (used to acidify the sample before solid-phase extraction (SPE)) (see Note 3).
8. Deuterated standard: deuterium-labeled isoprostane, [$^2H_4$] 15-F_{2t}-IsoP (8-iso-$PGF_2\alpha$) (Cayman Chemical, Ann Arbor, MI) (see Note 4).
9. Anhydrous Na_2SO_4: to dry ethyl acetate/heptane eluate from the C18 Sep-Pak (see Note 5).
10. Sep-Pak Plus C18 cartridge [(Waters Associates, Milford, MA), Silica Sep-Pak cartridge (Waters, Milford, MA) and 10-ml plastic syringe for normal-phase SPE].

11. 10% (v/v) Pentafluorobenzyl bromide (PFBB) solution in anhydrous acetonitrile.
12. 10% (v/v) *N,N*-diisopropylethylamine (DIPE) solution in anhydrous acetonitrile.
13. Thin-layer chromatography (TLC) plates: 5×20 cm glass plates covered with a 250 μm layer of silica gel particles 60 Å in diameter (Partisil LK6D; Whatman, Maidstone, England) and TLC developing chamber.
14. TLC standard: prostaglandin $F_2\alpha$ ($PGF_2\alpha$) methyl ester diluted in methanol (Cayman Chemical, Ann Arbor, MI).
15. 10% Phosphomolybdic acid in ethanol (to visualize sample migration on TLC plates wormed with hot plate).
16. Dimethylformamide (DMF) and undecane: stored over calcium hydride to prevent water accumulation (see Note 7).
17. Bis(trimethylsilyl)trifluoroacetamide (BSTFA, Supelco): stored at room temperature.
18. 15-ml Polypropylene culture tube with cap, 20-ml scintillation vial, 5-ml glass Reacti-Vial with Teflon-lined cap, and 1.5-ml microcentrifuge tube: for sample processing/chemical reactions.
19. Nitrogen gas and methane: for sample evaporations and mass spectrometry.
20. Temperature-controlled water bath, centrifuge, 95°C oven and hair dryer.
21. 15-m, 0.25-mm diameter, 0.25-μm film thickness DB1701-fused silica capillary GC column and gas chromatography (GC)/mass spectroscopy (MS) system.

3. Methods

Measurement of F_2-IsoPs has revolutionized our ability to quantify oxidative injury in cell/tissue samples. F_2-IsoPs are stable, robust molecules and are detectable not only in cells and tissues, but also in biological fluids, such as plasma, urine, cerebrospinal fluid, and bronchoalveolar lavage fluid. As F_2-IsoPs can be readily generated during purification/derivatization of biological materials containing arachidonoyl-containing lipids, it is important to process the samples immediately after isolation or assure their immediate storage at −80°C for later quantification. Formation of F_2-IsoPs does not occur, if a free radical scavenging agent such as BHT is added to the organic solvent during extraction of phospholipids or if the samples are rapidly frozen in liquid nitrogen prior to placement at −80°C.

3.1. Lipid Extraction and Hydrolysis of F_2-IsoPs-Containing Phospholipids in Cell/Tissue Samples

Formation of F_2-IsoPs occurs in situ in the phospholipid bilayer and then subsequently released in free form. This creates two forms of F_2-IsoPs, one that remains esterified in the membrane and a second that is hydrolyzed and released in free form. To quantify total F_2-IsoPs formation, both free and esterified F_2-isoPs are analyzed. It is necessary to extract the phospholiopids from the cell/tissue and release the F_2-isoPs from the phospholipids via base hydrolysis.

1. Add 0.05–0.25 g fresh or frozen samples (0.05–0.25 g) to 5 ml ice-cold Folch solution containing 0.005% BHT in a polypropylene culture tube with cap. Homogenize the sample with a blade homogenizer for approximately 30 s. Use the second aliquot of ice-cold Folch solution, added to a separate culture tube, to wash the blade homogenizer, and to ensure that all sample tissue is recovered, as tissue can adhere to or become lodged inside the blade of the homogenizer. Combine the two aliquots, cover with a nitrogen blanket, and mix every 10 min over 30 min at 25°C to allow maximal extraction of lipids from homogenized tissue (see Note 1).
2. Mix the lipid extracts vigorously with 2.0 ml 0.95 (w/v) NaCl, and separate the phases by centrifugation at $800 \times g$ for 10 min at 25°C. After centrifugation, discard the upper aqueous layer and carefully separate the lower organic layer from the intermediate semisolid proteinaceous layer. Evaporate the organic layer to dryness under a stream of nitrogen (see Note 2).
3. Dissolve total lipids in 0.5 ml methanol containing 0.005% BHT and store at –80°C or, if you process them further, add 0.5 ml of aqueous 15% KOH to the residue, so that lipid extracts are saponified to release esterified isoprostanes. Sonicate the mixture and mix vigorously until thoroughly suspended, and heat at 37°C for 30 min to affect hydrolysis and release of the F_2-IsoPs. Acidify the mixture to pH 3 with 1 M HCl (~1.2 ml) and dilute to a final volume of 10 ml with pH 3 water in preparation for the purification of F_2-IsoPs with SPE (see Note 3).

3.2. Sample Purification for Mass Spectrometric Analysis

1. Following acidification of the sample to pH 3 with 1 M HCl, add 200–1,000 pg deuterated standard. Vortex the mixture and isolate F_2-IsoPs using reversed-phase and normal-phase SPE (see Note 4).
2. Use a 10-ml plastic syringe to elute the sample and subsequent solvents through the Sep-Pak cartridge. For reverse phase, precondition Sep-Pak Plus C18 columns (each cartridge contains 500 mg of C18) with 5 ml methanol (flow rate, ~1.0 ml/min) and 7.0 ml H_2O (adjusted to pH 3.0 with 1 N HCl). Once the sample has been added, wash

the column sequentially with 10 ml water (pH 3) and 10 ml heptane, which removes nonpolar contaminates including unoxidized AA. The F_2-IsoPs are eluted with 10 ml of ethyl acetate/heptane (50:50, v/v) into a 20-ml scintillation vial.

3. Dry the ethyl acetate/heptane eluate from the C18 Sep-Pak over anhydrous Na_2SO_4 and apply to a silica Sep-Pak cartridge (each cartridge contains 500 mg of silica), which has been preconditioned with 5 ml of ethyl acetate. Once the sample has been added, wash the column with 5 ml ethyl acetate, and elute the F_2-IsoPs with 5 ml 50:50 (v/v) ethyl acetate/methanol into a 5-ml glass react-a-vial (with Teflon-lined cap). For normal-phase SPE, use Sep-Pak Plus Silica columns with a flow rate of ~0.5 ml/min throughout (see Note 5).

3.3. Conversion of F_2-IsoPs to Corresponding Pentafluorbenzyl Esters

Dry isoprostanes isolated in ethyl acetate/methanol eluate by SPE at 37°C under a nitrogen stream and derivatize them to pentafluorbenzyl (PFB) esters. Vigorously mix samples with 40 µl 10:90 (v/v) PFBB: anhydrous acetonitrile plus 20 µl 10:90 (v/v) diisopropylethylamine: anhydrous acetonitrile. Following reaction at 37°C for 20 min, dry the esters under a nitrogen stream and dissolve them in 50 µl 2:3 (v/v) chloroform:methanol.

3.4. Thin-Layer Chromatography

1. TLC is accomplished with 5 × 20 cm glass plates covered with a 250 µm layer of silica gel particles 60 Å in diameter. Just before use, wash the plates with ethyl 90:10 (v/v) acetate:ethanol, activate at 95°C for 20 min, and cool in a dessicator. A TLC chamber is lined with filter paper and conditioned 30 min with 100 ml 93:7 (v/v) chloroform: ethanol.
2. Apply dissolved samples in 50 µl chloroform: methanol to the upper half of pre-adsorbent in four prescored lanes and dry 5–10 s with a hair dryer. Add sample plates to both ends of the chamber. In contrast, TLC standard (5 µg methyl ester of $PGF_{2\alpha}$/5 µl CH_3OH) is applied to a separate plate that is positioned toward the center of the TLC chamber. After the chamber is rapidly closed, solvent is allowed to migrate 13 cm, and the plates removed.
3. Scrape samples from silica plates in the region of the TLC standard and visualize by spraying with a 10% solution of phosphomolybdic acid in ethanol followed by heating. Scrape the areas 1 cm below and 1 cm above $PGF_{2\alpha}$ (R_f ~ 0.15), and extract from the silica with 1 ml ethyl acetate.
4. Following centrifugation at 13,000 × *g* for 3 min at 4°C, transfer isoprostane pentafluorobenzyl esters in the ethyl acetate into a virgin microcentrifuge tube and store at –80°C, or further process samples for GC/MS analysis (see Note 6).

3.5. Formation of Trimethylsilyl Ether Derivatives and Quantification of F_2-IsoPs

1. After drying samples under a nitrogen stream, dissolve them in 8 µl DMF and mix with 20 µl bis(trimethylsilyl)trifluoroacetamide (BSTFA) to covert the residue to the trimethylsilyl ether derivatives.
2. After heating for 5.0 min at 37°C, dry silylated samples at 37°C under a nitrogen stream, redissolve in 20 µl of undecane, which has been dried over calcium hydride, and transfer into autosampler vial for GC/MS analysis (see Note 7).
3. For quantification of F_2-IsoPs, we routinely use a Hewlett Packard 5982A GC/MS system interfaced with an IBM Pentium computer. GC is performed using a 15-m, 0.25-mm diameter, 0.25-µm film thickness DB1701-fused silica capillary column. The column temperature is programmed from 190 to 290° at 20°/min. Methane is used as the carrier gas for NICI at a flow rate of 1 ml/min. Ion source temperature is 250°C, electron energy is 70 eV, and the filament current is 0.25 mA.

The major ions generated in the NICI mass spectra of the pentafluorobenzyl ester, tris–trimethylsilyl ether derivatives of F_2-IsoP are *m/z* 569 and corresponding ion for the [$^2H_4$]15-F_{2t}-IsoP internal standard, *m/z* 573 (Fig. 1). For quantification purposes, we compare the height of the pick containing derivatized F_2-IsoPs (*m/z* 569) with the height of the deuterated internal standard peak (*m/z* 573). The coefficient of variance for the assay is routinely less than 8% (see Notes 8 and 9).

4. Notes

1. Presence of BHT during extraction and hydrolysis is important to inhibit additional formation of F_2-IsoPs; since polystyrene is not resistant to chloroform and its subsequent interference with the analytical procedures, it is recommended that lipid extraction be carried out in polypropylene tubes; keep on ice.
2. The organic layer and the proteinaceous layer can be readily separated by carefully pouring off the organic layer into a new culture tube. If the proteinaceous layer is small, because of the type and size of the cell/tissue sample, it is often easier to remove the aqueous and proteinaceous layers simultaneously via suction. However, care must be taken not to compromise the organic phase, if this approach is used.
3. It is important to dilute the methanol in this solution to 5% or less to ensure proper column extraction of free F_2-IsoPs in the subsequent purification procedure.

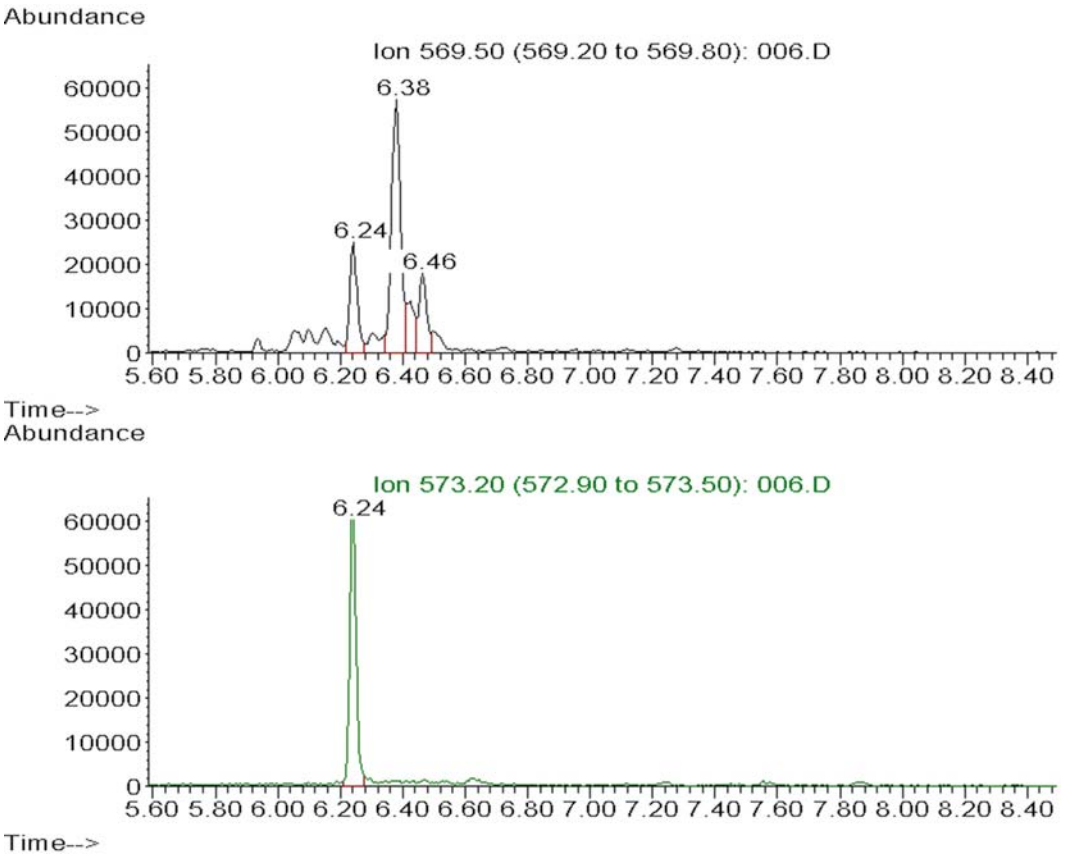

Fig. 1. Chromatograms of F_2-IsoPs from tissue sample. Chromatograms plot abundance vs. time (min) with *m/z* 569 chromatogram showing F_2-IsoPs and *m/z* 573 chromatogram showing internal standard.

4. The internal standard is a deuterium-labeled isoprostane, [$^2H_4$]15-F_{2t}-IsoP (8-iso-PGF_{2a}). The amount of internal standard added depends on the levels of F_2-IsoPs in the sample as well as the sensitivity of the mass spectrometer. For low-level samples such as cerebrospinal fluid (CSF), less internal standard needs to be added. Samples that consist of a particularly large amount of tissue will require more internal standard. This is because complex samples such as brain tissue, despite our best purification efforts, will still contain some unwanted compounds that may potentially have the same *m/z* (mass-to-charge) ratio as the internal standard when analyzed by GC/MS. Increasing the amount of internal standard to 1,000 pg in these samples minimizes the variability in the internal standard ion channel due to contamination in the tissue sample.
5. Drying of ethyl acetate/heptane eluate should be completed promptly, as Na_2SO_4 has been shown to adsorb lipids to some

degree. Care must be taken not to transfer any Na_2SO_4 to the silica Sep-Pak cartridge.

6. Ethyl acetate should be carefully removed without disrupting the silica pellet in the bottom of the tube (silica may affect the instrument's sensitivity). Avoid applying samples to the first 1 cm of the plate and do not spray the sample plate.
7. DMF should be stored over calcium hydride to prevent water accumulation. Similar to the amount of internal standard that is added to a sample, consideration should be given to the amount of undecane that is used to dissolve the derivatized sample. The amount added will depend on the levels of F_2-IsoPs. Samples that are rich in F_2-IsoPs will require greater amounts of undecane to keep them from overloading the column during GC. Likewise, low-level samples will require less undecane in order for the GC/MS signal to be of sufficient intensity for optimal quantification.
8. Quantification of the F_2-IsoPs levels may also be achieved by comparing the areas of the appropriate peaks in the *m/z* 569 SIM chromatogram of the F_2-IsoPs to that of the peak of the internal standard in the *m/z* 573 SIM chromatogram (Fig. 1).
9. In general, 12 samples can be assayed for F_2-IsoPs in approximately 10 h by an experienced investigator. Homogenization, lipid extraction, and hydrolysis of this number of samples require ~3 h; Sep-Pac purifications take ~2 h; drying, derivatization, and TLC purification require ~3 h; and drying and silylation require ~2 h. Though compared with other assays of oxidative stress, the time requirement for this assay is relatively large, it is noteworthy that the present assay has the greatest sensitivity and specificity for the detection of lipid peroxidation. Mass spectrometric analysis is automated and each sample requires ~15 min of instrument time. If the peak signal is low or if no peaks are detected by the mass spectrometer, the samples should be removed from the autosampler vial, washed by ethyl acetate, dried under nitrogen, and the conversion procedure to silylether derivative should be repeated. If the internal standard is detected at *m/z* 573, but there is low or nonexistent peak at 569, then levels of F_2-IsoPs are below the limit of detection.

Acknowledgments

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Chapter 14

Assessment of Glutathione Homeostasis

Gennaro Giordano, Collin C. White, and Lucio G. Costa

Abstract

The tripeptide glutathione (γ-glutamylcysteinylglycine; GSH) is the most abundant antioxidant thiol in the brain. GSH plays a critical role in protecting brain cells from oxidative stress and xenobiotics, as well as maintaining the thiol redox state. High levels of GSH are present in the central nervous system, particularly in astrocytes. GSH is synthesized into two enzymatic steps, the first, and the rate-limiting one, is catalyzed by glutamate–cysteine ligase (GCL) to form a dipeptide which is then converted to GSH by GSH synthetase. In this chapter, we describe assays for the measurements of GSH levels and GCL activity. The first spectrophotometric assay is based on the affinity of 2,3-naphthalenedicarboxaldehyde (NDA) for GSH. In the second assay, GSH levels are measured after being derivatized using the fluorescent thiol reactive compound monobromobimane (MBB) and are detected by high-performance liquid chromatography (HPLC). The third assay allows the assessment of GCL activity, also by HPLC.

Key words: Glutathione, Glutamate–cysteine ligase, HPLC

1. Introduction

The tripeptide glutathione (γ-glutamylcysteinylglycine; GSH) is a major antioxidant in the central nervous system (1), playing a critical role in protecting brain cells from oxidative stress and xenobiotics, as well as maintaining the thiol redox state. Concentrations of GSH in the brain are in the range of 2–3 mM, which is higher than that in blood (2), and GSH levels are higher in astrocytes than in neurons. GSH is taken up in the mitochondria where its content is higher than in the cytosol.

GSH is synthesized in the cytosol by the action of two enzymes: glutamate–cysteine ligase (GCL) and GSH synthetase. GCL is the rate-limiting enzyme in GSH synthesis. The enzyme is heterodimeric, with a large catalytic subunit (73 kDa) and a small modifier subunit (30 kDa). The modifier subunit modulates

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enzyme activity by affecting the affinity of the catalytic subunit for both substrates and inhibitors.

Oxidative stress is defined as the imbalance between the production and detoxification of reactive oxygen species (ROS) and can be of exogenous or endogenous origin. Exogenous stress can be caused by xenobiotics, drugs, and ionizing radiation, while endogenous oxidative stress results primarily from mitochondrial electron transport, and also from stimulated phagocytic cells undergoing oxidative burst, ischemia–reperfusion, inhibition of antioxidant enzymes, or induction of pro-oxidant enzymes. GSH plays an important role in the detoxification of ROS, particularly in brain, as brain cells are particularly vulnerable to oxidative stress. The brain utilizes 20% of the oxygen consumed by the body, despite being only 2% of the total body weight; this generates high quantity of ROS as result of the oxidative phosphorylation (3). The brain has lower superoxide dismutase and catalase activities, compared with other organs such as liver or kidney, and cell brain membranes are enriched with lipids containing unsaturated fatty acids that are targets of lipid peroxidation (4). Furthermore, some brain areas are enriched in iron content which can catalyze the generation of ROS (5). In addition, the loss of neurons caused by oxidative stress damage cannot be compensated by the generation of new neurons.

GSH acts through two types or reactions. GSH reacts nonenzymatically with radicals such as superoxide radical anions and hydroxyl radicals. GSH can also function as an electron donor, inducing the reduction of peroxides. The final product of the oxidation of GSH is glutathione disulfide (GSSG). Within cells, GSH is regenerated from GSSG by a reaction catalyzed by glutathione reductase (6). The ratio of GSH/GSSG in mitochondria under normal conditions is 18:1. A change in the GSH/GSSG ratio has adverse effects on mitochondria and subsequently on cells. Through *S*-glutathionylation, the reversible formation of mixed disulfides between protein thiols, GSH also prevents irreversible oxidation of proteins (7).

Intracellular GSH levels are tightly regulated by a complex mechanism involving control of synthesis, transport, and utilization. Failure to maintain adequate intracellular supply can be lethal for the cell and alterations in glutathione homeostasis have been shown to be involved in the pathogenesis of neurodegenerative and neuropsychiatric diseases such as Parkinson's disease (8), Alzheimer's disease (9), schizophrenia (10), or Huntington's disease (11).

The assays described in this chapter allow the measurements of GSH and GSSG levels and of GCL activity in any type of neuronal or astroglial cell in culture or in brain tissue. The first assay is based on the affinity of 2,3-naphthalenedicarboxaldehyde (NDA) for total GSH and combines the sensitivity of fluorometric detection and the convenience of a 96-well microtiter plate format. The second assay is based on high-performance liquid

chromatography (HPLC). The fluorescent thiol-reactive compound monobromobimane (MBB) is used to derivatize the reaction products, which are then separated by HPLC and quantified by fluorescence detection. Finally, the third assay allows the measurement of GCL activity, by relying on the derivatization of the reaction product. The sensitivity of this HPLC assay is sufficient to allow for measurements of GCL activity in cultured cells with low-GCL activity.

2. Materials

2.1. Measurement of Intracellular Total Glutathione Levels with the NDA Assay

1. Protein assay: bicinchoninic acid (BCA) assay kit (Pierce).
2. Locke's buffer: 10 mM HEPES, 5.5 mM KCl, 10 mM glucose, 5 mM $NaHCO_3$, and 130 mM NaCl.
3. 200 mM 5-Sulfosalicylic acid dehydrate (SSA) stock solution: dissolve 4.36 g in 100 ml of double-distilled water (dH_2O). Filter using a 0.2-μm filter.
4. Benchtop centrifuge.
5. 8 M 4-Ethylmorpholine (NEM) stock solution (Sigma).
6. 10 M Potassium hydroxide (KOH) stock solution: dissolve 56 g KOH in dH_2O. Bring volume up to 100 ml. Store at room temperature.
7. 1 M Potassium hydroxide (KOH) solution: dilute KOH stock solution 1:10 using water.
8. 0.2 M *N*-ethylmorpholine/0.02 M KOH solution: add 2.5 ml of NEM stock solution and 2.0 ml of 1 M KOH to 95 ml of dH_2O.
9. 10 mM glutathione (GSH) stock solution: dissolve 30.7 mg in 10 ml of 200 mM SSA.
10. 10 mM tris (2-Carboxyethyl) phosphine (TCEP) stock solution: dissolve 2.86 mg in 1 ml of dH_2O. Make it fresh before the experiment and keep it on ice until needed (see Note 1).
11. 10 mM NDA stock solution: dissolve 3.7 mg in 2 ml of dimethylsulfoxide.
12. Collection buffer: dilute Locke's buffer with (1:2) with dH_2O.
13. Microplate reader (e.g., Molecular Device).

2.2. Measurement of Intracellular Reduced and Oxidized Glutathione Levels by HPLC

1. 25 mM MBB stock solution: dissolve MBB in acetonitrile. Store in aliquots at –20°C (see Note 2).
2. 12.5 mM MBB solution: dilute 25 mM MBB solution in acetonitrile.

3. TES/SB homogenization buffer: 0.25 M sucrose, 20 mM Tris, 1 mM EDTA, 20 mM boric acid, and 1 mM L-serine. Adjust pH to 7.4 with 1 N HCl. Store up to 6 months at 4°C (see Note 3).
4. GSH standard solution: dilute GSH stock solution (10 mM) in TES/SB buffer to desired concentrations: 0.1, 0.25, 0.5, 0.75, and 1.0 mM.
5. pH paper: colorPhast pH 2–9 (EM Science).
6. 1 mM Tetrabutylammonium phosphate (TBAP; Regis) stock solution: adjust to pH 3.0 with 10% (v/v) phosphoric acid.
7. HPLC-grade methanol.
8. HPLC system, e.g., Shimadzu SCL-10A VP with autosampler, C-R8A integrator, and RF-10Axl fluorescence detector (or equivalent).
9. C18 guard column cartridge system (Phenomenex).
10. 150-mm reversed-phase synergy C18 HPLC column (Phenomenex).

2.3. Measurement of GCL activity by HPLC

1. Cysteine stock solution: dissolve 12.12 mg in 10 ml of dH_2O.
2. Microcon centrifugal filter device with a 10-kDa molecular mass cutoff filter.
3. GCL reaction cocktail solution (CS): add 1.2 g ATP, 1.7 g glutamic acid, 37 mg EDTA, 406 mg $MgCl_2$, 2.42 g Tris to 100 ml of dH_2O. Store in aliquots at –20°C.

3. Methods

3.1. Measurement of Intracellular Total Glutathione Levels by the NDA Assay

This method utilizes NDA, which binds GSH forming a cyclic reaction product with the cysteine sulfhydryl group and the glutamyl amino group of GSH. A cyclic compound with essentially equivalent fluorescence characteristics can also result from the reaction of NDA with γ-glutamate cysteine. The excitation and emission of these reaction products are similar to the ones for the fluorescein, and can be measured in instruments that are equipped with a argon–ion laser light. The assay allows measurement of total intracellular GSH levels and combines the sensitivity of fluorometric detection with the convenience of a 96-well microtiter plate format (12).

1. Collect cells using collection buffer. Save aliquots to carry out the BCA protein assay.
2. To complete cell lysis, dilute samples adding 1 volume of SSA stock solution to 1 volume of neuronal lysate. Use at least 20 μg of protein for each sample. Adjust the volume accordingly.

3. Keep samples on ice for at least 15 min, vortexing vigorously. Check samples under microscope to ensure lysis.
4. Centrifuge samples at 12,000×*g* for 3 min in a cold room. Discard the pellet and save the supernatant.
5. Add 25 µl of each sample and the standards, in triplicate, to black, flat-bottom, 96-well plates.
6. Add 100 µl of 0.2 M NEM/0.02 M KOH.
7. Assure that pH of samples is between 7 and 7.5. (see Note 4).
8. Add 10 µl of 10 mM TCEP to each sample; mix samples using a multichannel pipette.
9. Incubate for 20 min at room temperature.
10. Add 50 µl of 0.5 N NaOH to bring pH to 12.5.
11. Add 10 µl of NDA stock solution; mix samples using a multichannel pipette.
12. Incubate the plate in the dark for 30 min at room temperature.
13. Read fluorescence intensity using 472 (exitation) and 528 (emission) on a fluorescence plate reader.

3.2. Measurement of Intracellular Reduced and Oxidized Glutathione Levels by HPLC

Evaluation of cellular thiol status should include quantification of both the reduced (GSH) and the oxidized (GSSG) forms of glutathione (13). This protocol describes a method that can be used to quantify both GSH and GSSG. The fluorescent thiol-reactive compound MBB is used to derivatize the reaction products, which are then separated by HPLC and quantified by fluorescence detection. The assay is sensitive and allows the quantification of "total glutathione" (GSH and GSSG) in a sample; however, the assay must be performed twice on each sample to quantitate both GSH and GSSG.

1. Scrape cells in TES/SB homogenization buffer.
2. Use at least 20 µg of protein for each sample.
3. Keep samples on ice for at least 15 min, then centrifuge samples at 13,000×*g* for 10 min.
4. Add 200 µl of 0.2 M NEM/0.02 M KOH.
5. Assure that pH of samples is between 7 and 7.5.
6. Take two aliquots containing the same amount of protein from each sample; reduce one aliquot by adding 10 µl of 10 mM TCEP to determine total glutathione; add 10 µl of water to the second aliquot to determine reduced glutathione (GSH).
7. Incubate for 20 min room temperature.
8. Add 20 µl of 12.5 mM MBB and incubate in the dark for 30 min at room temperature.

9. Add 200 µl of 200 mM SSA to stop the reaction. Check the pH, to make sure that it is 2.0.
10. Spin the tube at 13,000 × *g* for 2 min.
11. Run the supernatant: analyze samples and standards using HPLC.
12. Set up an HPLC system with fluorescence detection (excitation at 375 nm and emission at 475 nm).
13. Prepare mobile phases. Use 1 mM TBAP (an ion-pairing agent), pH 3.0, as the aqueous phase, and HPLC-grade methanol as the organic phase.
14. Program the gradient.
15. Run the column using an autosampler, injection volume of 25–50 µl and a flow rate of 1.5 ml/min.
16. Average triplicates. Using standard curve, calculate the amount of total or reduced glutathione GSH in each sample. Figure 1 shows a typical chromatogram. The amount of GSSG in each sample is calculated by subtracting the value of GSH from that of total glutathione.

3.3. Measurement of GCL Activity by HPLC

Activity of GCL can be measured using spectrophotometric assays which are based on the detection of a secondary analyte or can be quantified through the analysis of thiols by HPLC. One of the most popular spectrophotometric assays to measure GCL activity is based upon the coupling of GCL enzyme activity to an ATP-generating system which, in turn, consumes NADPH which is monitored by absorption at 340 nm. HPLC-based assays are usually relying on UV, fluorometric, or electrochemical detection of the γ-glutamylcysteine (γ-GC) peak in the chromatogram.

Spectrophotometric assays can be particularly adapted to 96-well plate readers. However, they usually have a low sensitivity and do not allow the analysis of tissues/cell types with low-GCL activity. In contrast, HPLC assays are highly specific and very sensitive, allowing measurements of low-GCL activity, though they are time-consuming, and have a low throughput. Here we describe an HPLC-based fluorescence assay for the activity of GCL, based on fluorescence detection of its thiol product (previously derivatized with MBB) (14).

1. Collect cells in TES/SB buffer. Disrupt cells by two cycles of freezing and thawing at −20°C.
2. Homogenize cells using a 1-ml syringe with a 25 gauge needle (6–8 strokes). Keep tubes on ice during and after homogenization.
3. Spin at 12,000 × *g* for 10 min at 4°C, then place on ice.
4. Dialyze the entire supernatant at 4°C for 3 h by continuous-flow dialysis (e.g., with Microdialysis system, according to

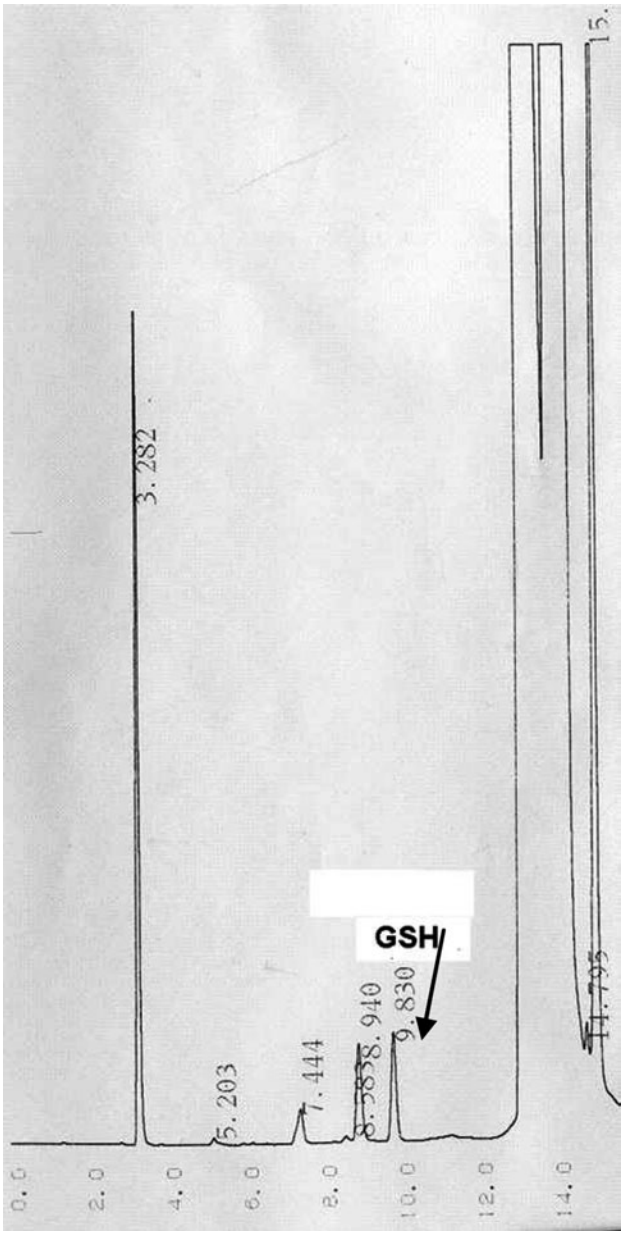

Fig. 1. Example of HPLC chromatogram. Cerebellar granule neurons were homogenized and intracellular GSH levels were measured by HPLC. Shown by the *arrow* is the GSH peak.

manufacturer's instructions), against TES/SB homogenization buffer (see Note 5). Alternatively, for small volumes the supernatant can be centrifuged through a microcon centrifugal filter device with a 10-kDa molecular mass cut-off filter, at 10,000 × *g* for 15 min at 4°C (see Note 6). Return samples to ice.

5. Prepare baseline samples (to determine background levels) by adding the following to microcentrifuge tubes: 80 µl ice-cold

GCL reaction cocktail, 30 µl water, 50 µl 200 mM ice-cold SSA, and 20 µl sample. Place on ice for ~15 min.

6. Prepare triplicate microcentrifuge tubes adding 80 µl ice-cold GCL reaction cocktail (see Note 7).
7. At 15–30-s time intervals, with the sample on ice, add 20 µl sample to each reaction tube and place in a 37°C water bath. Preincubate reaction tubes for 5 min at 37°C.
8. At the appropriate time, initiate reaction with 60 µl of 10 mM cysteine stock solution.
9. Stop each reaction at the appropriate time by adding 50 µl ice-cold 200 mM SSA and vortex. Place tubes on ice for ~15 min.
10. Prepare triplicate standard tubes for each standard dilution by adding the following to microcentrifuge tubes: 80 µl GCL reaction cocktail, 50 µl 200 mM SSA, and 20 µl standard solution.
11. Prepare one blank tube using 20 µl TES/SB homogenization buffer instead of a standard or a sample. Remember too not derivatize the blank.
12. Vortex samples and standards. Centrifuge at 13,000 × *g* in a benchtop microcentrifuge for 2 min. Transfer the supernatant to a different tube.
13. Add 200 µl of 0.2 M NEM/0.02 M KOH. Check pH of samples.
14. Add 30 µl of 12.5 mM MBB. Vortex standards and samples.
15. Leave tubes in the dark for 30 min.
16. Add 200 µl of 200 mM SSA. Vortex samples at maximum speed.
17. Spin for 2 min at 13,000 × *g*.
18. Run the supernatant: analyze samples and standards by HPLC.
19. Set up an HPLC system with a fluorescence detection (excitation at 375 nm and emission at 475 nm).
20. Prepare mobile phases. Use 1 mM TBAP (an ion-pairing agent), pH 3.0, as the aqueous phase, and HPLC-grade methanol as the organic phase.
21. Program the gradient.
22. Run the column using an autosampler injection volume of 25–50 µl and a flow rate of 1.5 ml/min.
23. Average triplicates. Using standard curves calculate the amount of GSH and γ-GC in each sample, subtracting blank levels (see step 5).

24. Express GCL activity in μmol of γ-GC formed per min/g tissue or in nmol/min/mg protein. To express activity per mg protein rather than per mg tissue, use the BCA method for protein determination (see Note 8).

4. Notes

1. TCEP is often used as a reducing agent to break disulfide bonds within and between proteins as a preparatory step for gel electrophoresis. When compared with the other two most common agents used for this purpose (dithiothreitol and β-mercaptoethanol), TCEP has the advantages of being odorless, and a more powerful and irreversible reductant; furthermore, it is more hydrophilic, and more resistant to oxidation by air.
2. MBB and bimane-derivatized thiols are photolabile. Reactions should be conducted under conditions that protect them from direct light exposure (e.g., in a darkened room or in brown glass reaction tubes).
3. The presence of both serine and borate in the homogenization buffer is necessary to inhibit any residual γ-glutamyl transpeptidase (GGT) activity. Otherwise, GGT will consume GSH and thus affect the calculation of baseline and sample GSH concentrations.
4. This range is the optimum pH for the derivatization process.
5. This step removes most of the endogenous GSH without affecting enzyme activity.
6. The centrifugal filter removes small molecules such as glycine, amino acid, and cofactors from the cell extracts, preventing the conversion of γ-GC to GSH during the course of the assay and other molecules interfering with the assay.
7. Reaction tubes should be prepared immediately prior to assay or stored on ice to prevent the degradation of ATP.
8. If activity is to be expressed per million cells, the cells should be counted prior to pelleting.

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Chapter 15

Assessing Neuronal Bioenergetic Status

Stephanie L.H. Zeiger, Jeannette N. Stankowski, and BethAnn McLaughlin

Abstract

Drug discovery and therapeutic development for disorders of the central nervous system (CNS) represents one of the largest unmet markets in modern medicine. We have increasingly recognized that the lack of stringent assessment of mitochondrial function during the discovery process has resulted in drug recalls, black box warnings, and an urgent need to understand the metabolic liability of small molecules in neural systems. Given that the brain is the most energetically demanding organ, even modest perturbations in neuronal energetic pathways have been shown to impact growth, signaling, connectivity, and the restorative capacity of the CNS. In this work, we describe several tools to assess metabolic activity of primary neuronal cultures and neural cell lines using an acute model of injury induced by oxygen glucose deprivation. Methods include the measurement of total ATP and NADH, enzymatic assessment of lactate production by anaerobic respiration, as well as viability assays. We also present a modified screening method for assessing aerobic respiration of immortalized cell lines using galactose challenge.

Key words: Mitochondria, Oxidative phosphorylation, Neuron, ATP, Lactate, Energetics, Oxygen glucose deprivation, Neurotoxicity

1. Introduction

There is an increasing awareness that appropriate metabolic tone is a key feature of biological processes including neurotransmission, cellular differentiation, and development as well as adaptation to stress (1–4). The brain represents the most energy demanding organ in the body accounting for 20% of total oxygen consumption and 25% of total body glucose utilization (5–7). Failure to maintain energetic status has been linked to a host of neurological disorders (8–15). Moreover, the drug discovery market has revealed that failure to account for off-target drug actions which impact aerobic respiration or mitochondrial function

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is a major source of liability (16, 17). In each of the acute and chronic disorders of CNS function, fundamental shifts in aerobic respiration, metabolic coupling, ionic homeostasis, and reactive oxygen species generation, which compromise neuronal health, have been observed. In the most simplified models, it is postulated that the ability to maintain sufficient energetic reserves even under stress redirects cell death from necrotic loss to apoptotic death and these features dictate the targets that are appropriate for drug discovery (18, 19). In this work, we discuss the advantages of several methods and model systems to assess metabolic function, adaptation, and survival in neural platforms in the context of an acute injury model of stroke. Methodologies are presented in a manner to facilitate cross purposing to assess energetic tone in cells exposed acutely or chronically to agents with possible neurotoxic action.

1.1. Essential Salient Features of the Krebs Cycle and Oxidative Phosphorylation

To accurately interpret the data acquired using the methods presented within this chapter, a basic understanding of the underlying principles of energetic status is required. In neurons, pyruvate is created within the cytoplasm through glycolysis and serves as the dominant mitochondrial substrate for aerobic respiration (20). It is transported into mitochondria and catalyzed into acetyl-CoA for use in the Krebs cycle to produce the reducing equivalents NADH and $FADH_2$.

The electron transport chain is located within the inner mitochondrial membrane and transfers electrons from NADH and $FADH_2$ to molecular oxygen forming a proton gradient that drives the formation of high-energy phosphate bonds on ATP from ADP. This aerobic pathway of energy also known as oxidative phosphorylation results in a combined total of 36 ATP. A far less efficient means of producing high-energy phosphate in the form of ATP involves pyruvate conversion to lactate setting the stage for glycolysis to occur by the liberation of NAD^+. Lactate is actively extruded from cells along with hydrogen and the accumulation of lactate is an excellent means to quickly assess dependence upon anaerobic metabolism. In the CNS, it is becoming increasingly clear that astrocytes, the predominate glia cell type in the brain, provide an essential metabolic support role for neurons by releasing lactate which can be converted back to pyruvate for aerobic respiration (Fig. 1).

1.2. Cell Line Usage and Energetics

Prior to attempting to manipulate the cellular energetic status following various stimuli, knowledge of the cell's baseline energetic profile is required. The use of tumor-derived, immortalized neuronal cell lines including PC-12, HT-22, and SY5Y cells has become increasingly popular due to their ease of passage and transfection, relative homogeneity, and low cost. As the tumor cells from which these lines are derived, these cell lines also

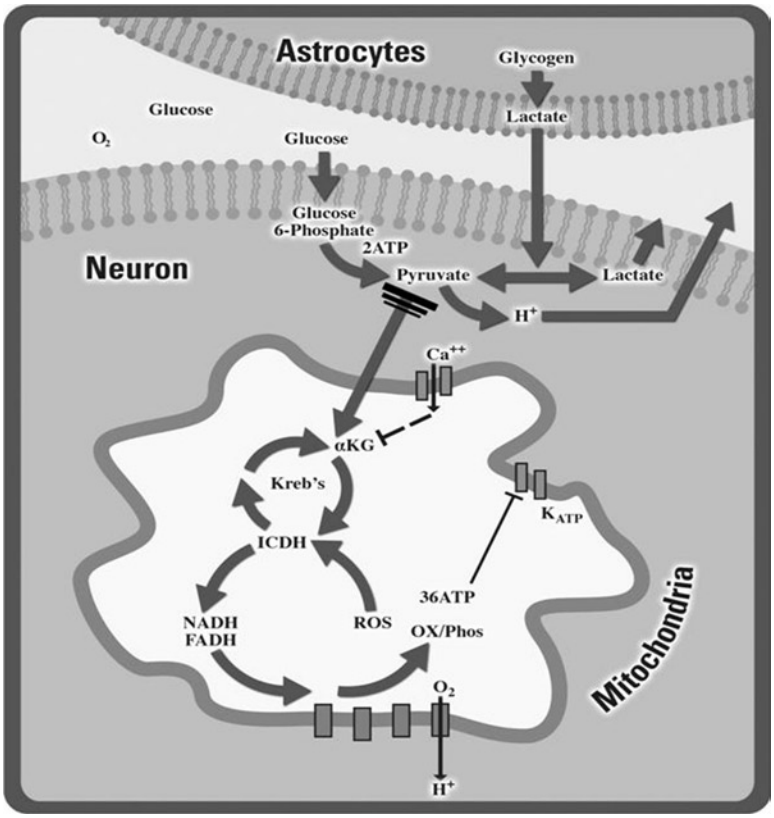

Fig. 1. Many factors play a role in determining a neuron's bioenergetic capacity. Through glycolysis, glucose is converted into pyruvate within the cytoplasm of the cell. In the presence of oxygen, this pyruvate is transported into the mitochondria and converted into acetyl-CoA to enter the Krebs cycle for further generation of NADH and FADH. The gradient formed by electron transfer from these reduced donors to oxygen leads to the production of ATP in the presence of ADP and phosphate via ATP synthase. When oxygen becomes limiting, anaerobic respiration can augment energetic capacity by converting pyruvate into lactate resulting in ATP production, albeit to a lesser extent than via aerobic respiration.

maintain the ability to adapt to low oxygen conditions and effectively use glycolysis to produce sufficient ATP (21, 22). Despite abundant oxygen present under normal culture growth conditions and fully functional mitochondria, these highly proliferative cells continue to depend on anaerobic pathways to generate ATP. Similarly, under conditions of high extracellular glucose, these cells often rely solely on anaerobic respiration to fuel ATP production, a condition commonly described as the Crabtree effect (21–23). Today's culture practices exacerbate these kinds of adaptations which normal neurons and glia could not endure *in vivo* as cell lines are often maintained in high-glucose medium. We believe that these kinds of adaptations at the energetic and molecular level are strongly linked to the poor predictive value of

these cells in the context of understanding mitochondria *in vitro*. This fact is starkly evidenced by the fact that many cell lines can survive hours in conditions of complete oxygen and glucose deprivation which would cause rapid death in both primary neuronal cultures and *in vitro* (21, 24–26). High throughput screens using primary cultures is impractical as the cells are extremely expensive to maintain, are time intensive, and exhibit low yield. In our experience, the best use of neural and other cell lines is to force cultures to rely more heavily upon aerobic respiration using a galactose challenge than expose them to secondary stressors.

1.2.1. Principles of the Galactose Challenge Assay

Galactose is a hexose that differs from glucose only by the configuration of its carbon four hydroxyl group. The replacement of glucose with galactose in the culture medium to force cells to rely more heavily on oxidative phosphorylation for ATP formation was initially described by H.G. Crabtree in 1935 (27) and has been used to determine underlying metabolic defects in a host of other cell lines (21–23). Cells are placed in medium containing 25–100% galactose and mitochondrial activity is assessed by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. Galactose metabolism promotes oxidative phosphorylation by promoting the use of glutamine and glutamate to fuel the Krebs cycle as opposed to pyruvate. In galactose-rich medium, cells which have increased proliferation and decreased production of lactate are the most aerobically active or adept at switching to an aerobic phenotype most readily. Establishing titering and proliferation curves for growth is essential to determine the set points at which proliferation is impeded, or in cells which have impaired oxidative phosphorylation, concentrations of galactose that cause cell death. Excellent reviews of establishing proliferation curves have been presented elsewhere (28, 29).

1.2.2. Principles of the MTT Assay

The MTT assay is used most often to assess cellular viability, but in the case of the galactose challenge, where mitochondrial activity is compromised, this technique can be used as an indirect measurement of cellular respiration. The method is based on the cleavage of the yellow tetrazolium salt MTT by active mitochondrial dehydrogenases to generate purple formazan crystals, a process that requires NADH and NADPH. The crystals are dissolved in acidified isopropanol and the purple solution is then measured spectrophotometrically. Other processes in addition to oxidative phosphorylation can consume the NADH as well as NADPH and combining this technique with other biochemical assays of total ATP reserves, lactate generation or pyruvate stores presents a fuller picture of the cell's metabolic profile and are typically considered next step strategies once proliferation curves for various galactose levels are established (30). ATP assays as

described in Subheading 1.4.1 of cells grown under galactose challenge vs. full glucose provides an excellent means to determine at what point metabolic compensations are brought online.

1.3. Assessing Primary Neuronal Survival Following Oxygen Glucose Deprivation

1.3.1. Principles of Primary Neuronal Cultures

Primary neuronal cultures are the gold standard for assessing neurotoxicity *in vitro*. They represent the most physiologically relevant metabolic model available for evaluating bioenergetic effects of a host of stressors including oxygen glucose deprivation (OGD) to model the loss of blood experienced during ischemic stroke. The composition of primary cultures can be altered by adding mitotic inhibitors at various times following isolation and plating. The default cellular program for isolated cells from CNS at embryonic day 16 or beyond in rat will promote the proliferation of glial components as they continue to proliferate following dissociation whereas the neuronal cell population is postmitotic. Glial inhibition in the 48 h following plating promotes formation of neuron-enriched cultures resulting in cultures which contain in the excess of 95% neurons and 5% astrocytes. Neurobasal medium provides a low serum base that can be supplemented with growth factors, neurotransmitters, antioxidants, vitamins, and fatty acid precursors to promote long-term survival and cell to cell communication.

1.3.2. Principles of OGD

Cell cultures exposed to OGD capture many of the essential features of the CNS when blood flow is halted in ischemic stroke. These cells undergo rapid energetic decline, release of glutamate, failure of the Na^+–K^+ ATPases, generation of oxygen-derived free radicals, glutamate transporter reversal, NMDA receptor hyperstimulation, ionic dysfunction, mitochondrial swelling, abnormal protein trafficking, and signaling cascades associated with both apoptotic and necrotic cell death. (31, 32).

The OGD method consists of placing neurons into glucose-free medium bubbled with an anaerobic mix to remove oxygen. A sealed hypoxic chamber containing the anaerobic gas mixture is used to maintain neurons in the oxygen deprived state at physiological temperatures for various durations. By conducting experiments 3 weeks following dissection (21–25 days *in vitro*), excitotoxicity is fully expressed as a consequence of neuronal expression of a full compliment of NMDA receptors (33, 34). Using this methodology, we have found that in culture neurons can survive 5 and 15 min of OGD, but longer exposures become increasingly toxic with maximal cell death observed 24 h after incubations longer than a single hour. Similar experiments in neural cell lines often require 4–6 h of OGD to elicit death (35–37), which highlights the glycolytic nature of these cells and their ability to survive anaerobic conditions for periods that are not physiologically relevant to humans.

1.3.3. Principles of the Lactate Dehydrogenase Assay

To assess neuronal survival following OGD exposure, we utilize a toxicity assay based on lactate dehydrogenase (LDH), an enzyme that catalyzes the formation of pyruvate to lactate. Upon cell damage or lysis, membrane integrity is compromised and LDH is released into the medium. Medium containing this enzyme is then used to catalyze the reduction of NAD^+ to NADH and H^+ by the oxidation of lactate to pyruvate. The resulting NADH reacts with a tetrazolium dye to form a pink color that can be measured spectrophotometrically. To account for variation in total LDH content between experiments, raw LDH values can be normalized to the toxicity caused by 100 μM NMDA plus 10 μM glycine, which is known to cause 100% cell death in this system (33, 34). To ensure predictability across culture preparations, all experiments should be performed using cells derived from multiple independent dissections.

1.4. Evaluating Neuronal Energetic Status (ATP, Lactate, and Pyruvate Assays)

1.4.1. Principles of the ATP Assay

The ATP assay is based on the bioluminescence formed from the interaction of the enzyme luciferase with luciferin and ATP present in the cell. Following OGD treatment, cells are lysed and mixed with the enzyme luciferase mixture to generate light in an ATP-dependent manner that can be measured with a luminometer. Care must be given to the appropriate handling of cells to ensure that external ATP and adenosine are removed and that hydrolysis of ATP is minimized by rapid sample preparation. While this common bench assay is widely available and amenable to equipment in nonspecialized labs, higher fidelity results are obtained by the use of high performance liquid chromatography to measure adenosine, ADP, and ATP to assess the total energetic reserves. This platform also allows for the assessment of other high-energy phosphate molecules including GTP. The bench assay does, however, provide a quick and inexpensive measure of total ATP which can be used in combination with a protein assay to account for variability in plating density that can arise in cell culture. This method alone does not discriminate between anaerobic or aerobic ATP content, but is rather a measure of total ATP.

1.4.2. Principles of the Lactate and Pyruvate Assays

As previously mentioned, pyruvate metabolism is a key determinate of metabolic efficiency. It can be converted into acetyl-CoA for use in the Krebs cycle or into lactate for anaerobic respiration. Measurements of pyruvate and lactate concentrations can be used to determine a cell's aerobic versus anaerobic respiration rates. An increase in lactate production over time with a decrease in pyruvate following a cellular stressor would suggest an increased anaerobic pathway of ATP generation. For each assay, cells are first lysed and lactate or pyruvate is enzymatically oxidized by the corresponding substrate to produce a color that can be measured

spectrophotometrically at the 570 nm wavelength or alternatively through fluorescence using an excitation of 535 nm and emission at 587 nm.

1.5. Future Methodologies for Assessing Cellular Energetic Status

Though beyond the scope of this chapter, new methodologies are emerging for noninvasively measuring cellular bioenergetic profiles. These technologies utilize live mammalian cells in a convenient, microplate format for fast and sensitive measurement of respiration and ATP utilization determined simultaneously and noninvasively by quantitative assessment of the oxygen consumption rate, extracellular acidification rate, lactate production, and carbon dioxide production (38, 39). This instrumentation has been adapted with high reliability using electrochemical sensors (21) and in a more mainstream platform using fluorophores and a microplate reader. Cost of the commercially available platforms range in the hundreds of thousands, while lab built devices require an extensive knowledge of mechanical and electrical engineering. However, this technology can be combined with measures of cell fate, protein expression, and traditional biochemistry to provide a comprehensive and powerful platform for basic research and drug discovery.

2. Materials

2.1. Galactose Challenge

1. Control medium: This medium should be the normal medium used for proper cell-line maintenance with its appropriate glucose concentration.
2. Galactose/glucose medium: This medium will consist of a percentage of galactose and glucose for a total concentration equal to the glucose concentration of the control medium. For example, HT-22 cell control medium contains 10 mM glucose. Using a glucose-free version of this control medium, a 25% galactose/75% glucose challenge medium will consist of 2.5 mM galactose and 7.5 mM glucose.
3. Sterile tissue culture plates and tissue culture hood.
4. Incubator appropriate for neuronal cell line.

2.2. MTT Assay

1. MTT solution: 15 mg MTT, 3 ml of culture medium used throughout the experiment. Solution is stable for at least 6 months when stored at –20°C.
2. MTT Solubilization solution: 10% Triton-X 100, anhydrous isopropanol, and 0.1 N HCl. For 50 ml, mix 5 ml Triton-X 100, 45 ml isopropanol, 1 drop of 12 M HCl.
3. Incubator appropriate for neuronal cell line.
4. Shaker and/or sonicator.

2.3. Neuronal Dissection and Maintenance of Cultures

1. Borate Buffer: 4.76 g (77 mM) boric acid, 2.54 g (6.66 mM) borax, 1,000 ml double-distilled H_2O, pH to 8.4 with NaOH. Sterile filter and store at 4°C (see Note 1).
2. Poly-L-ornithine (PLO, Sigma P3655): 1 mg PLO for each 2 ml of borate buffer (see Note 2).
3. Embryonic day 18 pregnant rat (see Note 3).
4. Plating medium: 80% Dulbecco's Modified Eagle's Medium (DMEM), 10% Ham's F12-nutrients, 10% bovine calf serum (iron-supplemented, Hyclone) with 24 U/ml penicillin, 24 g/ml streptomycin, and 2 mM L-glutamine. Store at 4°C.
5. Trypsin.
6. Culture medium: Neurobasal medium (Invitrogen) containing 50× B27 supplement (Gibco), 50× NS21 supplement (40), 24 U/ml penicillin, and 24 g/ml streptomycin. Store at 4°C (see Note 4).
7. Dissection tools: large scissors, fine tip dissecting scissors, and forceps.
8. 500× Cytosine Arabinoside (AraC) Stock: 100 mg Ara-C, 6 ml double-distilled H_2O. Sterile filter solution and store at −20°C in 0.5 ml aliquots.
9. Working AraC: 20 µl of 500× stock, 10 ml double-distilled H_2O. Sterile filter solution and store at 4°C.
10. 0.1N HCl: 248 ml double-distilled H_2O, 2 ml 12N HCl.
11. 95 and 70% Ethanol.
12. HBSS: 8.4 g NaCl, 224 mg KCl, 2.38 g HEPES, 1.0 g glucose, 1,000 ml double-distilled H_2O, pH to 7.3 with NaOH. Sterile filter the medium in the tissue culture hood and store at 4°C.
13. Trypan blue, microscope and hemocytometer for cell counts.
14. Tissue culture water.
15. Large coverslips (25 mm) and 6-well tissue culture plates.
16. Anesthesia for rat, Nembutal sodium solution.
17. Sonicator and orbital shaker.
18. Sterile Tissue culture hood.
19. Sterile incubator at 37°C and 5% CO_2.
20. 35-mm Culture dishes (sterile).
21. Pasteur pipettes.

2.4. Oxygen Glucose Deprivation

1. MEM/BSA/HEPES: 487.5 ml clear MEM, 12.5 ml 1 M HEPES, and 50 mg bovine serum albumin. Sterile filter and store at 4°C. Make up fresh medium before each experiment.
2. MEM/BSA/HEPES/2× N2: 98 ml MEM/BSA/HEPES and 2 ml N2 supplement (Gibco) (see Note 5).

3. Glucose-free balanced salt solution (GBSS): 5.26 g NaCl, 0.125 g KCl, 66.6 mg $CaCl_2$, 1.43 g HEPES, 600 ml double-distilled H_2O. pH to 7.3 using NaOH and HCl. Sterile filter and store at 4°C.
4. NMDA toxicity medium: MEM/BSA/HEPES/2× N_2, 10 μM glycine, and 100 μM NMDA.
5. Anaerobic gas mix (95% nitrogen and 5% CO_2).
6. Hypoxic chamber.
7. Sterile Incubator at 37°C and 5% CO_2.
8. 35-mm Culture dishes (sterile).
9. N_2 meter.

2.5. LDH and Lysis LDH Assay

1. LDH Assay kit (Sigma): LDH assay substrate, dye, enzyme, and lysis buffer. Enzyme is stored at –20°C. Lysis buffer is stored at room temperature, while all other components are stored at 4°C.
2. LDH assay mixture: Equal amounts of LDH assay substrate, dye and enzyme. Mix the appropriate volume to have 20 μl for each well that will be measured in a 96-well plate plus 10% extra. Both the enzyme and mixture is light sensitive so combine reagents in the appropriate lighting conditions. Make up fresh before each experiment.
3. Phosphate-buffered saline solution (PBS): 1 l double-distilled H_2O, 80 g NaCl, 2 g KCl, 2 g KH_2PO_4, 115 g Na_2HPO_4. pH to 7.5 using NaOH or HCl. Dilute this 10× PBS to 1× using double-distilled H_2O.
4. PBS/lysis buffer: 150 μl of lysis buffer and 950 μl of PBS. Make 1.5 ml of buffer for each well of a 6-well tissue culture plate plus 40 μl for each blank well of the 96-well plate.
5. 96-Well clear transparent plate.
6. Repeater pipette.
7. Spectrometer to measure absorbance at a wavelength of 492 nm.

2.6. ATP Bioluminescent Assay

1. Ice cold PBS.
2. ViaLight® Plus Kit (Lonza): Assay buffer, cell lysis reagent, ATP monitoring reagent (AMR). Store at 4°C.
3. AMR: Place 10 ml of assay buffer into lyophilized AMR. Mix to reconstitute the AMR and then add mixture to the assay buffer bottle. Store reagent in polypropylene tubes at –20°C for up to 2 months.
4. 96-Well transparent plate with white walls.
5. Spectrometer to measure luminescence.

2.7. Lactate and Pyruvate Assays

1. Ice cold PBS.
2. Lactate assay kit (BioVision): Lactate assay buffer, standard, probe, and enzyme mix.
3. Pyruvate assay kit (BioVision): Pyruvate assay buffer, standard, probe, and enzyme mix.
4. Teflon cell scrapers.
5. Microcentrifuge at 4°C.
6. 96-Well transparent plates.
7. 1.5-ml Tubes.
8. Spectrometer to measure absorbance at 570 nm or fluorescence with an excitation of 530 nm and emission of 587 nm.

3. Methods

3.1. Galactose Challenge

1. In a sterile tissue culture hood, plate your cells in the appropriate size tissue culture plates containing the galactose/glucose medium at various percentages or control medium for comparison heated to the necessary temperature for the cell line.
2. Cells can be maintained in the galactose/glucose medium percentages for the appropriate time depending on your cell type. At a minimum, expose cells to this medium for 24–48 h (see Note 6).
3. Proceed with the MTT assay (Subheading 3.2) to evaluate mitochondrial respiration and compare with control medium exposed cells.
4. Alternatively, cells can be exposed to increasing percentages of galactose over time. Upon splitting cells, place half of the cells in a higher galactose percentage medium. MTT assays should be performed at similar exposure times for each galactose/glucose percentage medium to determine differences in mitochondrial respiration.

3.2. MTT Assay

1. Add MTT solution in an amount equal to 10% of culture medium volume to each well in a sterile tissue culture hood. For example, for a 6-well tissue culture plate which contains 1.5 ml of medium in each well, add 150 µl of MTT solution (see Note 7).
2. Incubate the culture plate for 2 h depending on cell density. Longer incubation times (2–4 h) may be necessary to compensate for low cell densities and metabolic activity.

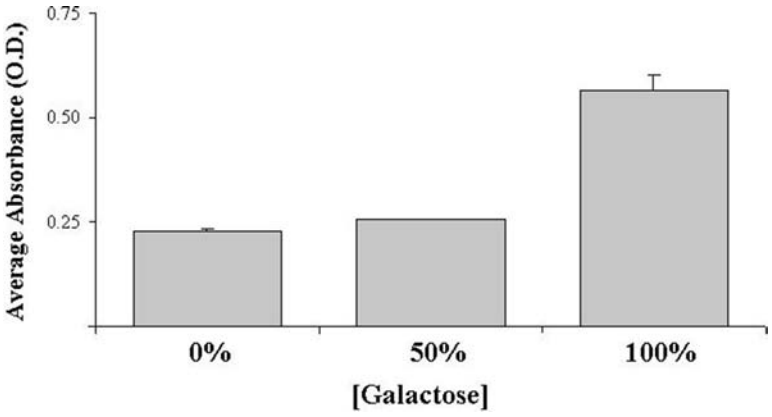

Fig. 2. The HT-22 cell line exhibits an increase in mitochondrial activity upon exposure to galactose. The HT22 mouse neuroblastoma cell line was exposed to varying concentrations of galactose for 48 h and reducing capacity of the cells was assessed using an MTT assay. Complete replacement of glucose with galactose (100%) led to an increase in MTT absorbance due to enhanced mitochondria activity. Slow metabolism of galactose altering viability and ATP production reveals underlying dependence upon oxidative phosphorylation. This data suggests that even with half the normal amount of glucose, HT-22 cells can successfully produce sufficient ATP for survival and proliferation.

3. Remove the plate and add an amount of MTT solubilization solution equal to the original medium volume to dissolve the formazan crystals. For 6-well tissue culture plates, add 1.5 ml to each well.
4. Gently shake, pipette up and down, or use a sonicator to completely dissolve the crystals.
5. Using the spectrometer, measure absorbance of the samples directly in the plate using a wavelength of 570 nm (Fig. 2).

3.3. Neuronal Dissection

3.3.1. Coverslip Preparation

1. Place one large box of coverslips and 250 ml of double-distilled H_2O into a 500 ml beaker and swirl the contents gently for 2 min. Decant the water into the sink making sure no coverslips pour out.
2. Add 250 ml of new double-distilled H_2O and sonicate for 15 min.
3. Repeat steps 1–2. Following the last step, decant the water, and add 250 ml of 0.1N HCl to the coverslips. Cover the beaker with parafilm and place on the orbital shaker for 1 h.
4. Decant the 0.1N HCl into the appropriate hazardous waste drum and wash the coverslips three times with double-distilled H_2O.
5. Add 250 ml of 95% ethanol to the coverslips and place the beaker on the orbital shaker for an additional hour.
6. Decant the 95% ethanol into the appropriate hazardous waste drum and wash the coverslips twice with double-distilled H_2O.

7. Add 250 ml of 95% ethanol to the coverslips, place parafilm over the beaker, and store the cleaned coverslips until future use.

3.3.2. Embryonic Dissection

1. At least 2 days before dissection, in a sterile tissue culture hood place cleaned coverslips into 6-well tissue culture plates (one coverslip per well). Parafilm the tissue culture plate and store until future use.
2. One day before dissection, place culture plates containing coverslips into sterile tissue culture hood. Remove the lid of each plate and expose to ultraviolet (UV) light for 1 h (see Note 8).
3. Place 2 ml of freshly made PLO/borate buffer solution into each well following UV treatment. After a minimum of 1 h, wash each well twice with 2 ml of tissue culture water. Remove the water and lids from each plate and allow the plate to dry in the hood for 2–4 h. Add 1.5 ml of plating medium to each well and place plates in the incubator until dissection.
4. For dissection preparation, weigh out 3 mg of trypsin and dissolve in 5 ml of HBSS. Sterile filter the solution into a 35-mm culture dish and label. Add 3 ml of HBSS to seven of the 35-mm culture dishes. Add 450 μl of trypan blue to a 1.5 ml Eppendorf tube. Clean dissection tools with 70% alcohol. Aspirate the coating medium from the culture plates. Add 5 ml of plating medium to two 15-ml tubes. Set out three more tubes for tituration procedure. Place plating medium in hood to bring to room temperature.
5. Inject the rat with the sedative, Nembutal, by intraperitoneal injection (~2 ml), then wait a few minutes for her to relax and lay down. Check her "blink" reaction and squeeze her foot to determine if she is properly sedated.
6. Place the rat on the absorbent pad belly up and spray the belly with 70% alcohol. Use the large forceps to lift the belly skin and cut open the rat with the heavy-duty dissecting scissors along the midline up to the diaphragm area.
7. Next, cut the muscle along the midline to expose the embryos and the diaphragm and cut both sides of the rat's diaphragm to stop respiration.
8. Use the large forceps to pull all the embryos out of the rat. Cut the embryos open with fine-tip dissecting scissors and cut off the head of the embryo into the HBSS culture dish.
9. Transfer one embryo head into a new dish of HBSS that is now under the microscope. Hold the head straight up, puncture the skin and membrane outside of the brain with the dissecting forceps. Peel the skin and membrane toward the front of the head until the brain pops out. Turn the head around and peel toward the back of the head until the brain

stem is exposed. Put the dissecting forceps under the brain stem and pull toward the back of the head until the brain is separated with the skull. Pinch cut the brain stem to separate the brain completely.

10. Place the brain in a new HBSS dish. With the dorsal of the brain facing up, hold the brain stem gently with forceps and separate the hemispheres with the dissecting forceps. Pull the cerebral cortex away from the brain stem.
11. Place one cortex in a new dish of HBSS. Flip the cerebral cortex so that the outside is facing up. Pinch cut the olfactory bulb with the dissecting forceps and peel the meninges from the front toward the back. Try to peel it off in one piece (see Note 9).
12. Pinch cut any mid-brain structures underlying the cortex and transfer the cortex to a new dish with HBSS. Repeat for the other cortices (see Note 10).
13. Transfer the dish with all the cortices to the hood and place the cortices into the trypsin and HBSS solution with the forceps, making sure to take as little HBSS as possible with them. Allow cortices to sit in the solution for 30 min in the hood at room temperature.
14. Transfer the cortices from the trypsin to a new dish with HBSS with a Pasteur pipette, one at a time. Do not break the cortices. Gently swish them around to wash off the trypsin. Repeat once more.
15. Transfer the cortices with a new Pasteur pipette to the first tube of plating medium. Slowly triturate the cortices with the Pasteur pipette to maximize the total cell number and viability. Let the chunks settle down to the bottom of the tube.
16. Transfer only the cell suspension (not the larger pieces) to a new 15-ml tube with a Pasteur pipette. Again, slowly triturate with cortices. Continue this process until the suspension looks even (see Note 11).
17. When triturations are complete, add 50 µl of the cell suspension to the Eppendorf tube with trypan blue (a 1:10 dilution). Mix well by flipping the tube upside-down several times. Remove 50 µl of trypan blue mixture, and add it to the hemocytometer (it takes ~15–20 µl to fill each side).
18. Count the number of live cells and dead cells in three of the 4×4 quadrants (one on one side and two on the other side). Use this series of formulas to determine the needed cell to medium dilution.
    1. (Live cells)/(three squares) = y
    2. $y \times 10 = z$ (for 1:10 Dilution in Trypan blue)
    3. $z \times 10{,}000 =$ (hemocytometer volume correction)

19. Dilute the cell suspension to the desire plating density with plating medium (350,000 cell/ml). Plate 2 ml/well, and change the pipette after every two plates. Make sure to gently swirl medium bottle to insure proper homogeneity of cell suspension in between plates. Carefully return dishes to the incubator, making sure not to splash medium on lids or disrupt cells.
20. Add 1–2 μM working Ara-C solution directly into each culture well 2 days after dissociation to inhibit glial proliferation. On the following day, replace the plating medium with 2 ml growth medium. Medium will need to be partially replaced every 2–3 days. All neuronal experiments should be conducted 3 weeks after dissociation (Fig. 3).

3.4. Oxygen Glucose Deprivation

1. Warm MEM/BSA/HEPES, MEM/BSA/HEPES/2× N2 and GBSS to 37°C. Place the hypoxic chamber with lid off into sterile tissue culture hood and treat with ultraviolet light for 10 min.
2. Place appropriately labeled 35-mm dishes into the chamber with their lids off. By adjusting the N_2 meter in the hood to 5 PSI, degas the chamber for 5 min with the anaerobic

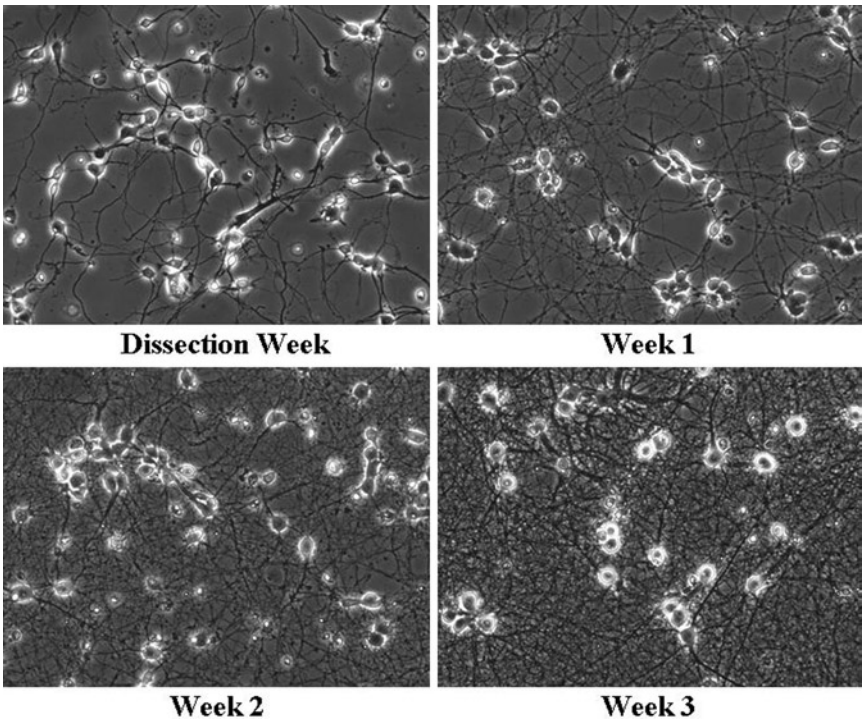

Fig. 3. Healthy neuronal cultures following dissection. Neuronal architecture was monitored following initial plating until 3 weeks following dissection. Healthy neurons display phase bright somas early on following the dissection with many neurites. Older neurons demonstrate increasing well-defined processes along with phase bright somas.

mixture by connecting the N_2 tube to the entrance tube of the chamber. Check the exit tube on the chamber to ensure that the anaerobic mix is flowing properly by covering up the exit tube on the chamber for ~5 s. When released, there will be a hissing sound and a slight rise and fall in the lid of the chamber. Close the clasp on the exit tube followed by closing the entrance tube and place the chamber in the incubator for a minimum of 10 min (see Note 12).

3. Place the GBSS medium into the hood and degas it for 5 min by placing a 2-ml pipette in the bottle with the anaerobic mix flowing through it. Make sure to check for proper flow (5 PSI).
4. Return the chamber from the incubator to the hood and add 3 ml GBSS medium to each dish. Carefully add one coverslip of neurons to each dish using the forceps. Make sure that the medium covers the coverslips. Replace the chamber lid and allow the anaerobic mixture to flow for 5 min. Close bath clasps and return to the incubator for the appropriate incubation time.
5. Following the incubation, return the chamber to the hood, remove the lid and using a 25-ml pipette, remove the GBSS medium from each dish. Gently wash each dish with 2 ml of MEM/BSA/HEPES. Add 1.5 ml MEM/BSA/HEPES/2× N2 to each dish, place the lid on the dish, and return to incubator for the remaining post time.
6. For normalizing to total kill, add NMDA toxicity medium to wells containing 1–2 coverslips.

3.5. LDH and Lysis LDH Assay

1. In a 96-well transparent plate, fill all wells in column A with 40 µL of control medium. In the case of our primary neuronal culture OGD experiments, we use MEM/BSA/HEPES/2× N2.
2. Pipette 40 µl of each sample from your toxicity experiment in triplicate into the 96-well plate. Using a repeater, add 20 µl of the LDH assay mix to each well. The reaction is light sensitive so cover the plate with paper towel and take to the spectrometer (see Note 13).
3. Shake the plate for 5 s to mix components and store the plate in a dark location for 20–30 min at room temperature. Measure absorbance at a wavelength of 490 nm using the spectrometer.

   Alternatively or in combination with the LDH assay, a lysis LDH assay can be performed. Lysis LDH is a modification of the assay which allows you to wash off the "released" LDH, then lyse healthy cells to determine cell viability.

4. Wash each well twice with 2 ml of PBS. Add 1.5 ml of PBS/lysis buffer to each well and incubate the plate for 45 min at 37°C. Following the lysis, proceed with the LDH protocol at step 1. The PBS/lysis buffer will be used for the blank wells instead of MEM/BSA/HEPES/2× N2.
5. Data can be normalized to LDH and lysis LDH values from NMDA-treated cells to obtain a percentage of death or viability (Fig. 4).

3.6. ATP Bioluminescent Assay

1. Remove culture plate from the incubator for 5 min to allow plate to cool to room temperature. Set up new 6-well tissue culture plates by adding 300 μL of cell lysis reagent to each well. After cooling, remove coverslips from initial plate and place in the plate containing the cell lysis reagent (see Note 14).
2. Allow cells to lyse for 10 min. During that time, remove an AMR aliquot from the −20°C refrigerator to thaw, and program the plate reader with the following parameters: measurement mode luminescence, integration time of 1,000 ms, and gain of 150.

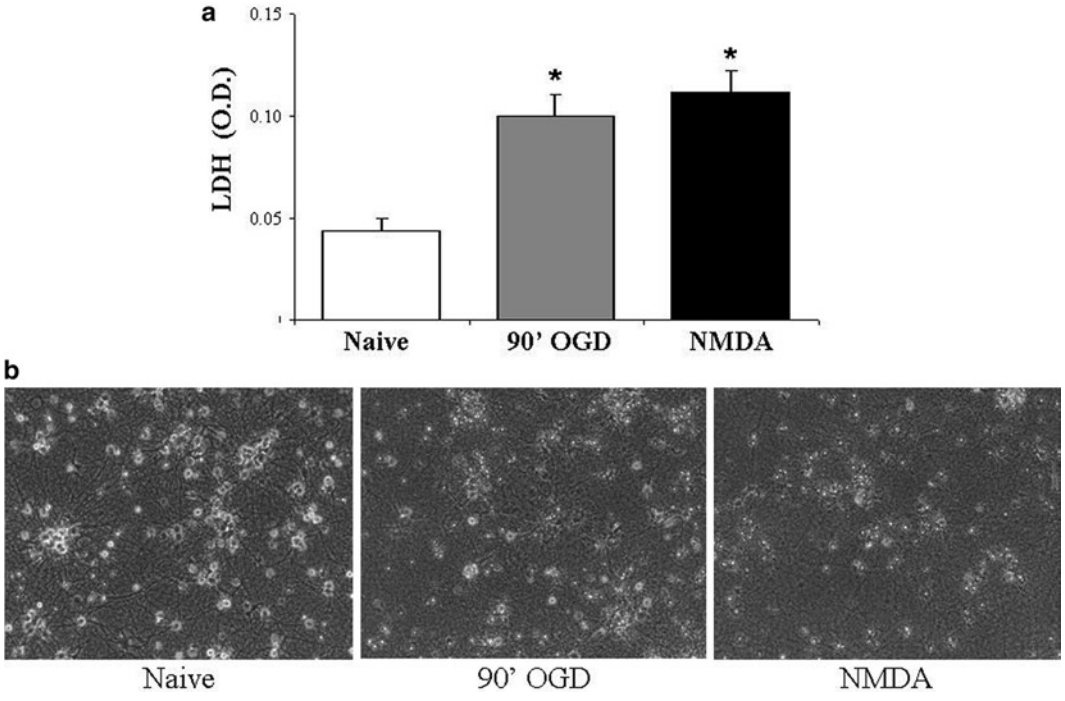

Fig. 4. Neurons cannot survive long periods of OGD or NMDA toxicity. (**a**) Primary neuronal cultures were either naïve, exposed to 90 min of OGD (90′OGD) or NMDA (100 μM for 60 min) and cell death was assessed 24 h later by measuring LDH release. The higher LDH values signify a release of this enzyme into the medium from ruptured cells observed with 90 min OGD or NMDA exposure. Data represent the mean ± SEM from six-independent experiments. *Asterisk* denotes statistical significance by two-tailed *t* test with $p < 0.05$. (**b**) Representative photomicrographs taken 24 h after the exposures illustrate the effect of OGD or NMDA exposure on neuronal architecture. Neurons from control conditions have phase bright somas with well-defined processes, whereas the 90 min OGD and NMDA exposed neurons show signs of cell death with small debris evident and widespread neurite beading and retraction.

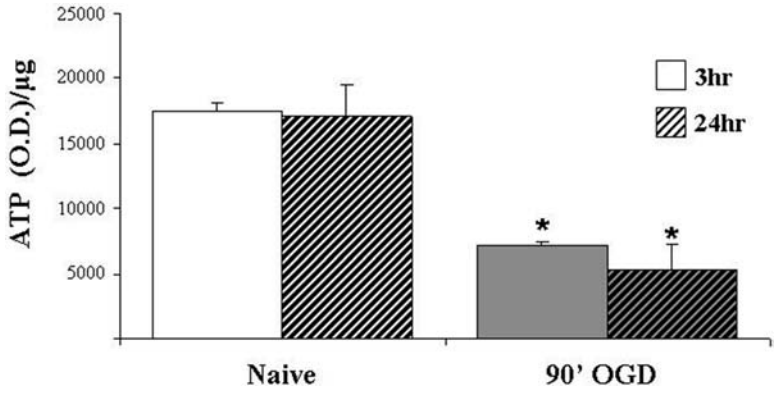

Fig. 5. ATP generation collapses following 90 min of OGD. ATP levels from neuronal cultures were measured 3 or 24 h after exposure to 90 min of OGD (90′OGD) or control medium (Naïve). ATP levels are significantly reduced 3 h following OGD exposure and do not recover even 24 h later. Data were normalized for total protein and are expressed as ATP/µg of protein ± SEM from three-independent experiments. Statistical significance compared with control as determined by two-tailed *t* test with $*p < 0.05$.

3. Transfer 80 µl of cell lysate from each well to a 96-well transparent white plate. Add 100 µl of AMR to each well using a repeater and let the plate sit at room temperature for 2 min before measuring on the spectrometer. Use cellular lysate to determine protein concentrations of each well. It is necessary to normalize ATP values to protein concentrations to account for differences between cultures (see Note 15, Fig. 5).

3.7. Lactate and Pyruvate Assays

The procedure for both the lactate and pyruvate assays is similar except for using the corresponding appropriate enzyme, probe, standard, and assay buffer within the kit.

1. Wash each well twice with ice cold PBS and harvest in 300 µl of assay buffer, transferring the same buffer to each well and scraping to combine the contents of all the wells into a 1.5-ml tube.
2. To fully lyse the cells, pipette cells up and down several times. Save 50 µl of each lysate in a separate tube for protein assay. The protein assay sample can be stored at −80°C.
3. Spin the cellular lysate at 700×*g* in a microcentrifuge for 10 min at 4°C to clear extract.
4. During the spin, prepare the standard curve by diluting the appropriate standard 100-fold to 1 nmol/µl in assay buffer. In the first column of the 96-well plate, add 0, 2, 4, 6, 8, and 10 µl of standard into each well individually. Adjust the volume of each well to 50 µl with the appropriate assay buffer to generate 0, 2, 4, 6, 8, and 10 nmol/well of the standard. Repeat this in the second column of the 96-well plate for a duplicate standard.

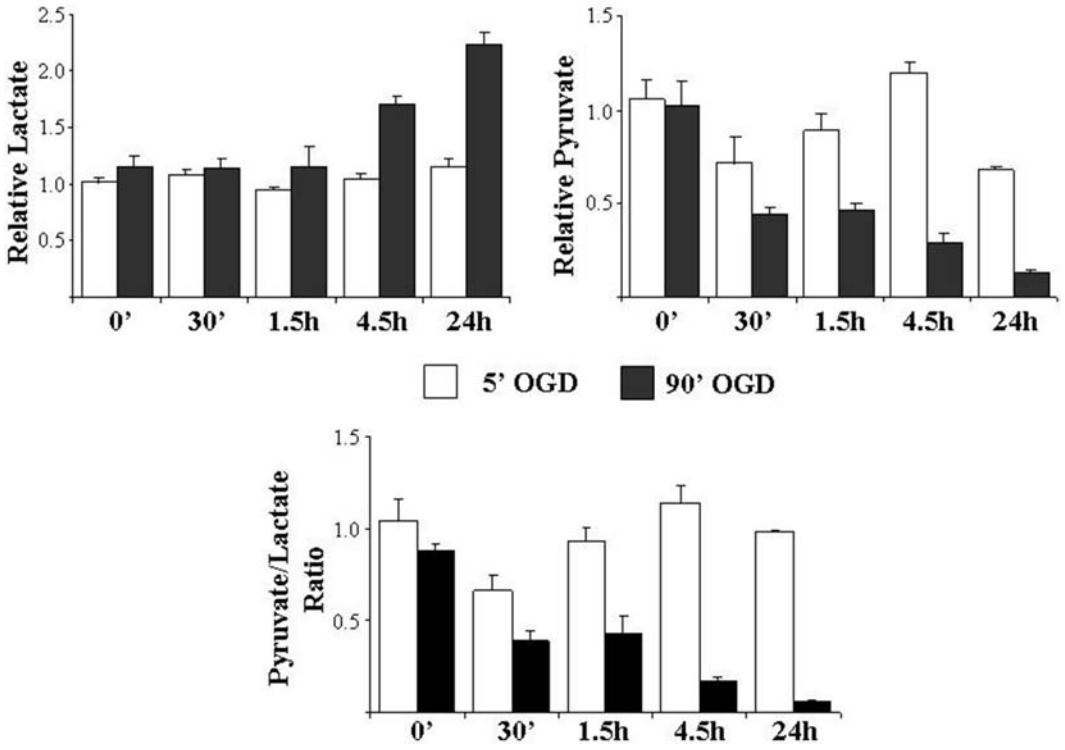

Fig. 6. Neurons are able to metabolically adapt following a brief OGD exposure. Lactate and pyruvate levels were measured at various times following an exposure to 5 or 90 min of OGD. The nonlethal 5 min OGD exposure did not significantly impact the production of lactate and pyruvate. In contrast, a lethal 90 min exposure results in a dramatic increase in lactate production and a loss of pyruvate over time indicating an inability for these neurons to adapt metabolically. Data were normalized to control cells and represent the mean from four-independent experiments ± SEM.

5. Add the appropriate volume of each cleared extract in duplicate to the 96-well plate. To determine the volume that is within the standard curve, use two or three initial volumes of 50 µl or less. Bring the volume of each sample well to 50 µl using the assay buffer.
6. For each well, prepare a 50 µl total volume reaction mix consisting of 46 µl of assay buffer, 2 µl of probe, and 2 µl of enzyme mix. Add 50 µl of the reaction mix to each well containing the standard or test samples. Protect plate from light as reaction is light sensitive.
7. At the spectrometer, shake the plate for 5 s to mix well. Incubate the samples at room temperature for 30 min protected from light. Measure the absorbance at 570 nm or fluorescence with an excitation of 535 nm and emission of 587 nm. Normalize values to protein concentrations as determined using a protein assay (Fig. 6).

4. Notes

1. Borate buffer should be made up ahead of time as it takes approximately 8 h with gentle mixing for all components to go into solution.
2. PLO: borate buffer should be made up fresh before use.
3. The use of older embryos results in less neuronal plasticity and higher cell death following dissections.
4. We have found that a combination of the commercially available B27 supplement and a lab made NS21 supplement provides enhanced long-term health of neuronal cultures (40).
5. The N2 supplement is a chemically defined, serum-free supplement necessary for the growth and maintenance of primary neurons. Without the N_2 supplement, the neurons will die within 24 h.
6. Longer exposures may allow cells to make proper adaptations necessary for aerobic pathways. If longer exposure times are used, make sure to feed cells in a similar manner as if cultures were in the control medium.
7. Phenol red and microbial contamination can lead to erroneous measurements during the MTT assay.
8. Longer ultraviolet exposures can lead to the leaching of chemicals from the plate into the culture medium and result in cellular toxicity.
9. If not removed, cells of the meninges will proliferate and overtake the neurons within the culture.
10. All cortices should be in trypsin within 30 min from the start of the dissection due to activity of cellular proteases compromising neuronal membrane integrity and culture health.
11. Trituration steps are critical. If too harsh, neuronal health will be compromised.
12. This warming procedure is done to minimize any stress to neurons due to temperature differentiations during the OGD experiment.
13. Pop any bubbles with a needle or forceps as they will interfere with absorbance readings.
14. The transfer of the cover slips to a new plate is due to ATP released into the current medium. The new well contains the cell lysis reagent and takes only the cells on the cover slip into account for the ATP, instead of ATP released into the medium.
15. The ATP lysis reagent is not compatible with the BioRad protein assay kit. Rather, we use the Pierce BCA protein assay kit to determine protein concentrations.

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Part III

Signaling in Neurotoxicity

Chapter 16

Fluorescent Assessment of Intracellular Calcium in Individual Cells

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Abstract

Calcium is an important intracellular ion involved in numerous cell processes. There are multiple factors that contribute to the release of Ca^{2+}. Some factors induce release as part of intracellular signaling cascades, while others result in unwanted changes to both basal and inducible Ca^{2+} levels. The accurate measurement of intracellular Ca^{2+} is, therefore, an important tool in neurotoxicology for assessing compounds/substances that disrupt Ca^{2+} homeostasis. Fluorescent, Ca^{2+}-sensitive probes Indo-1 and Fluo-3 allow for the quantification of intracellular Ca^{2+} in individual cells to determine what effects neurotoxins have on both basal and stimulus-dependent Ca^{2+} concentrations.

Key words: Calcium, Neurotoxicology, Astrocytes, Fluorescence, Indo-1/AM, Fluo-3/AM, Confocal imaging

1. Introduction

Calcium is one of the most ubiquitous and versatile divalent cations in cellular biology. Cells utilize Ca^{2+} in a wide array of signaling cascades that regulate a multitude of common processes such as cell growth and proliferation, transcription, contraction, and apoptosis (1, 2). In the central nervous system, subsets of neurons utilize action potentials to increase intracellular Ca^{2+} via voltage-gated ion channels. Ca^{2+} influx can also occur through the synaptic input of different neurotransmitter receptors (3–5). The timing and duration of intracellular calcium release is, therefore, an important factor for assessment in both neurophysiology and neurotoxicology as exposure to toxins/toxicants can disrupt this integral process. Previous work has demonstrated that exposure

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to ethanol can disrupt muscarininc receptor-induced Ca^{2+} release in primary rat cortical astrocyte, and human 132 1N1 astrocytoma cells (6, 7).

Commercially available fluorometric dyes are available for calcium imaging that are structurally based on the Ca^{2+}-specific chelator 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) (8). Two fluorescent probes, ratiometric Indo-1 (9) and nonratiometric Fluo-3 (10) are described in the following chapter, with Indo-1 serving as the primary, and Fluo-3 as the alternate protocol depending on instrumental limitations. Scanning confocal laser microscopic fluorescence detection allows for measurement of changes in both basal and inducible intracellular $[Ca^{2+}]$.

2. Materials

2.1. Cell Culture

1. Complete 5% Serum Medium: Dulbeccos' Modified Eagle's Medium (DMEM), low glucose with HEPES, supplemented with 5% (v/v) fetal bovine serum, 1 U/mL penicillin, and 1 µg/mL streptomycin, stored at 4°C for up to 1 month (see Note 1).
2. Serum-free Medium: DMEM, low glucose with HEPES, supplemented with 1% (w/v) fatty acid-free fraction V bovine serum albumin (BSA), 1 U/mL penicillin and 1 µg/mL streptomycin, stored at 4°C for up to 1 month.
3. 1 mM Carbachol in Krebs bicarbonate buffer (see Subheading 2.2. Carbachol solutions are not stable for long-term storage and should be prepared fresh just prior to experimental use.

2.2. Indo-1/AM and Fluo-3/AM Calcium Fluorophore Loading

1. Krebs Bicarbonate Buffer (10× Stock solution): 70.14 g NaCl, 3.5 g KCl, 1.91 g $CaCl_2$, 1.63 g KH_2PO_4, 2.96 g $MgSO_4 \bullet 7H_2O$, and 21.08 g glucose in a total volume of 1 L of deionized H_2O. This 10× Stock solution can be stored at 4°C for 1–2 months. Dilute to a 1× working solution and add 2.1 g/L of $NaHCO_3$ and pH to 7.4 with 0.1N HCl daily before using with dye loading protocols.
2. 2 mM Indo-1 acetoxymethyl ester (AM) and Fluo-3/AM (Molecular Probes, Invitrogen) stock solutions (1,000×) in sterile, high-quality anhydrous dimethyl sulfoxide. Aliquot into small volumes and store under desiccated, low-light conditions at ≤–20°C (see Note 2).
3. Krebs Loading Buffer : 1.0 g fraction V BSA in 100 mL of the 1× Krebs' bicarbonate buffer working solution. Adjust pH to 7.4 with HCl, aliquot, and store at –20°C for up to 1 year.

4. 2 μM Indo-1/AM and Fluo-3/AM working solutions: Prepare a 1:1,000 dilution of the 1,000× Indo-1/AM, or the Fluo-3/AM stock solution in Krebs loading buffer. Working solutions should be prepared daily on an experimental basis.
5. Probenecid Stock Solution (Optional): 28.5 mg/mL in sterile DMSO, aliquot, and store at –20°C. Working solutions are prepared daily on an experimental basis by making a 1: 100 dilution in Krebs loading buffer (see Note 3).
6. Liquid aspiration equipment: this should be assembled for use with multiple washes during fluorescent dye labeling. Components for this device should include a vacuum and trap flask set up in series with inline filters to prevent liquid from entering the vacuum system. Attach one end of some flexible PVC tubing to the vacuum flask. A glass Pasteur pipette attached to the opposite end of the PVC tubing will serve as an interface and regulator for aspiration of all liquids from cell cultures.

2.3. Scanning Confocal Laser Microscopic Measurement of Intracellular Calcium Fluorescence

1. Lab-Tek two-well coverglass chamber slides (Thermo Fisher Sci., Rochester, NY).
2. Inverted, scanning confocal microscope, equipped with a mercury arc lamp or argon ion laser power source capable of an emission wavelength of 351–365 nm, and two photomultiplier tubes (PMT) capable of quantifying fluorescence in the range of 400–410 nm and 500–530 nm simultaneously (see Note 4).
3. 445 nm Long-pass dichroic filter (Omega Optical, Brattleboro, VT).
4. Neutral density filters (Omega Optical).

2.4. Analysis of Fluorescence Data

1. Data acquisition system for collecting and storing digital images.
2. Spreadsheet software: Microsoft Excel© or available software of choice.
3. Statistical analysis software: Prism 4 GraphPad© Statistical Software or available software of choice.

2.5. Calcium Calibration Curve

1. Calcium Buffer Calibration Kit (Molecular Probes): 10 mM K_2EGTA zero-free calcium buffer, and 10 mM CaEGTA high-calcium calibration buffer (see Note 5).
2. 22% (v/v) ethanol: in all calcium calibration buffers just prior to standards preparation (see Note 6).
3. 10 μM Indo-1/AM or Fluo-3/AM: in all 22% ethanol calcium calibration buffers (see Note 7).

3. Methods

Measurement of intracellular calcium concentration is an important assessment technique in neurotoxicology. Fluorescent kinetic analysis allows for the testing of neurotoxins in models of both acute and chronic exposures not only for effects on basal [Ca^{2+}], but also for the effects on stimulus-dependent [Ca^{2+}] changes. The protocol described here uses the ratiometric fluorescent Ca^{2+} probe Indo-1 acetoxymethyl ester (Indo-1/AM) to measure both basal and stimulus-dependent changes of Ca^{2+} in individual cells over time using scanning confocal laser microscopy (SCLM). Baseline [Ca^{2+}] is measured by scanning prior to the addition of any stimuli, followed by the assessment of stimulus-dependent [Ca^{2+}] changes in the same cell. Treatment of the human astrocytoma cell line 132 1N1 with 1 mM carbachol acts as a positive control for a stimulus-dependent release of Ca^{2+} (7, 11). Indo-1/AM is a cell membrane permeant dye; once inside cells the acetoxymethyl esters are cleaved by esterases to yield free Indo-1. As previously mentioned, Indo-1 is a ratiometric fluorescent Ca^{2+} probe. When not bound to Ca^{2+}, it has an emission wavelength maximum at 470 nm (blue), with significant emission in the green (530 nm). Once Indo-1 binds with Ca^{2+}, the emission wavelength maximum shifts to 405 nm (violet), and much lower fluorescence emissions in the blue/green wavelengths. This allows for a ratio measure of Ca^{2+}-bound versus unbound Indo-1 in individual dual cells.

An alternative protocol for the measurement of intracellular [Ca^{2+}] is included outlining the use of the non-ratiometric Ca^{2+} fluorophore Fluo-3/AM. This alternate protocol can be used in the place of Indo-1, if a laser with UV excitation capability is not available or the imaging system does not have the ability to detect fluorescence simultaneously at two different wavelengths. Fluo-3 displays an increase in fluorescence when bound to Ca^{2+}. This method, however, is greatly dependent on consistent dye loading technique, as any variation will result in differences in fluorescent quantum yield. This is in stark contrast to Indo-1 that serves as its own loading control. Both protocols are described using the human astrocytoma cell line 132 1N1.

Generation of a Ca^{2+} calibration curve is performed in parallel to determine the absolute concentration of Ca^{2+} that corresponds to ratio fluorescence of Indo-1 or non-ratiometric Fluo-3. For Indo-1, this curve will also need to be determined as the ratio of the probe in the Ca^{2+}-bound versus unbound state. Results from ratiometric plots can also be calculated to determine the percentage of cells responding, the average amplitude of response (%above control), and the area under the curve for individual cells ($\mu M \times s$) (6, 11).

3.1. Cell Culture and Instrumental Preparation

1. Immortalized human astrocytoma 132 1N1 cells are maintained in 75-cm^2 flasks. Flasks are fed every 2–3 days with complete, 5% serum medium (see Note 8) and passaged once a week with trypsin to further propagate maintenance cultures in 75-cm^2 flasks. Experimental cultures are plated at a density of 2.0×10^4 cells/cm^2 (2 mL at 5×10^4 cells/mL) in 2-well chamber slides (see Note 9). Multiple wells can be set up for each experimental group, however, because of the total time involved in fluorescent dye labeling and analysis for individual wells it is suggested to not set up more than ten wells for any given experiment. Allow cells to grow for 2 days or until desired confluency is reached, then serum-deprive the cells for two additional days by substituting an equivalent 2 mL volume of serum-free medium.
2. All stock solutions and buffers required for fluorescent labeling should be made prior to the experiment and stored accordingly. Reagents and buffers that require an incubation temperature of 37°C should be pre-warmed. Liquid aspiration set-up should be assembled and ready for use.
3. The ion argon laser/mercury lamp should be warmed up and aligned prior to the start of the dye labeling procedure. Tune the laser/lamp to excite in the range of 351–365 nm with a minimum illumination output of 60 mW. Insert the 455 nm long-pass dichroic filter set so that fluorescent emission is split and can be collected simultaneously by the two detectors at 405 (violet emission) and 530 nm (green emission).
4. Set up the data acquisition software and create a directory folder for saving images and data. Multiple image files will be created during analysis so ensure that an abundant amount of hard-disk drive space is available.
5. Alternate Fluo-3/AM Protocol: Experiments utilizing Fluo-3/AM, follow the instructions given in Subheading 3.1, steps 1–4 with the following exceptions; prepare and treat cell cultures in 35-mm plastic tissue culture dishes instead of 2-well chamber slides. Tune the laser to excite at 488 nm. Insert appropriate filter sets to quantify emission intensities at 530 nm.

3.2. Indo-1/AM and Fluo-3/AM Calcium Fluorophore Loading

1. Aspirate media from the coverglass chambers and rinse once with 2 mL of Krebs bicarbonate buffer, and once with 2 mL loading buffer (see Note 10). If more than two wells are being analyzed, dye loading should be staggered over time. This will be dependent on the analysis time per well and the dye loading procedure. For the purposes of this protocol, a 5-min scan is used and cell loading should be staggered ≥20 min between coverglass chambers.

2. Incubate the cells for 1 h at 37°C in 2 mL of the Indo-1/AM or Fluo-3/AM working solution (see Note 8).
3. Remove the dye labeling solution and replace with 2 mL of Krebs loading buffer. Allow the cells to incubate an additional 15–30 min to allow for full conversion of Indo-1/AM to Indo-1 by esterase activity. This additional incubation time will need to be optimized for a given cell line as nonspecific esterase activities will vary between different cell and tissue types.
4. Remove the loading buffer and perform one additional rinse with Krebs bicarbonate buffer. This is to ensure removal of any residual/unloaded dye that could influence background fluorescence.
5. Add 1 mL of Krebs bicarbonate to the coverglass chamber and leave cells in this volume for the duration of the fluorescent measurements.
6. Alternate Fluo-3/AM Protocol: Follow the instructions given in Subheading 3.2, steps 1–5, substituting Fluo-3/AM for Indo-1/AM.

3.3. Scanning Confocal Laser Microscopic Measurement of Intracellular Calcium Fluorescence

1. Place the chamber slide securely on the microscope platform and bring the cells into focus using incandescent illumination. Initiate a test-scan of the chamber slide with laser/mercury light illumination with fluorescence detection to verify that the cells are in focus, and if necessary update the data acquisition settings (see Subheading 3.1, step 3). Adjust the focus as necessary to ensure that fluorescence emission intensity is optimal for imaging.
2. Adjust the PMT voltage so as to appropriately set the baseline. If the expectation is an increase in intracellular [Ca^{2+}], then baseline fluorescence should be set at a threshold setting such that signals emitting at 405 nm (λ_{em} of Indo-1 when bound to calcium) are not at/near the upper detection limits of the detector. Conversely if the expectation is a decrease in intracellular [Ca^{2+}], then baseline fluorescence should be set at a threshold setting such that signals emitting at 530 nm (λ_{em} of Indo-1 when not bound to calcium) are not at/near the lower detection limits of the detector. The PMT gain/voltage can be changed before experimental analysis begins, however, these settings must remain constant throughout experimentation and measurement of the standard curve. This is to ensure that comparison of ratio values between experiments and the standard curve remains a constant (see Note 11).
3. Set the number of desired scans and the scan delay. The delay setting will be dependent on the scan speed and the field size. Once these settings have been determined, they should remain constant throughout the analysis.

4. Set the step (pixel size) of the image to the desired resolution, and set the number of pixels (in the X and Y dimensions) to accommodate the desired field size.
5. Begin scanning and data collection. Perform at least three scans to determine baseline [Ca^{2+}]. Figure 1a shows representative gray-scale photomicrographs of Indo-1 fluorescence at three different time points presented as unbound Indo-1, Ca^{2+} Indo-1, and the ratio-merged image of the two fluorescent signals. Baseline calcium values were collected from 0 to 45 s. Each baseline scan was performed consecutively at 15 s intervals. Figure 1b represents the corresponding [Ca^{2+}] plot over time in individual cells. Once baseline [Ca^{2+}] has been determined, stimuli for calcium release can be added manually

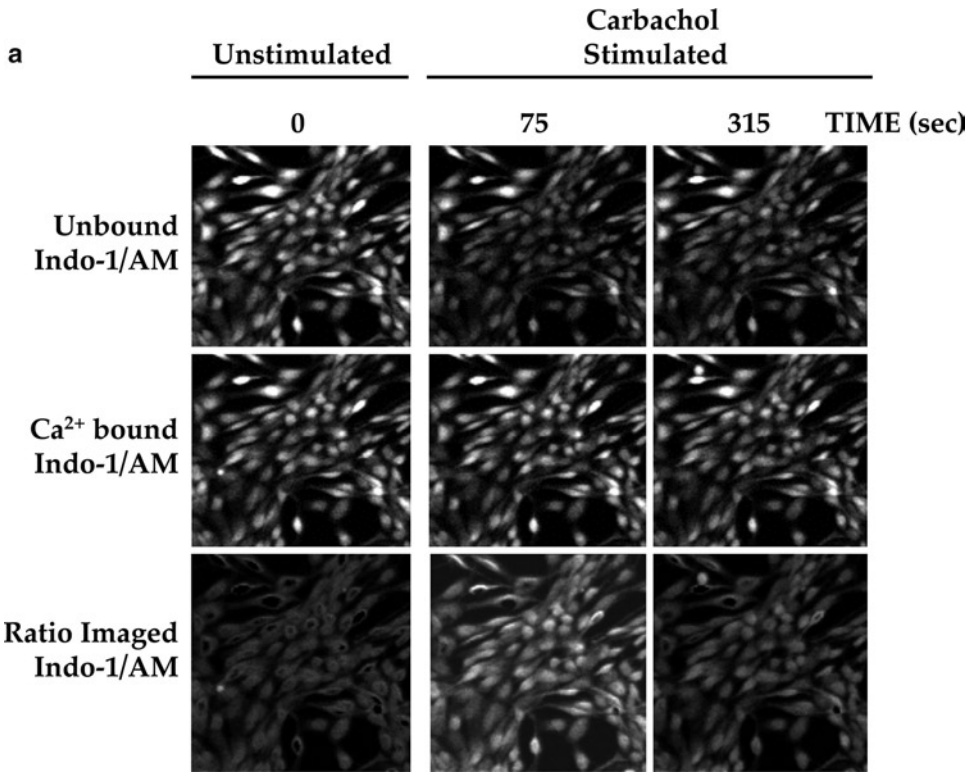

Fig. 1. Representative gray-scale images and associate time-course plots of 132 1N1 human astrocytoma cells. (**a**) Shown are gray-scale images of calcium levels loaded with 2 μM Indo-1/AM under basal conditions in the first image column, and following the addition of 1 mM carbachol in the second and third image columns. Each column contains an image of unbound Indo-1 in the *top* row, calcium-bound Indo-1 in the *middle* row, and the image ratio of bound vs. unbound Indo-1 in the *bottom* row. Images were collected at time points 0, 75, and 315 s to demonstrate Indo-1 ratiometric fluorescence under basal conditions, carbachol-stimulated conditions, and recovery from stimulation, respectively. (**b**) Representative time-course plots of 20 individual 132 1N1 human astrocytoma cells before and after the addition of 1 mM carbachol. The *y*-axis represents the calculated calcium concentration, *x*-axis represents sequential time points collected from 0–315 s. Calcium concentrations at each of the 20 time points were calculated based on the ratio fluorescence collected from individual cells from two independent fields. Individual data points were connected by lines to generate the presented time-course plot. The *dashed vertical line* represents the addition of 1 mM carbachol.

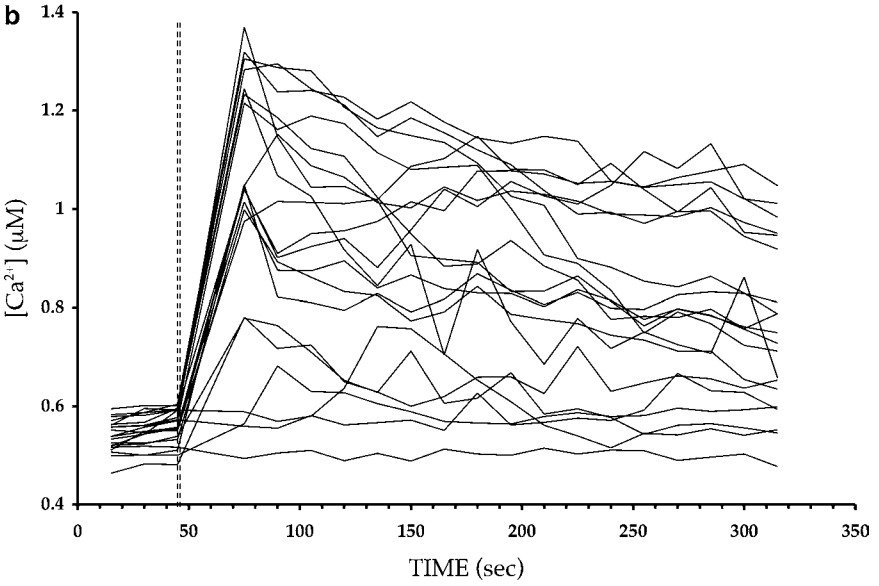

Fig. 1. (continued)

in 1 mL volumes using a pipette. Figure 1 shows increased Indo-1 violet/green fluorescence ratio subsequent to the addition of 1 mM carbachol, consistent with an increase in intracellular Ca^{2+}. Measurement begins at time point 75 s allowing for a 30 s delay from the end of baseline scans to accommodate the addition of stimuli. During this step, care must be taken to not disturb the chamber slide and cause the image to go out of focus. Collect an optimal number of time points as determined by your experimental parameters. Figure 1b demonstrates additional time points collected at 15 s intervals.

6. Alternate Fluo-3/AM Protocol: Follow the instructions given in Subheading 3.2, steps 1–5, substituting Fluo-3/AM for Indo-1/AM.

3.4. Analysis of Fluorescent Data

1. Start with a baseline image for analysis by opening the image file in the appropriate software analysis program (see Note 12).
2. Using an area or polygon selection tool encompass an individual cell within the image field. Save these newly drawn areas/polygons as a template to use with subsequent images of the same field collected from your scans.
3. Measure the total fluorescence in each cell polygon area by determining the integrated fluorescence intensity of the selected cell for images collected at both wavelengths. Perform this measurement for each collected wavelength in each

sequential image using the template you saved for the individual cells.

4. Transfer the data to a spreadsheet program (e.g., Microsoft Excel©) and organize the measured intensities. Calculate the ratio fluorescence by dividing the Ca^{2+}-bound Indo-1-integrated fluorescence intensity by the unbound Indo-1-integrated fluorescence intensity. Compile these ratio values and calculate absolute [Ca^{2+}] using the equation generated from the [Ca^{2+}] calibration curve (see Subheading 3.5).
5. Alternate Fluo-3/AM Protocol: Follow the instructions given in Subheading 3.4, steps 1–4 with the following exceptions; Use the computer software to integrate fluorescence intensity within each polygon/area over time. Final data is reported as relative fluorescence intensity vs. time. Save and export to appropriate spreadsheet program.

3.5. Calcium Calibration Curve

1. Generation of a calcium calibration curve is needed to determine the absolute [Ca^{2+}] corresponding to a given ratio of Indo-1 dye fluorescence in the bound and unbound states. Set up the microscope as described above (see Subheading 3.1, steps 3 and 4). Again, it is important to reiterate that the PMT settings for the two detectors must remain consistent throughout the measurement of both the [Ca^{2+}] standard calibration curve and experimental groups. Any alteration of these settings will modify the ratio for a given concentration of calcium (see Note 11).
2. Scratch the internal surface of an unused 2-well coverglass chamber slide using a sharp metal object. This step aids in proper focusing of the objective on the Indo-1 calibration solution/glass interface in the chamber slide.
3. Adjust the calibration buffers to a pH of 7.4. Use deionized H_2O and plastic containers for all calibration solutions (see Note 7).
4. Add ethanol to standard buffer solutions at a final concentration of 22% (v/v). Additionally add Indo-1 to a final concentration of 10 μM (see Note 7).
5. Use the dissociation constant for EGTA at the experimental conditions of pH = 7.4, temperature of 37°C (K_d^{EGTA} = 43.7 μM), and the provided calculation instructions from the Molecular Probes Calcium Calibration kit to determine free [Ca^{2+}]. Measure and calculate [Ca^{2+}] subsequent to serial dilutions of the high [Ca^{2+}] solution.
6. Secure the chamber slide on the microscope stage and use the scratch previously made in the chamber slide to focus on the internal surface plane of the solution. Adjust the microscope stage until the scratch is no longer in view. Conduct a preview

Calcium (μM)	Ratio
0.000	0.335
0.004	0.342
0.009	0.359
0.015	0.390
0.023	0.420
0.034	0.488
0.051	0.562
0.079	0.689
0.136	0.941
0.307	1.497
0.420	1.712
0.648	2.073
1.329	2.481
9.014	2.846

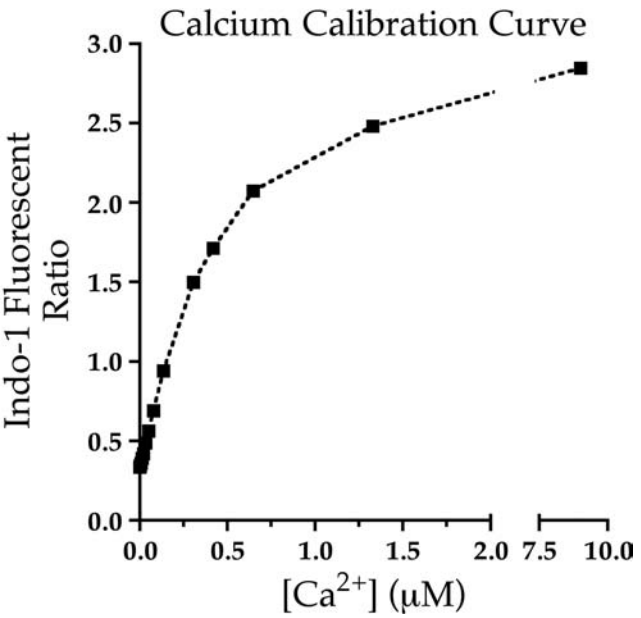

Fig. 2. Representative calcium calibration curve of Indo-1/AM ratio fluorescence versus calcium concentration (μM) in 22% ethanol.

scan using the zero Ca^{2+} solution to focus the objective for maximum fluorescence. Adjust the analysis parameters as discussed in Subheading 3.3, step 2. This will allow for analysis parameters to remain within the linear range of the detection limits of the system.

7. Measurement of Ca^{2+} standards can then be generated using the zero and high [Ca^{2+}] buffers, respectively, by means of the instructions provided with the kit. Fluorescent ratios of Ca^{2+}-bound Indo-1 vs. unbound Indo-1 can be calculated corresponding to the known concentrations of free Ca^{2+}. Use the generated [Ca^{2+}] curve to calculate absolute [Ca^{2+}] corresponding to the ratio fluorescence of Indo-1 collected from experimental groups.
8. Alternate Fluo-3/AM Protocol: Follow the instructions given in Subheading 3.5, steps 1–7, substituting Fluo-3/AM for Indo-1/AM (see Fig. 2).

4. Notes

1. Cell culture systems are composed of living cells. Therefore, sterilization of all equipment and solutions must be performed prior to contact, and aseptic techniques should be used accordingly when handling cultures.

2. Fluorescent labeling dyes are photo-sensitive and their efficacy can be compromised in the presence of excess light. All manipulations with these dyes, and cells loaded with these dyes should be carried out in low-light conditions.
3. Probenecid is useful as an inhibitor of organic anion transporters. Its use may be beneficial to increase dye-loading efficiencies. This is dependent on the cell type and its dye retention capacity (12, 13). The cytotoxicity of probenecid, if any, should be assessed in your cell type prior to use.
4. Preassembled instrumental systems, although ideal, are not necessary for this type of fluorescent analysis. The necessary components are composed of an inverted, scanning confocal microscope equipped with a mercury arc lamp or argon ion laser capable of λ_{em} of 351–365 nm. PMT's or other fluorescent detectors capable of measuring and quantifying simultaneous signals in the ranges of 400–410 and 500–530 nm. Fluorescent filter sets can be obtained from a variety of vendors dependent on their compatibility with the microscope and detectors.
5. It is suggested to use calcium standards from a vendor because of inherent issues with quality control and variability that may arise from producing your own Ca^{2+} standards.
6. Concern over the differences in viscosity between buffer and cells, which could affect the responsiveness of Indo-1/AM or Fluo-3/AM to changes in calcium concentration has been a criticism of using a cell-free method for construction of a calcium calibration curve. To address this issue, the addition of ethanol to a final concentration of 22% in all calibration buffers has been shown to correct for some of the viscosity differences between the cells and buffers (14).
7. Prepare all standards and calibration buffers using deionized H_2O and plastic containers to avoid any external contamination, or leaching of calcium into the standard solutions. A higher concentration of 10 μM Indo-1 or Fluo-3 is used in generating the calcium curve versus the 2 μM used for experimental cell labeling. This is to accommodate for differences in dye loading, and volume concentration that exist between cellular compartments and instrumental cuvettes.
8. All incubations should be carried out in a humidified 37°C, 5% CO_2 incubator unless otherwise stated.
9. Coverglass chamber slides are extremely fragile. Hold the chamber slide by the flanges/edges to avoid breakage and leaks. Depending on the cell type used, there may be different requirements for cell attachment that are not included with the chamber slides. This may involve precoating the chamber slides with artificial extracellular matrices such as poly-D-lysine or collagen.

10. Aspirate liquids from the chamber slides by gently placing the tip of the Pasteur pipette into the corner of the chamber. Take care to not make contact with the cell monolayer, and ensure that the rate of liquid aspiration is not too rapid as cell detachment could occur during this process.
11. Care should be taken to optimize excitation output of the laser/mercury lamp so as to result in enough Indo-1 fluorescence emission for analysis, and not cause damage to the cells or cause Indo-1 dye bleaching. This can be accomplished by adjusting the amperage to the laser or through the use of neutral density filters in front of the excitation beam.
12. Different software packages are available in different price ranges. Image J is public domain software. It is a java-based image processing and analysis program provided for free by the National Institutes of Health.

Acknowledgements

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Chapter 17

Homeostatic Regulation of Glutamate Neurotransmission in Primary Neuronal Cultures

Victor Briz and Cristina Suñol

Abstract

Glutamate is the mayor excitatory neurotransmitter in vertebrate nervous system. It has a crucial role in most brain functions under physiological conditions through the activation of both ionotropic and metabotropic glutamate receptors. In addition, extracellular glutamate concentration is tightly regulated through different excitatory amino acid transporters (EAAT). Glutamate neurotransmission is also involved in the neurotoxic effects of many environmental chemicals and drugs. Furthermore, homeostatic changes in glutamate neurotransmission appear in response to prolonged block/enhancement of electrical activity. Here, we describe different approaches to evaluate alterations in glutamate neurotransmission regarding glutamate receptors and glutamate transporters by using primary cultures of neurons and astrocytes. The methods are based on the increased fluorescence of calcium-sensitive probes in response to glutamate agonists, on radioligand binding to glutamate receptors and transport sites, and on inmunocytochemistry visualization of glutamate receptors.

Key words: Glutamate, Receptor function, Receptor binding, Transport, Intracellular calcium, Fluorescence, Radioligand, Primary cultured neural cells

1. Introduction

Glutamate is the mayor excitatory neurotransmitter in vertebrate nervous system. Consequently, it has a crucial role in most brain functions under physiological conditions through the activation of both ionotropic and metabotropic glutamate receptors. In addition, extracellular glutamate concentration is tightly regulated through different excitatory amino acid transporters (EAAT). However, excessive glutamate receptor activation leads to neuronal death, also known as excitotoxicity (1), which is involved in the etiopathology of ischemia and several neurodegenerative

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disorders, such as Alzheimer's or Huntington's diseases. Glutamate neurotoxicity is calcium (Ca^{2+})-dependent and it is mainly mediated through the activation of *N*-methyl-D-aspartate (NMDA) receptor (2). In contrast, a reduced glutamatergic synaptic activity has been related to learning and memory deficits (3) and to the development of schizophrenia (4). Glutamate neurotransmission is also involved in the neurotoxic effects of many environmental chemicals and drugs. For instance, methylmercury blocks glutamate transporters both in neurons and astrocytes (5, 6), increasing extracellular glutamate concentration. Organochlorine pesticide's acute toxicity can be prevented by glutamate receptor antagonists (7), whereas long-term exposure to low concentrations of these pollutants reduces glutamate receptors functionalities (8, 9). In addition, the NMDA receptor is the molecular target of several drugs of abuse, such as ketamine or phencyclidine (10).

Homeostatic changes in glutamate neurotransmission appear in response to prolonged block/enhancement of electrical activity. Chronic blockade of neural activity results in increased synaptic clustering and activity of ionotropic glutamate receptors (11, 12). On the contrary, a decrease of functional glutamate receptors is seen when neural activity is enhanced by the inhibition of GABAergic neurotransmission (8, 9).

In this chapter, we describe different approaches to evaluate alterations in glutamate neurotransmission regarding glutamate receptors and glutamate transporters by using primary cultures of neurons and astrocytes. Primary cultures of cortical neurons and cerebellar granule cells express glutamate receptors and respond to glutamate agonists by increasing intracellular calcium (8, 13). Primary cultures of cerebellar granule cells also express the neuronal EAAT3 (6), whereas primary cultures of astrocytes express glial EAAT1-2 (14). These cultures constitute in vitro models that are extensively used in neuropharmacological and neurotoxicological studies involving glutamate neurotransmission, neurodegeneration, and neuroprotection mechanisms.

Activation of ionotropic NMDA and AMPA/Kainate receptors induces Ca^{2+} influx, either directly through their own channel or through the activation of voltage-gated calcium channels (2). Furthermore, activation of type-I metabotropic glutamate receptors (mGLUR) also increase $[Ca^{2+}]_i$ by stimulating its release from intracellular stores (15). Therefore, their functionalities can be evaluated by measuring the increase in $[Ca^{2+}]_i$ in response to agonist's treatment with the fluorescent probe Fluo-3AM. On the other hand, [^{3}H]-MK801 binding assay may be useful to test compounds with potential affinity for the NMDA receptor and moreover to quantify functional NMDA receptor expression. By using [^{3}H]-aspartate uptake, we can measure the activity of glutamate transporters. Finally, an example is given on how to measure (qualitatively and quantitatively) NMDA receptor scaling in response to long-term exposure to the organochlorine pesticide

dieldrin, by using the combination of Fluo-3AM fluorescence, [^{3}H]-MK801 binding, and confocal immunofluorescence for the NR1 subunit of the NMDA receptor.

2. Materials

2.1. Cell Cultures

1. Pregnant NMRI mice (16th day of gestation), 7-day-old NMRI mice, and newborn NMRI mice are used for the preparation of primary cultures of cortical neurons, cerebellar granule cells, and astrocytes, respectively.
2. Dulbecco's Modified Eagle's Medium (DMEM) (Biochrom AG, F0455) pH 7.0 supplemented with 26.2 mM $NaHCO_3$, 25 mM glucose, 0.2 mM L-glutamine, 100 mU/L insulin, and 7 μM *p*-aminobenzoic acid. Filter under sterile conditions and add 10% fetal bovine serum (FBS). For cerebellar granule cell cultures, DMEM contains 25 mM KCl.
3. Krebs buffer: 120.9 mM NaCl, 4.83 mM KCl, 1.22 mM KH_2PO_4, 25.5 mM $NaHCO_3$, 12 mM glucose, 3 g/L bovine seroalbumin, and 0.015 g/L phenol red. Prepare sterile and store at 4°C.
4. Trypsin solutions. Prepared in Krebs buffer containing 1.2 mM $MgSO_4$, 50 mg/L deoxyribonuclease I (DNAase), and 250 mg/L trypsin. To stop the trypsinization process, prepare a solution containing 500 mg/L soybean trypsin inhibitor and 50 mg/L DNAase in Krebs buffer. Make fresh as required. Prepare sterile.
5. Mitotic inhibitor solution. Prepare a solution containing 250 μM 5-fluoro-2′-deoxyuridine and 1 mM uridine (50×) in distilled water. Prepare sterile.
6. 50 mg/L poly-D-lysine solution in distilled water. Prepare sterile.

2.2. Measurement of $[Ca^{2+}]_i$ by Fluo-3AM Fluorescence

1. Fluo-3AM fluorescent dye (Molecular Probes, Eugene, OR, USA) is a lyophilized and light sensitive product. Store at –20°C. Make fresh as required.
2. Pluronic F-127 (Molecular Probes) is dissolved in dimethyl sulfoxide (DMSO) at 167 mg/ml, by incubating for 10 min at 50°C. Make fresh as required.
3. Hank's buffer solution: 137 mM NaCl, 1.3 mM CaCl, 5.4 mM KCl, 0.4 mM KH_2PO_4, 0.5 mM MgCl, 0.4 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 8 mM HEPES, and 5.5 mM glucose (adjusted to pH 7.4 with NaOH if necessary). Store at 4°C. pH is stable at 4°C up to 2 weeks.
4. Magnesium-free (Mg-free) Hank's buffer solution: 137 mM NaCl, 1.3 mM CaCl, 5.4 mM KCl, 0.4 mM KH_2PO_4, 4.2 mM

$NaHCO_3$, 0.3 mM Na_2HPO_4, 8 mM HEPES, and 5.5 mM glucose, adjusted to pH 7.4. Store at 4°C. pH is stable at 4°C up to 2 weeks.

5. L-Glycine solution is prepared in water at 5 mM. Store at 4°C (see Note 1).
6. L-Glutamic acid, kainic acid, and (*R,S*)-3,5-dihydroxyphenylglycine (DHPG, Tocris Cookson, Bristol, UK) solutions are prepared in Hank's buffer all at 1 mM. Store at 4°C. Stable at 4°C up to 1 month.
7. NMDA solution is prepared in Magnesium (Mg)-free Hank's buffer at 1 mM. Store at 4°C. Stable at 4°C up to 1 month (see Note 2).
8. (+)-MK-801 hydrogen maleate and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) disodium salt (Tocris) are dissolved in water at 2 mM. Store in aliquots at –20°C.
9. 6-Methyl-2-(phenylethynyl) pyridine (MPEP, Sigma, St. Louis, MO, USA), 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCOOEt, Sigma) are dissolved in DMSO at 6 mM. Store in aliquots at –20°C.
10. A23187-free acid or calcimycin (Invitrogen, Barcelona, Spain) is reconstituted in DMSO at 25 mM. Store in aliquots at –20°C. Working solution is made fresh as required in Hank's buffer at 125 µM.
11. $CuSO_4$ solution (5 mM) is prepared in water containing 0.9% NaCl. Store at 4°C.
12. Multichannel pipette.
13. Fluorimetric plate reader (Ex 485/Em 530; SpectraMax GeminisXS; Molecular Devices, Sunnyvale, CA, USA).

2.3. [^{3}H]-MK801 Binding

1. Phosphate buffer solution (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4 (adjust to pH 7.4 with HCl if necessary). Store at room temperature.
2. Solution containing 100 µM L-glycine and 100 µM L-glutamic acid is prepared in PBS. Store at room temperature.
3. [^{3}H]-MK801 (Perkin Elmer, Boston, MA, USA) (1 mCi/ml in ethanol) must be stored at –20°C. Prepare fresh [^{3}H]-MK801 solution in PBS at 105 nM for each experiment.
4. (+)-MK-801 hydrogen maleate (Sigma) is dissolved at 100 µM in PBS containing 100 µM L-glycine and 100 µM L-glutamic (see Note 3). Make fresh as required. Manage with care. Hazard information: harmful by contact, ingestion, and inhalation; irritant, slightly neurotoxic, flammable.
5. NaOH is dissolved at 0.2 M in water. Store at room temperature. Manage with care; corrosive.

6. Optiphase 'Hisafe'2 (Perkin Elmer) liquid scintillation cocktail. Store in the unopened container, protected from the light at room temperature.
7. Ultra clear polypropylene tubes of 3 ml (Delta Lab, North Huntingdon, PA, USA).
8. Liquid scintillation counter (Wallac 1414 Winspectral™; Wallac Oy, Turku, Finland).

2.4. Measurement of Glutamate Transport by [^{3}H]aspartate Uptake

1. HEPES-buffered saline solution (HBSS): 136 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 1.4 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 10 mM HEPES, and 9 mM glucose, adjusted to pH 7.4.
2. 170 µM Glutamate solution: Dilute 60 µL of 2 mM glutamate solution in a total volume of 700 µL with HBSS. This solution is used to prepare the [^{3}H]aspartate solution.
3. [2,3-^{3}H]-D-aspartate (Perkin Elmer, 1 mCi/ml) must be stored at 4°C. Prepare fresh [^{3}H]-D-aspartate solution at 70 nM in the above 170 µM glutamate solution. Take the solution up and down with a pipette for a complete mixing or use the vortex very gently.

2.5. Immunocytochemistry for the NR1 Subunit of the NMDA Receptor

1. Permanox chamber slides (Nunc™, Roskilde, Denmark).
2. Pure methanol pre-tempered at –20°C. Manage with care: toxic and highly flammable.
3. Lysis buffer solution: 0.03% Triton X-100 in PBS. Triton X-100 is irritant and harmful.
4. Blocking buffer solution: 5% bovine serum albumin (BSA) in PBS.
5. Primary antibody solution: goat polyclonal anti-NMDAR1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) is diluted in blocking buffer solution at 1:50.
6. Secondary antibody solution: chicken anti-goat Alexa 488 (Molecular Probes) is diluted in PBS at 1:1,000.
7. Mowiol 4–88 (Calbiochem, EMD Bioscience, Inc., La Jolla, CA, USA).
8. Confocal fluorescence microscope.

3. Methods

3.1. Preparation of Primary Neural Cell Cultures

1. Obtain the cerebral cortex from E16 embryos or the cerebellum from postnatal P7 mice to prepare primary cultures of cortical neurons and cerebellar granule cells, respectively. Glial cells are prepared from cerebral cortex of P1–P2 mice.

2. Mince the tissue and digest the cells with the trypsin solution, under mild agitation at 37°C. Stop digestion by adding the trypsin inhibitor solution.
3. Disperse the cells with a cannula or pipette, centrifuge at $200 \times g$ for 5 min and resuspend in DMEM.
4. Seed the cell suspension (1.6×10^6 cells/ml for neuronal cultures and 4×10^5 cells/ml for astrocyte cultures) in 24-well plates (0.5 ml/well) and 96-multiwell plates (0.1 ml/well), pre-coated with poly-D-lysine, and incubated for 6–9 days in a humidified 5% CO_2/95% air atmosphere at 36.8°C without changing the culture medium (neuronal cultures) or changing the medium every 4–6 days.
5. After 24–48 h in culture, add the mitotic inhibitor to halt glial proliferation for neuronal cultures.

3.2. Measurement of $[Ca^{2+}]_i$ by Fluo-3AM Fluorescence

Fluo-3AM is a quite sensitive and membrane permeable fluorescent dye, which is cleaved by intracellular esterases. Fluo-3 fluorescence (F) is directly proportional to $[Ca^{2+}]$ present in the cultures. Therefore, it can be used as an indicator of changes in $[Ca^{2+}]_i$ induced by glutamate (nonspecific endogenous agonist of glutamate receptors) or by specific agonists of either NMDA receptor, AMPA/Kainate receptors (kainic acid), or type-I mGLUR (DHPG) (see Note 4). Figure 1 shows the effects of glutamate receptor agonists and antagonists on $[Ca^{2+}]_i$. Specificity of the assay is demonstrated by the complete reversion of agonist effects by specific antagonists (Fig. 1b). The ionophore calcimycin and the quenching agent $CuSO_4$ are used to obtain the maximum (F_{max}) and the minimum (F_{min}) fluorescence values, respectively. $[Ca^{2+}]_i$ was calculated for each well as: $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$, where K_d is the dissociation constant of Fluo3AM/Ca^{2+} (320 nM) (16).

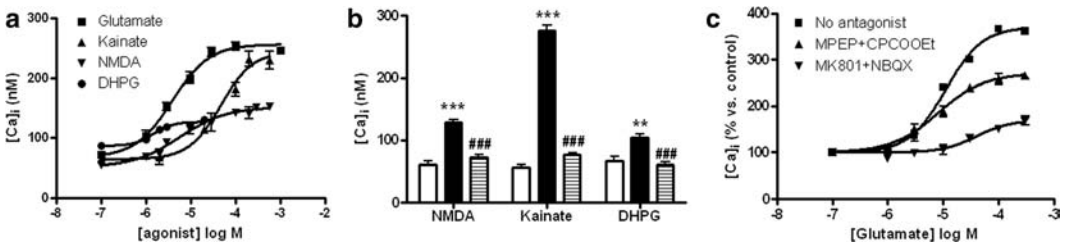

Fig. 1. Effects of glutamate receptors agonists/antagonists on $[Ca^{2+}]_i$ measured by fluo-3 fluorescence in primary cultures of cortical neurons. (**a**) Concentration–response curves for glutamate and the specific agonists NMDA, Kainate, and DHPG. (**b**) Agonist-induced $[Ca^{2+}]_i$ rise (*black bars*) is completely reversed by their respective antagonists (*stripped bars*) (see Note 4). *White bars* represent $[Ca^{2+}]_i$ in the absence of agonist. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. nontreated cultures (*white bars*); $^{\#\#\#}p < 0.001$ vs. agonist-treated cultures. (**c**) Glutamate-induced increase on $[Ca^{2+}]_i$ is reduced by either ionotropic (MK801 and NBQX) or type-I metabotropic (MPEP and CPCOOEt) glutamate receptor antagonists.

1. Reconstitute each aliquot of Fluo-3AM in 15 µl of Pluronic-F127 solution and incubate at 37°C for 5 min. Transfer it into a new tube (protected from the light) containing 10 ml of Hank's buffer solution (final concentration of Fluo-3AM = 9 µM).
2. Remove the culture media from the 96-well plates where the cells are grown (see Note 5). Wash twice with 100 µl/well of Hank's buffer solution and add 80 µl/well of Fluo-3AM solution (see Note 6). Incubate for 1 h at 37°C.
3. Rinse away the excess of Fluo-3AM solution and wash three times with 100 µl/well of Hank's buffer (or Mg-free Hank's buffer).
4. Add 100 µl/well of the agonist solution (see Note 7) with the multichannel pipette and immediately read the fluorescence (F) (Ex 485 nm/Em 530 nm).
5. Add 20 µl/well of calcimycin solution without removing the agonist solution. Incubate for 30 min at room temperature and read the fluorescence (F_{max}).
6. Add 20 µl/well of $CuSO_4$ solution. Incubate for 12 min at room temperature and read the fluorescence (F_{min}).
7. Determine $[Ca^{2+}]i$ for each well by using the above equation.

3.3. [^{3}H]-MK801 Binding

[^{3}H]-MK801 binding can be used to evaluate both the agonist and antagonist properties of several compounds not only within the ion channel but also at allosteric sites on NMDA receptors (17, 18). In addition, it could be used to determine alterations on NMDA receptor activity or cell surface expression after chronic exposure to neurotoxicants or drugs (8, 19). In the first case, a concentration–response curve would be the proper way to test the modulatory effects of the selected compound on [^{3}H]-MK801 binding. The concentration that afford the half inhibition/potentiation on [^{3}H]-MK801-specific binding (IC50/EC50) will indicate the potency of the compound at the NMDA receptor. On the other hand, it is possible to determine the number of functional NMDA receptors from [^{3}H]-MK801 binding assay by fitting the binding values to a competitive one-site binding curve as previously described by De Blasi et al. [20] (see Note 8). From apparent K_d and B_{max} calculation, we will obtain information about the affinity of MK801 (activity of NMDA receptor) and the number of functional NMDA receptors, respectively.

1. Remove the culture media from the 24-well plates where the cells are grown (see Note 5). Wash three times with 500 µl/well of PBS at 37°C.
2. Add 500 µl/well of PBS at 37°C containing 100 µM L-glycine, 100 µM L-glutamic and different concentrations of

the selected compound in the case of concentration–response curve or of non-labeled MK-801 (0–10 μM) to determine apparent K_d and B_{max} (see Note 9). Nonspecific binding is determined in the presence of 100 μM non-labeled MK-801.

3. Add 25 μl/well of PBS containing 105 nM [^{3}H]-MK801 (final concentration in the well 5 nM) and incubate for 15 min at 37°C.
4. Wash out three times with 1.5 ml/well of ice-cold PBS.
5. Add 250 μl/well of 0.2 M NaOH and incubate 4 h at room temperature with agitation.
6. Collect the disaggregated cells from each well and mix them with 2 ml of Optiphase 'Hisafe'2 in polypropylene tubes. After gently agitation radioactivity is measured in a liquid scintillation counter (see Note 10).

3.4. Measurement of Glutamate Transport by [^{3}H]-D-Aspartate Uptake

The assay has been reported to be useful to evaluate alterations in the functionality of the EAATs in the presence of environmental neurotoxicants such as methylmercury and manganese in primary neuronal and glial cultures (6, 21). EAATs catalyze Na^+- and K^+-coupled transport of L-glutamate as well as L- and D-aspartate with similar affinities. Therefore, the uptake of radioactive-labeled D-aspartate (as a tracer of glutamate) can be used to determine the inhibitory potency (IC50) of the selected compound at the glutamate transporter, by performing a concentration–response curve. Primary cultures of cerebellar granule cells are a nearly homogenous population of glutamatergic neurons that express the glutamate transporter EAAT3 (6), whereas primary cultures of astrocytes express different levels of GLAST (EAAT1) and GLT1 (EAAT2) (14, 26) (see Note 11).

1. Remove the culture media from the 24-well plates where the cells are grown (see Note 5).
2. Wash out three times with 500 μl/well of HBSS at 37°C.
3. Add 250 μl/well of HBSS and 250 μl/well of the test compound (2×). Nonspecific uptake is determined in the presence of 1 mM glutamate. Mix solutions gently by drawing a virtual eight with the plate.
4. Incubate 10 min at 37°C.
5. Add 25 μl/well of [^{3}H]-d-aspartate solution PBS containing (final concentration in the well 5 nM). Mix solutions gently and incubate 5 min at 37°C.
6. Wash out three times with 1.5 ml/well of ice-cold HBSS.
7. Add 250 μl/well of 0.2 M NaOH and incubate 4 h at room temperature with agitation.
8. Collect the disaggregated cells from each well and mix them with 2 ml of Optiphase 'Hisafe'2 in polypropylene tubes.

After gently agitation radioactivity is measured in a liquid scintillation counter (see Note 12).

3.5. Homeostatic Scaling of NMDA Receptors Visualized by Immunocytochemistry

Changes on NMDA receptor localization can be monitored by performing immunolabeling of the NR1 subunit of the NMDA receptor followed by confocal immunofluorescence detection. NR1 immunofluorescence have been used to confirm the reduction of NMDA receptor cell surface expression after long-term exposure to the organochlorine pesticide dieldrin in primary cultures of cortical neurons and cerebellar granule cells (8, 9). Figure 2 shows an example of how the reduction of NMDA receptor functionality can be observed by three different techniques described here: Fluo-3 fluorescence, [^{3}H]-MK801 binding, and immunocytochemistry against the NR1 subunit of the NMDA receptor.

1. Remove the culture media from Permanox chamber slides where the cells are grown and wash twice with 200 µl/well of PBS.

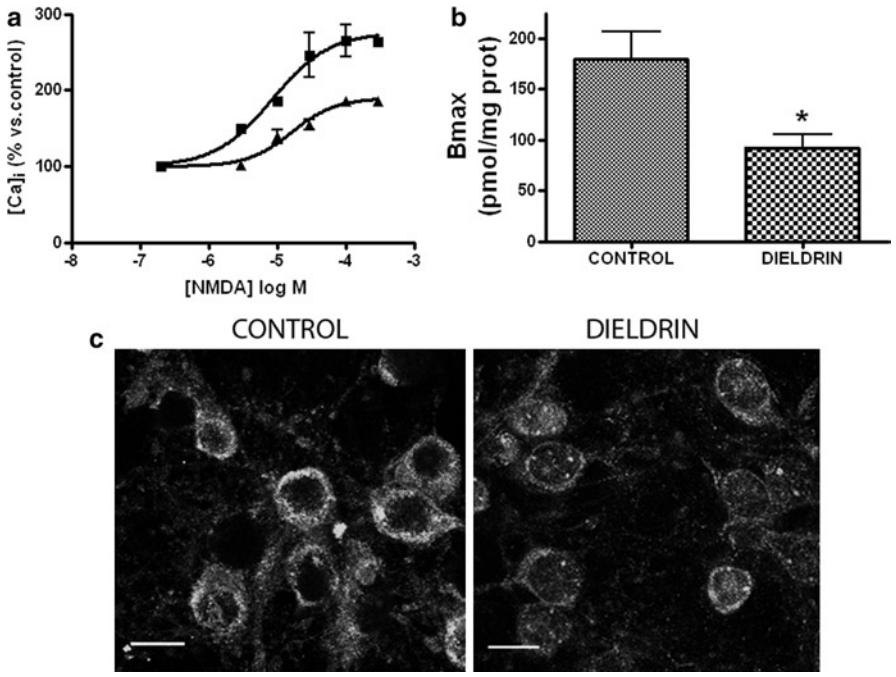

Fig. 2. Effects of long-term exposure to the insecticide dieldrin (60 nM for 6 days in vitro) on NMDA receptor functionality and cell surface expression in primary cultures of cortical neurons. (**a**) Concentration–response curve for NMDA-induced $[Ca^{2+}]_i$ rise in nontreated (*squares*) and dieldrin-treated cells (*triangles*). (**b**) The number of functional NMDA receptors is significantly lower ($p < 0.05$) in dieldrin-treated cells with respect to control cells, as measured by determining B_{max} values from [^{3}H]-MK801 binding. (**c**) Confocal immunofluorescence of the NR1 subunit of the NMDA receptor revealed a reduced immunostaining of NR1 at the plasma membrane of the cells after long-term exposure to dieldrin. *Bar size* = 10 µm.

2. Fix with methanol at −20°C for 10 min.
3. Wash twice with 200 µl/well of PBS. Fixed cells can be stored at 4°C for several days.
4. Incubate with 200 µl/well of lysis buffer for 5 min at room temperature and then wash three times (5 min each rinse) with PBS.
5. Incubate with 200 µl/well of blocking buffer for 30 min at room temperature.
6. Incubate over night at 4°C with 150 µl/well of the primary antibody solution.
7. Wash three times (×5 min) with PBS and incubate with 150 µl/well of the secondary antibody solution for 1 h at room temperature.
8. Wash three times (×5 min) with PBS and coverslip the slides with Mowiol.
9. Examine the cells in a confocal fluorescence microscope (see Note 10).

4. Notes

1. L-glycine is coagonist of the NMDA receptor and dramatically potentiates NMDA receptor activation (22). Therefore, it is convenient that both NMDA and glutamate solutions contain 5 µM L-glycine to observe maximal agonist response.
2. NMDA solutions must be prepared in the absence of Mg because it blocks NMDA receptor under basal conditions. In contrast, for glutamate solutions, this is not necessary since glutamate-induced depolarization remove Mg from NMDA receptor (23). Nevertheless, the sensitivity for glutamate-induced $[Ca^{2+}]_i$ rise at low concentrations of agonist could be improved in the absence of Mg.
3. Glutamate and glycine must be present at such high concentration during the binding assay because both neurotransmitters are needed to maintain the open-channel conformation of the NMDA receptor, which allow MK801 to bind to the channel pore (24). Despite the EC50 for glutamate and glycine binding to NMDAR are around 0.15 µM, it is common to use saturating concentrations of glutamate and glycine for [^{3}H]-MK801 binding in membrane preparations as well as in cultured neurons (8).
4. Eventually, the antagonists of NMDA receptor (MK801), AMPA/Kainate receptors (NBQX), or type-I mGLUR (CPCOOEt for mGLUR1 and MPEP for mGLUR5) may be useful to evaluate the contribution of each glutamate receptor to the glutamate-induced $[Ca^{2+}]_i$ rise in the given culture system (see Fig. 1c).

5. To evaluate NMDA receptor function in primary neuronal cultures, cells should be grown first for at least 7 days in vitro to be sure that they express functional NMDA receptors (25).
6. From here until the end of the experiment 96-well plates must be protected from the light. Furthermore, it is convenient to work and place the fluorimetric plate reader in the maximum darkness conditions.
7. The proper way to study the functionality of glutamate receptors is by performing a concentration–response curve with at least 4–5 different concentrations of agonist, and fitting the values to a sigmoid curve. An appropriate broad range of concentrations for glutamate, NMDA, and DHPG is from 1 to 100 μM, whereas for kainic acid is from 5 to 500 μM (8).
8. In brief, from [^{3}H]-MK801 binding curve, we will obtain two parameters: B_0 (specific binding = total binding – nonspecific binding) and IC50. Now, we can calculate apparent K_d and B_{max} as follows: $K_d = \mathrm{IC50} - L$; $B_{max} = B_0 \times \mathrm{IC50}/L$, where L is the concentration of [^{3}H]-MK801.
9. If the affinity of the selected compound for the NMDA receptor is unknown, use a broad range of concentrations (from 0 to 1 mM) to assure whether the compound has effect or not.
10. Before collecting the cells, 10–20 μl can be taken from each well for protein content determination to express radioactivity as pmol of [^{3}H]-MK801/mg of protein, as follow: DPM/*KAP*. In this equation, DPM is the radioactivity in decompositions per minute, K is the conversion factor from DPM to nanocuries (nCi) (2.24×10^3 DPM/nCi), A is the specific activity of [^{3}H]-MK801 in Ci/mmol, P is the protein content in mg/well. Eventually, the radioactivity of 25 μl of PBS containing 105 nM [^{3}H]-MK801 can be straight measured to calculate the actual concentration of [^{3}H]-MK801 in the well, as follows: ([^{3}H]-MK801 (nM) = DPM/*KAV*), where V is the total volume of the well (0.525 ml).
11. It is worth noting that pure astroglial cultures expressed only GLAST (EAAT1), whereas astrocytes grown in the presence of neurons expressed both GLAST (at increased levels) and GLT1 (EAAT2) (14, 26). Consequently, mixed cultures of astrocytes and cerebellar neurons will express EAAT1, EAAT2, and EAAT3. Thus, mixed cultures may be useful to study general effects on glutamate transport and pure cultures to evaluate the effects of the test compound on one specific EAAT.
12. Since the NMDA receptor is mainly expressed at the cell surface of the cells, the use of confocal fluorescence microscope is needed to discriminate the NR1 immunostaining between the plasma membrane and the cytosol of the neurons.

Acknowledgments

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Chapter 18

Detection of Nitric Oxide Formation in Primary Neural Cells and Tissues

Ronald B. Tjalkens, David L. Carbone, and Guoyao Wu

Abstract

Nitric oxide (NO) is a free radical molecule with a short half-life (<5 s). Because its synthesis from L-arginine by constitutive NO synthase (NOS) is low in many cell types, including neurons and endothelial cells, direct detection of NO in biological systems is a difficult task. During pathological conditions in the CNS, the inducible form of NOS (iNOS or NOS2) is expressed in activated astrocytes and microglial cells and can result in higher levels of NO. However, it may still be difficult to detect NO in these cell types using typical spectrophotometric methods. Of particular note, NO is readily oxidized to nitrite and nitrate (relatively stable products) in cells and medium, which can be measured as a valid indicator of NO synthesis. The conversion of NO to peroxynitrite leads to the formation of stable protein adducts that can be detected by immunohistochemical or immunofluorescence methods. Additionally, intracellular levels of NO can be detected in real time using fluorescence imaging and NO-specific, cell permeable indicator dyes.

Key words: Nitric oxide, Peroxynitrite, HPLC, Immunofluorescence

1. Introduction

To circumvent the problem of detecting low levels of NO in neural cells and tissues, we present several methods based upon analytical, immunohistochemical, and flourescence imaging approaches. The analytical approach describes a simple, rapid, sensitive, and specific high performance liquid chromatography (HPLC) method for detecting picomole levels of nitrite and nitrate in biological samples (1). In this method, nitrite reacts with 2,3-diaminonaphthalene (DAN) under acidic conditions to yield 2,3-naphthotriazole (NAT), a highly fluorescent product, which is stable in alkaline solution (2). Reversed-phase HPLC effectively separates NAT from DAN and other fluorescent

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compounds in samples, permitting fluorescence detection of NAT. Separation of these products is critical to overcoming issues surrounding poor limits of detection inherent in spectrophotometric analysis of DAN/NAT.

NO rapidly reacts with superoxide to form peroxynitrite, a potent electrophile that readily forms covalent adducts with proteins, leading to profound mitochondrial inhibition and neurotoxicity (3). Immunohistochemical detection of 3-nitrotyrosine (3-NTyr) protein adducts thus enables region- and cell-specific detection of NO production relevant to CNS pathology. Immunofluorescence detection of 3-NTyr protein adducts offers the additional advantage of employing antibodies to identify particular cells or structures in conjunction with 3-NTyr adducts, thus allowing a finer level of discrimination of the precise cell type in which NO/peroxynitrite adducts are being formed. We have employed this technique to identify protein adducts in both neurons in vivo (4) and astrocytes in vitro (5).

Finally, a method is presented for the detection of intracellular production of NO in real time using fluorescence imaging. The application of cell permeable NO-specific fluorescent indicator dyes, such as 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM), permit the detection of NO in live cultured cells. The fluorescence signal of DAF-FM is low until reaction with NO, which produces a stable covalent benzotriazole product with much greater quantum yield of emitted fluorescence (6). Using this approach, steady-state increases in NO caused by inflammatory stimuli or rapid increases in NO caused by drugs or endogenous agonists can be reliably detected in real time (7, 8).

2. Materials

2.1. Analytical Determination of NO by High Performance Liquid Chromatography

1. Nitrate reductase (Roche).
2. 2,3-Diaminonaphthalene (DAN, Sigma).
3. C_8 column (15 cm×4.6 mm, 5 μm) and C_{18} column (5 cm×4.6 mm, 40 μm) (both from Supelco, Bellefonte, PA).
4. HPLC-grade methanol and HPLC-grade water (both from Fisher Scientific, Houston, TX).
5. Waters HPLC apparatus including a Model 600E Powerline multisolvent delivery system with 100-μl heads, a Model 712 WISP autosampler, a Model 474 fluorescence detector, and a Millenium-32 Workstation (Waters, Milford, MA).

2.2. Determination of NO/ONOO Formation by Immunofluorescence in Fixed Cells

1. Cell culture plates and compatible thin microglass coverslips (e.g., 24-well culture plate and 12-mm round glass cover slips).

2. Wash buffer: phosphate-buffered saline (PBS) 10× stock containing 1.37 M NaCl, 0.027 M KCl, 0.01 M Na_2HPO_4, and 0.02 M KH_2PO_4; adjust the pH to 7.4 with HCl. Stock may be sterilized by filtration or autoclave if desired. 1× Solutions may then be easily prepared from this stock.
3. 100% Methanol, stored at −20°C.
4. Triton X-100, diluted to 0.1% v/v in PBS.
5. Blocking and antibody dilution buffer: 1% w/v bovine serum albumin (BSA) in PBS.
6. Primary antibody (e.g., anti 3-nitrotyrosine, Chemicon, Temecula, CA).
7. Secondary antibody (e.g., Alexa Fluor-labeled secondary antibody, Molecular Probes, Eugene OR).
8. Microscope slides.
9. Coverslip mounting media (Vectashield mounting media with DAPI, Vector Laboratories, Burlington, CA).
10. Clear nail polish.
11. Fluorescence microscopy detection and quantification capabilities (see Note 1).

2.3. Qualitative Real-Time Detection of NO Formation in Live Cells Using Fluorescence Imaging

1. Round glass coverslips for microscopy, 30 mm diameter (Warner Instruments, Hamden, CT). These are suitable for use with the Zeiss POC Mini incubation chamber (Carl Zeiss Microimaging, Thornwood, NY). Alternately, adherent cells may be subcultured onto chambered coverglass slides; typically 4-well chamber slides are used (Nalge Nunc International, Rochester, NY).
2. 2 mM DAF-FM diacetate (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; Invitrogen, Carlsbad, CA); working stock solution in DMSO (see Note 2).
3. Imaging Medium: Hank's buffered salt solution (HBSS), with HEPES: 0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, and 10 mM HEPES (pH 7.4). Do not add sodium bicarbonate to this solution.
4. 10 mM Adenosine triphosphate, sodium salt (Sigma-Aldrich, St. Louis, MO); working stock solution in 18 Ω MilliQ water. Make fresh and keep on ice.
5. 1–10 mM *S*-nitroso-*N*-acetylpenicillamine (SNAP), (Invitrogen, Carlsbad, CA); working stock solution in DMSO at concentrations appropriate for the specific experiment to be performed.
6. Phosphate-buffered saline (PBS), pH 7.4.
7. Fluorescence microscopy detection and quantification capabilities (see Note 1).

3. Methods

3.1. Analytical Determination of NO by High Performance Liquid Chromatography

1. Filter all samples to be tested through 10-KDa cutoff ultrafilters to remove large molecular weight proteins. Wash filters four times with deionized and double-distilled water (DD-H_2O) prior to use. Dilute filtered samples with DD-H_2O (e.g., 2–3 times for medium from neuron or endothelial cell culture medium) depending on concentrations of nitrite and nitrate.
2. Reaction of nitrite with DAN. This is performed as follows: 100 µl of diluted sample, diluted blank medium, or sodium nitrite standard (0–2 µM) is incubated with 100 µl of DD-H_2O and 20 µl of 316 µM DAN (in 0.62 M HCl) at room temperature for 10 min, followed by the addition of 10 µl of 2.8 M NaOH. This solution is vortexed before analysis by HPLC.
3. Analysis of the nitrite-DAN derivative (NAT; see Fig. 1). 15 µl of the nitrite-DAN derivatization solution is directly injected into a 5-µm C_8 column guarded by a 40-µm C_{18} column for chromatographic separation of NAT. The mobile phase (1.3 ml/min) is 15 mM sodium phosphate buffer (pH 7.5) containing 50% methanol (1 l of 30 mM Na_2HPO_4 and 125 ml of 30 mM NaH_2PO_4 mixed with 1.125 l of 100% methanol) (0.0–3.0 min), followed sequentially by 100% HPLC-grade water (3.1–5.0 min), 100% methanol (5.1–8.0 min), 100% HPLC-grade water (8.1–10.0 min), and the initial 15 mM sodium phosphate buffer (pH 7.5)–50% methanol solution (10.1–15.0 min). All chromatographic procedures are carried out at room temperature. Fluorescence is monitored with excitation at 375 nm and emission at 415 nm. NAT is rapidly eluted from the column and its retention time is approximately 4.4 min (Fig. 2).
4. Analysis of nitrate. Nitrate is converted to nitrite as follows: 200 µl of diluted sample or nitrate standard (0–2 µM), 10 µl of 1 U/ml nitrate reductase, and 10 µl of 120 µM NADPH are mixed and incubated at room temperature for 1 h. This solution is then used directly for nitrite analysis as described for step 3, above. (The conversion of nitrate to nitrite is 98% as determined with known amounts of both standards).

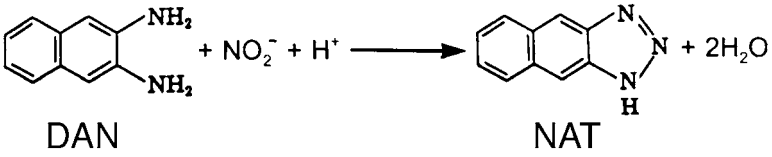

Fig. 1. Reaction of nitrite with 2,3-diaminonaphthalene (DAN) to form 2,3-naphthotriazole (NAT) under acidic conditions. Reprinted from ref. 1, with permission from Elsevier.

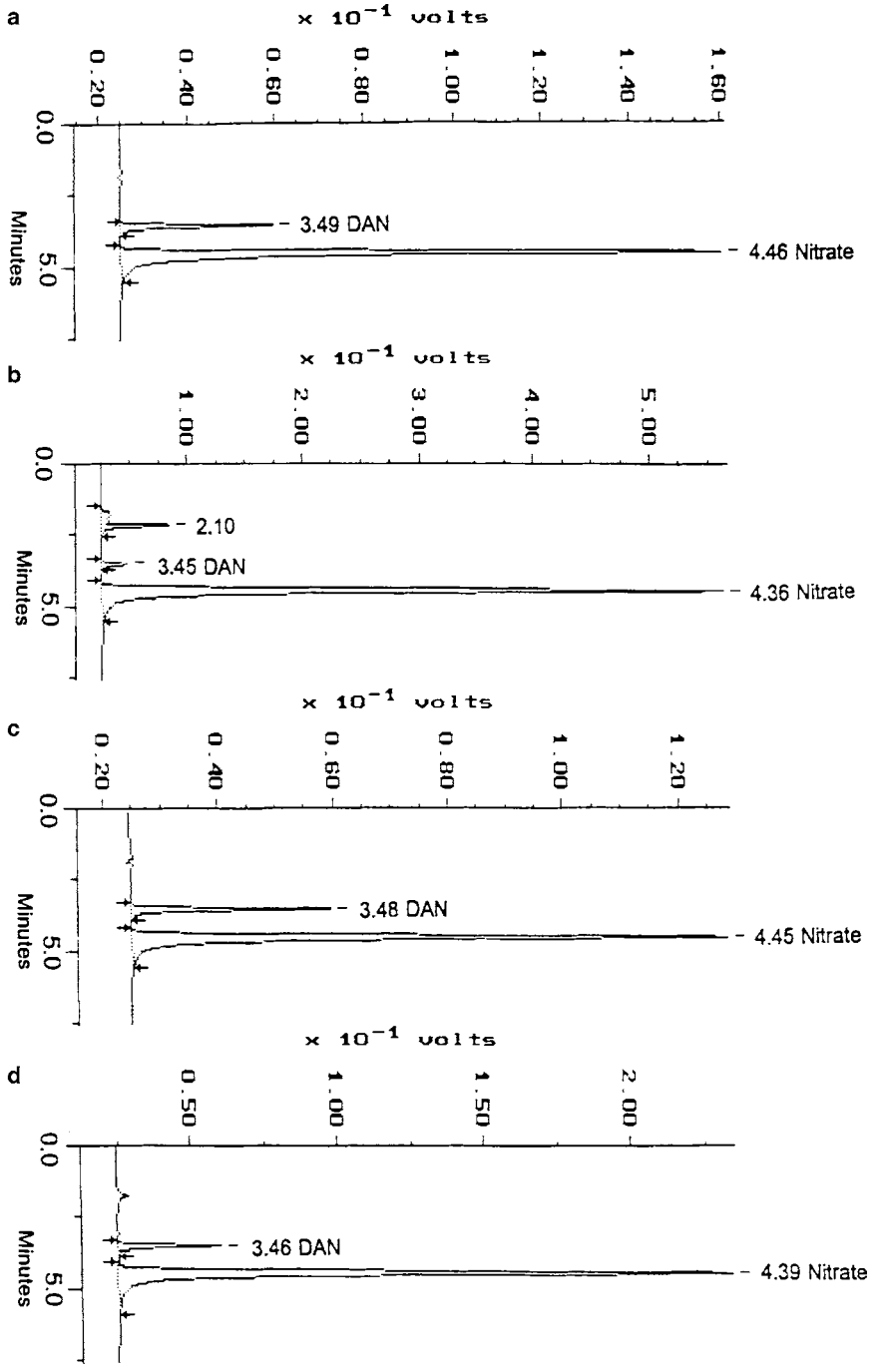

Fig. 2. Analysis of nitrate in biological samples using the HPLC-DAN method. Nitrate was reduced to nitrite by nitrate reductase. Nitrite reacts with 2,3-diaminonaphthalene (DAN) to yield under acidic conditions. DAN was separated from NAT by reversed-phase HPLC followed by fluorescence detection at 375 nm excitation wavelength and 415 nm emission wavelength. (**a**) 200 nM nitrate standard; (**b**) endothelial cell culture medium; (**c**) plasma; (**d**) urine. Reprinted from ref. 1 (with permission from Elsevier).

3.2. Determination of NO/ONOO Formation by Immunofluorescence in Fixed Cells and Tissues

1. Remove the cell culture media and rinse the coverslips with 1× PBS by adding 0.5–1 ml of PBS, and then aspirating (see Note 3).
2. Fix the cells by covering the glass coverslip with methanol prechilled to –20°C, and store at –20°C for 10 min. This step will precipitate proteins and remove lipids.
3. Remove the methanol and wash the cells by adding 0.5–1 ml of 1× PBS.
4. Remove the PBS and permeabilize the membranes by adding 0.1% Triton X-100 for 30 s.
5. Remove the Triton X-100 and perform three, 5 min washes using 1× PBS with gentle agitation using a plate shaker.
6. Remove the final PBS wash, add 1% BSA (w/v) in PBS, and incubate with gentle agitation for 1 h to block.
7. Remove the blocking solution, add the primary antibody, and incubate overnight at 4°C with gentle agitation. We have found our optimal dilution with this antibody to be 1:600; however, this will likely vary based on cell type and other parameters. Furthermore, using a standard 24-well cell-culture plate, 250 ml of antibody solution is typically sufficient to coat the coverslip, provided the plate is agitated using a rotary shaker rather than a plate rocker.
8. Remove the primary antibody and perform three, 5-min washes using 1× PBS.
9. Remove the final wash, add the secondary antibody, and incubate for 1 h in a light-proof container. Because the fluorescent label on the secondary antibodies is light sensitive, limit the exposure to ambient light. The secondary antibody incubation and all subsequent wash steps should be conducted in a light-proof container, such as foil-wrapped plastic ware.
10. Perform three, 5 min washes in 1× PBS, using a light-proof container.
11. One standard microscopy slide can easily accommodate two 20 mm coverslips. Place a small drop (e.g. 3–5 μl) of mounting media on the slide, and place the coverslip cell-side down on top of the drop, taking care to minimize air bubbles.
12. Blot excess mounting media gently with a Kimwipe, and seal the edge of the coverslip by gently applying a bead of clear nail polish around the perimeter of the coverslip.
13. Place the slides in a slide box, and store at 4°C, allowing several hours (overnight) for the nail polish to dry.
14. Fluorescent imaging is conducted using a Zeiss 20× air Plan Apochromatic objective (Carl Zeiss, Inc., Thronwood, NY) using Slidebook v 5.0 (Intelligent Imaging Innovations, Inc., Denver, CO). Regardless of the imaging system employed,

image saturation, or photobleaching should be avoided by using the smallest possible exposure time. An example of immunofluorescence detection of 3-NTry adducts is shown in Fig. 3a.

15. Multiple approaches regarding signal quantification may be employed; however, our laboratory prefers to record the total signal per field, normalized to the number of cells, thus giving a representation of immunoflourescence signal per cell. In our experience, this method has proven more reliable than quantifying mean signal per field, as this latter approach may not accurately represent the robustness, or number of cells adversely affected, by a given treatment. An example of quantifying the fluorescence intensity of 3-NTyr adducts is shown in Fig. 3b.

3.3. Qualitative Real-Time Detection of NO Formation in Live Cells Using Fluorescence Imaging

1. Prior to imaging, allow cells cultured on microscopic grade cover glass to reach approximately 75% confluency.
2. Add DAF-FM (from the 2 mM working stock solution) to 2 μM final concentration in culture medium and incubate 15 min at 37 C, 5% CO_2.
3. Replace the dye incubation solution with Imaging Medium prewarmed to room temperature. All subsequent steps and imaging may be performed at room temperature. Do not wash the cells; simply removed the dye incubation solution and replace directly with Imaging Medium.
4. Mount the coverslip holder or chambered slide on the stage of the inverted microscope and focus using a 20× objective.
5. Imaging parameters for detecting steady-state production of NO:
   - Filter set – fluorescein/FITC (490 nm excitation/515 nm emission). For confocal imaging, a 488 nm laser line is used for excitation. If imaging using a confocal system, attenuate the laser power as much as possible to avoid photobleaching the dye.
   - To minimize exposure time, resolution is typically reduced, e.g., 2 × 2 binning on a charge-coupled device (CCD)-based wide field system. Exposure times should be limited to 20–50 ms if at all possible.
   - Set up parameters for multiple exposures over time to collect one image per minute for 20 min, which allows a steady state to be reached between evolution of NO and reaction with DAF-FM to yield the highly fluorescent benzotriazole derivative.
   - Initiate the imaging sequence as rapidly as possible after mounting cells on the microscope stage to avoid saturation of the DAF-FM signal.

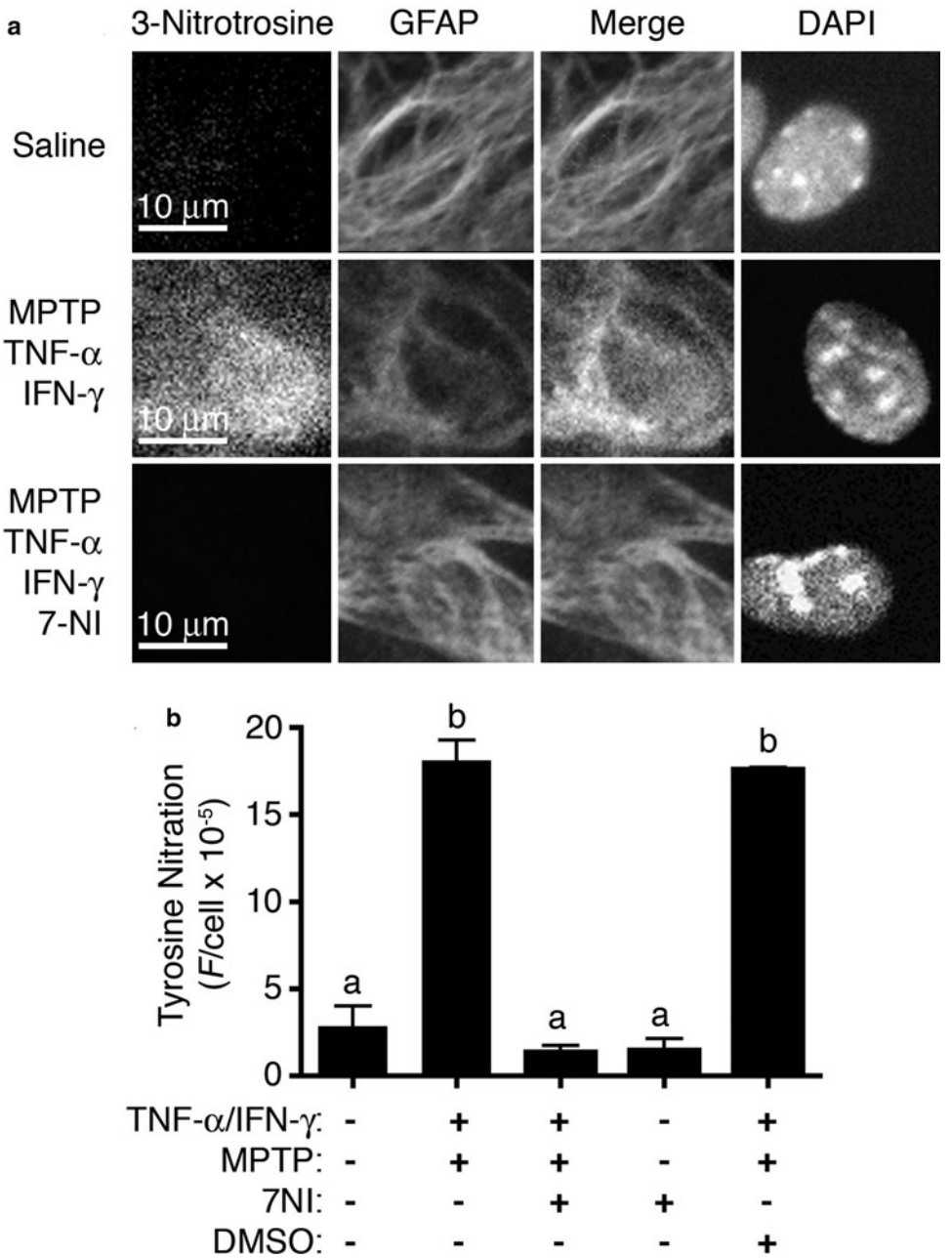

Fig. 3. Inflammatory stimulation astrocytes increase total cellular protein nitration. Astrocytes were exposed for 8 h to 10 μM 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in combination with the cytokines tumor necrosis factor alpha (TNF-α, 10 pg/ml) and interferon gamma (IFN-γ, 1 μg/ml). (**a**) Immunofluorescence detection of 3-nitrotyrosine adducts was performed in conjunction with antibodies against the astrocyte-specific marker glial fibrillary acidic protein (GFAP) to confirm the identity of the cell type. Nuclear morphology was detected using the fluorescent DNA stain, 4′,6-diamidino-2-phenylindole (DAPI, 10 μM in mounting medium) to permit cell counting. (**b**) Total 3-nitrotyrosine signal per field was normalized to the number of cells by counting nuclei and presented as a ratio. Quantitative analysis demonstrates an elevated presence of adducts following the inflammatory stimulation, and prevention of this response with the selective neuronal nitric oxide synthase inhibitor 7-nitroindazole (7-NI). Reprinted from ref. 5 (with permission from Elsevier).

- An example of steady-state imaging of NO production in astrocytes induced by the NO donor, SNAP, is shown in Fig. 4a.

6. Imaging parameters for detecting rapid increases in NO induced by treatment with drugs or agonists:
   - Filter set – fluorescein/FITC (490 nm excitation/515 nm emission). For confocal imaging, a 488 nm laser line is used for excitation. If imaging using a confocal system, attenuate the laser power as much as possible to avoid photobleaching the dye.
   - To minimize exposure time, resolution is typically reduced, e.g., 2×2 binning on a CCD-based wide field system. Exposure times should be limited to 20–50 ms if at all possible.
   - Set up parameters for multiple exposures over time to collect one image per second for 60–300 s.
   - Initiate the imaging sequence and add drug or agonist at the desired time, typically after 10 s (per the parameters given here, this would be the tenth exposure after initiation of the imaging sequence). It is important to collect images for at least the first five exposure times before adding drug or agonist to achieve a steady baseline of fluorescence signal.
   - An example of detecting rapid increases in NO in astrocytes induced by treatment with ATP is shown in Fig. 4b.
7. Analysis. Using the masking feature of the imaging software, place a mask on each cell in the microscopic field to define individual regions of interest for analysis. Export data for the background-subtracted fluorescence intensity of each region

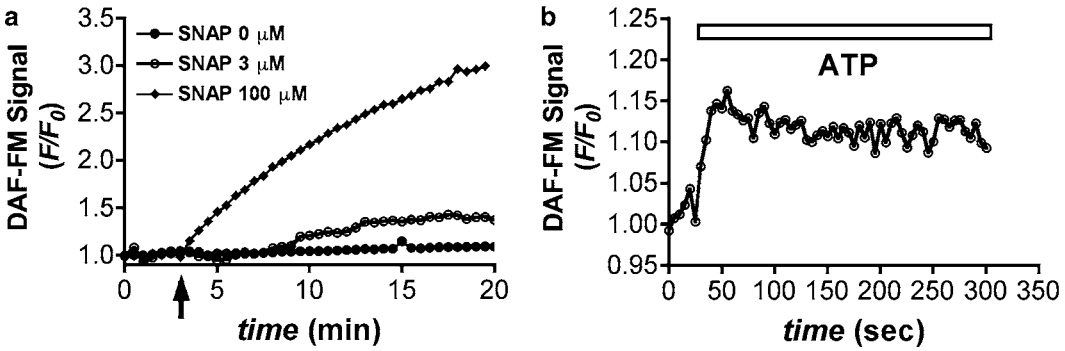

Fig. 4. Real-time detection of NO in live primary cultured astrocytes. (**a**) Primary cortical murine astrocytes were loaded with DAF-FM and examined for increases in NO following addition of increasing concentrations of the NO donor, *S*-nitroso-*N*-acetylpenicillamine (SNAP). (**b**) Rapid changes in intracellular NO were detected using DAF-FM imaging in astrocytes following the addition of ATP to stimulate purinergic receptor-mediated calcium transients.

of interest. Data are expressed as the mean fluorescence intensity of each region of interest relative to the baseline image for that region. This value is expressed as F/F_0, where F is the fluorescence intensity of a region of interest (e.g., a single cell) at any given exposure time divided by F_0, the fluorescence intensity of the same region of interest in the first image collected for the series, the "time zero" or baseline image. Data can then be expressed as the fold-change in DAF-FM signal from the first to the last image in the series, or from the first image to whichever image represents the appropriate point of steady-state production of NO, based upon the shape of the curve (see Note 4).

4. Notes

1. Fluorescence images may be acquired by wide field or confocal microscopy using either an upright or inverted microscope equipped with analysis software suitable for the quantitation of individual cellular fluorescence in multiple channels. The system used to collect the images presented here consists of a Zeiss Axiovert 200M inverted microscope with 20–100× Plan Apochromat objectives, ORCA-ER CCD camera (Hammatsu), DG-4 xenon flash lamp rapid filter changer (Sutter), and a motorized *xy* stage (Proscan stage by Prior). The software used for both acquisition and analysis is Slidebook (v5.0, Intelligent Imaging Innovations, Denver, CO).
2. Fluorescent indicator dyes for live cell imaging are typically easily oxidized and very light sensitive. It is, therefore, highly recommended to purchase the catalog number listed for DAF-FM, which is 1 mg total solid material packaged in ten individual 50 μg amounts. DMSO is then added immediately before the experiment and the vial should be wrapped in aluminum foil to protect from light. It is acceptable to leave this stock solution at room temperature during the experiment. If the working stock solution is to be used for more than one experiment, it should be stored at –20°C. If the working stock solution is to be stored for longer than 1 week, it is helpful to gently flush the headspace with nitrogen and then wrap the tightly capped tube in parafilm before storing at –20°C.
3. These instructions are optimized for primary cortical astrocytes subcultured onto 12-mm coverslips and grown to confluence in a standard 24-well cell culture plate. This protocol is easily adapted to different cell types and coverslip sizes; however, reagent volumes and antibody dilutions will need to

be empirically optimized. Unless otherwise noted, all steps may be performed at room temperature.

4. When calculating F/F_0, it is useful to represent the baseline fluorescence intensity as the average of the fluorescence intensity of each region of interest of the first three images in the sequence. This helps to correct for variations in fluorescence signal that often occur when performing single cell imaging. To calculate the F/F_0 value, divide the fluorescence intensity of a given region of interest at time (t) by the average intensity of the same region for the first three images in the sequence.

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Chapter 19

JNK3-Mediated Apoptotic Cell Death in Primary Dopaminergic Neurons

Won-Seok Choi, Heather M. Klintworth, and Zhengui Xia

Abstract

Investigation of mechanisms responsible for dopaminergic neuron death is critical for understanding the pathogenesis of Parkinson's disease, yet this is often quite challenging technically. Here, we describe detailed methods for culturing primary mesencephalic dopaminergic neurons and examining the activation of c-Jun N-terminal protein Kinase (JNK) in these cultures. We utilized immunocytochemistry and computerized analysis to quantify the number of surviving dopaminergic neurons and JNK activation in dopaminergic neurons. TUNEL staining was used to quantify apoptotic cell death. siRNA was used to specifically inhibit JNK3, the neural specific isoform of JNK. Our data implicate the activation of JNK3 in rotenone-induced dopaminergic neuron apoptosis.

Key words: Dopaminergic neuron, JNK, Primary culture, Immunocytochemistry, TUNEL, siRNA

1. Introduction

Much effort has been put toward understanding mechanisms of cell death in neurodegenerative diseases. In Parkinson's disease, cell death of dopaminergic neurons is responsible for motor deficits; investigation of the underlying cell death mechanisms is important for a better understanding of Parkinson's disease pathogenesis (1). Many studies used model dopaminergic cell lines in the past. Although studies using model cell lines are clearly informative, it is important to confirm major observations in primary cultures because cell lines are immortalized and quite different from postmitotic, differentiated neurons. Primary dopaminergic neurons are most often prepared from embryonic day 14 (E14) embryos from rats or mice. However, the mesencephalon of an E14 mouse brain is very small; one can obtain approximately 150,000–300,000 cells from each embryo, of which only 1–5%

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are tyrosine hydroxylase (TH)-positive, dopaminergic neurons (2). Thus, this primary neuron culture system is technically challenging and it is important to develop techniques to maximize the use of these cells and identify cell-specific molecular responses to stimuli. The detailed methods for preparing and culturing primary dopaminergic neurons are described here. These methods can be adapted to prepare cultures from a single mouse embryo which can be extremely useful for studies involving transgenic knock-out animals (3, 4).

There are three major forms of cell death: autophagy, necrosis, and apoptosis. Autopagy relies on the cell's own lysosomes to breakdown the cell from inside out. Necrotic cells generally burst, releasing their cellular contents into the surrounding extracellular space triggering an inflammatory response. Apoptosis is an energy-dependant programmed form of cell death and can be distinguished from necrosis, an uncontrolled form of cell death, in several ways. Characteristics of apoptosis include nuclear condensation, DNA fragmentation, membrane blebbing, and phagocytosis by neighboring cells (5). Unlike necrosis, apoptosis does not cause an immune response. Apoptosis can be identified by assaying nuclear morphology and Terminal dUTP Nick-End Labeling (TUNEL). Both of these methods can be used to identify apoptotic dopaminergic neurons (6, 7) and are described here.

There are several signaling pathways involved in apoptosis including c-Jun N-terminal protein kinase (JNK), a member of the mitogen-activated protein (MAP) kinases. The JNK signaling pathway is activated by phosphorylation and can work together with mitochondrial Bcl-2 family proteins to activate caspases, thus causing apoptosis (8, 9). JNK is of special interest since it is activated in response to toxicants and oxidative stress and is often associated with apoptotic cell death (10–12). Most importantly, JNK activation has been implicated in several Parkinson's disease models (7, 11, 13, 14). JNK signaling activation can be measured in dopaminergic neurons using immunocytochemistry (ICC) and quantification techniques described in this manuscript.

The ten JNK isoforms are encoded by three genes: *Jnk1*, *Jnk2*, and *Jnk3* (15). While *Jnk1* and *Jnk2* are expressed ubiquitously, *Jnk3* is the only neural-specific isoform of JNK. Though several drug inhibitors have been used to inhibit JNK, none is isoform specific. Small interfering RNAs (siRNAs) are 20–25 nucleotides of double-stranded RNA that interfere with specific gene expression by cleaving and degrading messenger RNAs with perfect matching complementary sequence or blocking translation of the target mRNA (16, 17). siRNAs have been widely used to silence the expression of a specific gene. Recently, specific siRNAs for *Jnk1*, *2* or *3* have been used in primary cultured cells to prevent the expression of each isoform of JNK (3, 18). The methods for siRNA transfection into primary cultured dopaminergic neurons and verification by PCR are described in this manuscript.

2. Materials

2.1. Primary E14 Ventral Mesencephalic Culture

1. Embryonic day 14 (E14) Sprague-Dawley rats or mice.
2. Aclar sheets (Electron Microscopy Sciences, Hatfield, PA).
3. Poly-D-lysine (PDL) and laminin aliquoted for single use and stored at −80°C.
4. Dissection tools: large, medium, and small scissors, and fine forceps.
5. Dissection scope.
6. Phosphate-buffered saline (PBS), pH 7.2.
7. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin, 25 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], and 30 mM glucose. Supplements are aliquoted and stored at −20°C (see Note 1). Fresh media is made for each culture.
8. DMEM-F12 supplemented with 1% N2 supplement and 10 μg/mL bovine serum albumin (BSA). Supplements are aliquoted and stored at −20°C. Fresh media is made for each culture.
9. Rotenone is dissolved in dimethylsulfoxide (DMSO) to 1 mM and paraquat is dissolved in water to 400 mM. Both solutions were aliquoted and stored at −20°C. Aliquots are diluted in culture medium to desired concentration before drug treatment.

2.2. ICC and Quantification of JNK Activation in Dopaminergic Neurons

1. Fixing solution: 4% paraformaldehyde/4% sucrose in PBS, pH 7.5. Heat 70 mL water with 0.3 ml of 0.5% Phenol-red to 60°C in fume hood. Add 4 g paraformaldehyde and stir. Add 5N sodium hydroxide drop wise until solution is clear to help dissolve. Add 4 g sucrose and stir until dissolved. Add 10 mL of 10× PBS. Adjust water to about 100 mL. Adjust pH to 7.5 and bring to a final volume of 100 mL. Solution can be aliquoted and stored at −20°C. A 1 L stock of 10× PBS can be prepared by dissolving 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, and 2.4 g KH_2PO_4 in 0.8 L of distilled water and bringing the final volume to 1 L.
2. 10 mM Glycine in 0.1% Triton X-100 in 1× PBS (PBST). For 1 L of 1× PBS buffer dissolve 8.00 g NaCl, 0.20 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in 800 mL of water and adjust the pH to 7.4. Add water to bring the final volume to 1 L.
3. Permeablization solution: 0.5% IPEGAL in 1× PBS.
4. Blocking solution: 2.5% BSA, 5% horse serum, and 5% goat serum in PBST. Serum stored in small aliquots at −20°C.

5. Humidified container: This can consist of a 10-cm petri dish lined with damp filter paper or paper towel.
6. Primary antibodies: mouse monoclonal antibody against tyrosine hydroxylase (TH; 1:500), *gamma*-Aminobutyric acid (GABA; 1:1,000), and rabbit polyclonal antibody against TH (1:5,000), phospho-JNK (1:100), active caspase-3 (0.2 μg/mL).
7. Secondary antibodies: Alexa Fluor 488, 568, 594, or 660 goat anti-rabbit IgG and Alexa Fluor 488, 568, 594, or 660 goat anti-mouse IgG (1:200).
8. Nuclear stain: 2.5 μg/μL Hoechst 33258 in PBST. Solution can be stored at 4°C for several months.
9. Aqua-Poly/Mount.
10. Fluorescent microscope equipped with a digital camera.
11. NIH Image J program (http://rsb.info.nih.gov/ij/).

2.3. TUNEL

1. Fixing solution: 4% paraformaldehyde in PBS, pH 7.5. Heat 70 mL water with 0.3 ml of 0.5% Phenol-red to 60°C in fume hood. Add 4 g paraformaldehyde and stir. Add 5N sodium hydroxide drop wise until solution is clear to help dissolve. Add 10 mL of 10× PBS. Adjust water to about 100 mL. Adjust pH to 7.5 and bring to a final volume of 100 mL.
2. 1× PBS.
3. Permeablization solution: 1× PBS containing 0.1% Triton X-100 and 0.1% sodium citrate.
4. Terminal deoxynucleotidyl Transferase with 5× buffer (TdT, Promega, Madison, WI).
5. Biotin-11-dUTP.
6. Streptavidin-FITC.
7. Fluorescent microscope.

2.4. Transfection of JNK siRNAs into Primary Cultured Neurons

1. siRNA against *Jnk1*, *2*, or *3* and scrambled control, non-silencing siRNA (18) (QIAGEN, Valencia, CA).

   Jnk1 siRNA sequence is 5′ GAAGCUCAGCCGGCCAU UUdTdT 3′;

   Jnk2 siRNA 5′ GCCUUGCGCCACCCGUAUAdTdT 3′;

   Jnk3 siRNA 5′ GCCAGGGACUUGUUGUCAAdTdT 3′;

   Scrambled siRNA 5′ UUCUCCGAACGUGUCACGUd TdT 3′.
2. TransMessenger™ Transfection Reagent (QIAGEN) with Enhancer R and Buffer EC-R included.

3. Methods

3.1. Primary E14 Ventral Mesencephalic Culture

1. Two days prior to culture, punch out Aclar coverslips 1 cm in diameter (for 24-well plates). Collect coverslips in a 50-mL conical tube and add 70% ethanol (EtOH) to completely cover the coverslips. Vortex several times and soak overnight. Coverslips can be kept in EtOH until ready to use.
2. Wash coverslips five times in autoclaved water. To do this, aspirate EtOH. Add water to completely cover coverslips, vortex, and aspirate water. Repeat five times.
3. Place coverslips in 24-well plate and coat plates with 50 μg/mL PDL and 5 μg/mL laminin in water, 600 μl per well. Ensure that coverslips are completely covered in solution.
4. Incubate plates at 37°C overnight.
5. On day of culture, wash plates twice with autoclaved H_2O, ensuring that coverslips are immersed with each wash. Allow to dry completely in tissue culture hood with lid off.
6. Sanitize all dissection tools in 70% EtOH.
7. Euthanize dam in accordance with Institutional Animal Care and Use.
8. Extract embryos by making a longitudinal cut from the sternum to the tail and two lateral cuts across the lower abdomen using large scissors. Cut through the muscle wall to expose the uterine horn. Remove both sides of the uterine horn and carefully cut away the embryonic sacs from each embryo using medium to small scissors. Decapitate the embryo with small scissors and store on ice for dissection.
9. Using a dissecting microscope, excise ventral mecencephalon (VM) on ice using fine forceps in cold PBS, pH 7.2. To isolate VM, make two vertical cuts, one separating the diencephalon from the midbrain and one at the isthmus separating the midbrain from the hindbrain. Next make one horizontal cut separating the dorsal mesencephalon from the ventral portion. See diagram in Fig. 1 for details.
10. Mechanically cut tissue blocks with fine forceps into smaller pieces.
11. Wash with DMEM. Allow tissue pieces to settle and draw off excess medium with pipet.
12. Add 5 ml of DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, 25 mM HEPES, and 30 mM glucose.
13. Dissociate tissue into single cells with 1-mL pipet tip by pipetting up and down 5–7 times.

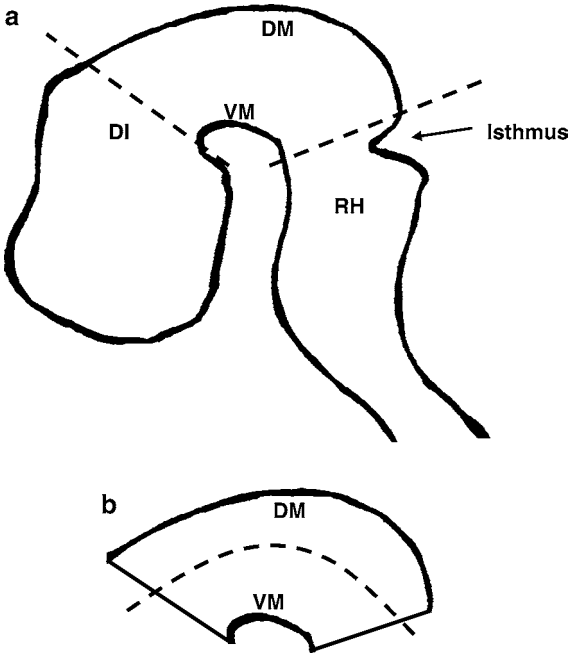

Fig. 1. Diagram of E14 rodent brain. (**a**) To dissect out the mesenchephalon, make two diagonal cuts shown as *dashed lines*. (**b**) Separate ventral from dorsal mesencephalon by cutting along *dashed line*. *DI* diencephalon, *VM* ventral mecencephalon, *DM* dorsal mesencephalon, *RH* rhombencephalon, *Arrow* isthmus.

14. Suspend cells at a density of 10^6 cells/mL; plate 100 μL in one drop per well to form microislands (i.e., 100,000 cells/well). Use care to ensure drop stays on aclar coverslip.
15. Incubate overnight at 37°C, 7% CO_2.
16. Add 400 μL DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, 25 mM HEPES, and 30 mM glucose next morning (Day in vitro 1, DIV1). Always pre-warm media to 37°C in a water bath before adding to cells. Always add media carefully to the side of the tissue culture well as to not disturb cells.
17. Change 1/2 media at DIV3. Remove 250 μL of media and discard. Add 250 μL of fresh pre-warmed (37°C) media.
18. At DIV5, change 1/2 media to DMEM-F12 supplemented with 1% N2 supplement and 10 μg/mL BSA.
19. Change 1/2 media on DIV7 with DMEM-F12 supplemented with 1% N2 supplement and 10 μg/mL BSA with appropriate drug treatments. In experiments described here, rotenone or paraquat was used to induce apoptosis specific to dopaminergic neurons.

3.2. ICC

1. After drug treatment, aspirate media. Fix cells with 4% paraformaldehyde/4% sucrose in PBS, pH 7.5 at room temperature for 30 min. If cells are not well attached, 8% paraformaldehyde/8% sucrose in PBS, pH 7.5 can be added directly to the media in a 1:1 ratio.
2. Wash cells three times for 5 min each with 10 mM glycine in PBST (0.1% Triton X-100 in 1× PBS). Cells can be kept in last wash and stored at 4°C till staining.
3. To permeablize cells, add 0.5% IPEGAL in 1× PBS at room temperature for 30 min.
4. Rinse once with PBST.
5. Block with 2.5% BSA, 5% horse serum, 5% goat serum in PBST 1–4 h at room temperature or overnight at 4°C.
6. Remove coverslips from tissue culture dish and lay on parafilm in a humidified container. Add 40 μL of primary antibody diluted in blocking solution per coverslip. For double immunostaining, dilute two desired antibodies of different species origin in blocking buffer. For example, Fig. 2c uses mouse monoclonal TH and rabbit polyclonal active caspase-3 primary antibodies (see Note 2). Incubate for 2 h at room temperature or overnight at 4°C.
7. Wash three times for 10 min each with 500 μL of PBST per coverslip.
8. Add 40 μL of secondary antibody diluted in blocking solution per coverslip. For double immunostaining, dilute two appropriate secondary antibodies in blocking buffer. For example, Fig. 2c uses goat anti-mouse Alexa 660 and goat anti-rabbit 594 antibodies. Incubate for 1 h at room temperature.
9. Wash three times for 10 min each with 500 μL of PBST per coverslip.
10. To stain nuclei, incubate with 2.5 μg/μL Hoechst 33258 in PBST for 10 min.
11. Wash for 10 min with 500 μL of PBST per coverslip.
12. Mount coverslips on slides with one drop per coverslip of Aquapoly mount (see Note 3). Air dry in dark.
13. Immunostaining is monitored using fluorescence microscopy. See Figs. 2–5 for examples.

3.3. Quantification of JNK Activation in Dopaminergic Neurons

1. Fix and double stain the cells with anti-TH and anti-phosphorylated JNK antibody.
2. Capture images of eight random fields from each well using a fluorescent microscope equipped with a digital camera.
3. Open the images using NIH Image J program.

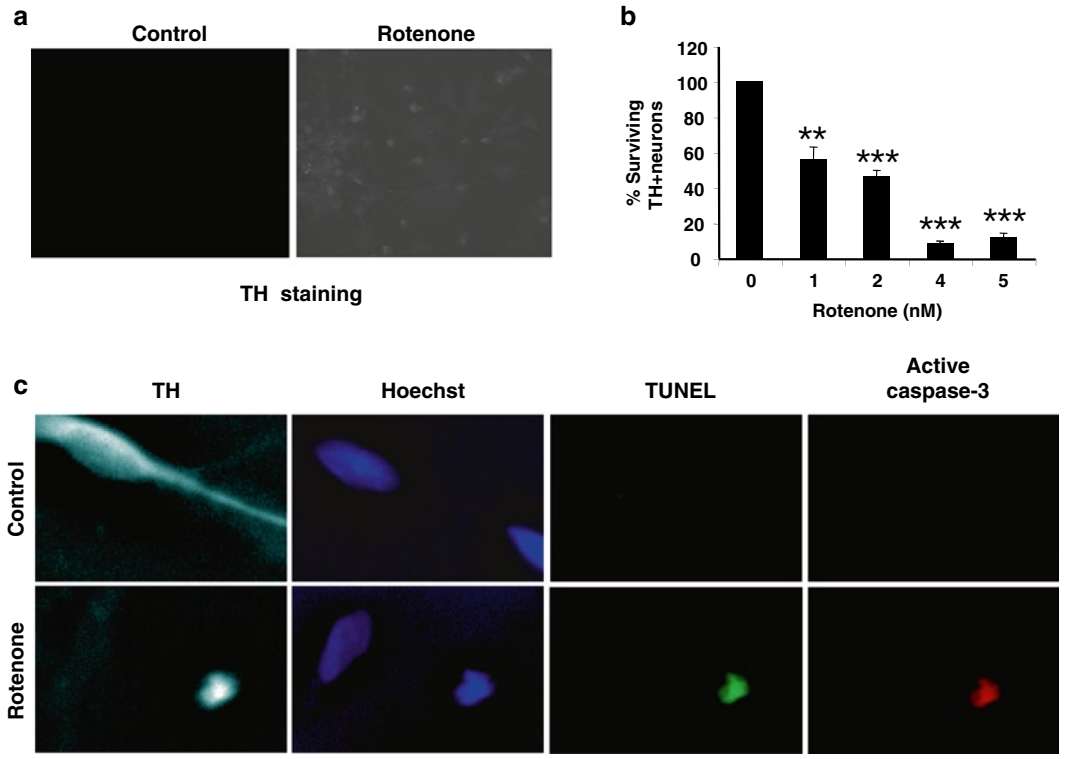

Fig. 2. Rotenone induces dopaminergic neuron apoptosis in E14 ventral mesencephalic primary cultures. Primary ventral mesencephalic cultures were prepared from E14 rats and treated with rotenone for 24 h. (**a**) Representative photomicrographs of cells treated with 5 nM rotenone or vehicle control for 24 h and immunostained for tyrosine hydroxylase (TH), a marker for dopaminergic neurons. (**b**) Rotenone induces a dose-dependant cell loss of TH+ neurons. All TH+ cells on each coverslip were counted. Vehicle control represents 100% survival, which normally has approximately 100 TH+ neurons per coverslip. (**c**) Representative deconvolution photomicrographs of cell treated with 2 nM rotenone or vehicle control for 18 h. Cells were TUNEL labeled followed by immunostaining for active caspase-3 and TH. Data are representative of at least two independent experiments with duplicate determinations. *Error bars* are SEM. ***$p < 0.001$ (ANOVA); compared with vehicle control-treated group. (Reproduced from ref. 6 with permission from the Society for Neuroscience).

4. Using freehand selection tool, draw the margin of phosphorylated JNK and TH double-stained cells.
5. Measure the mean staining intensity of p-JNK using Analyze tools.
6. Score all transfected TH^+ neurons in captured images and calculate the average p-JNK level per TH^+ neuron.
7. Normalize the intensity of p-JNK staining to vehicle control-treated group as relative intensity. See Fig. 4 for an example.

3.4. TUNEL

1. Aspirate media. Fix cells with 4% paraformaldehyde for 30 min.
2. Wash cells two times with 1× PBS 10 min each.

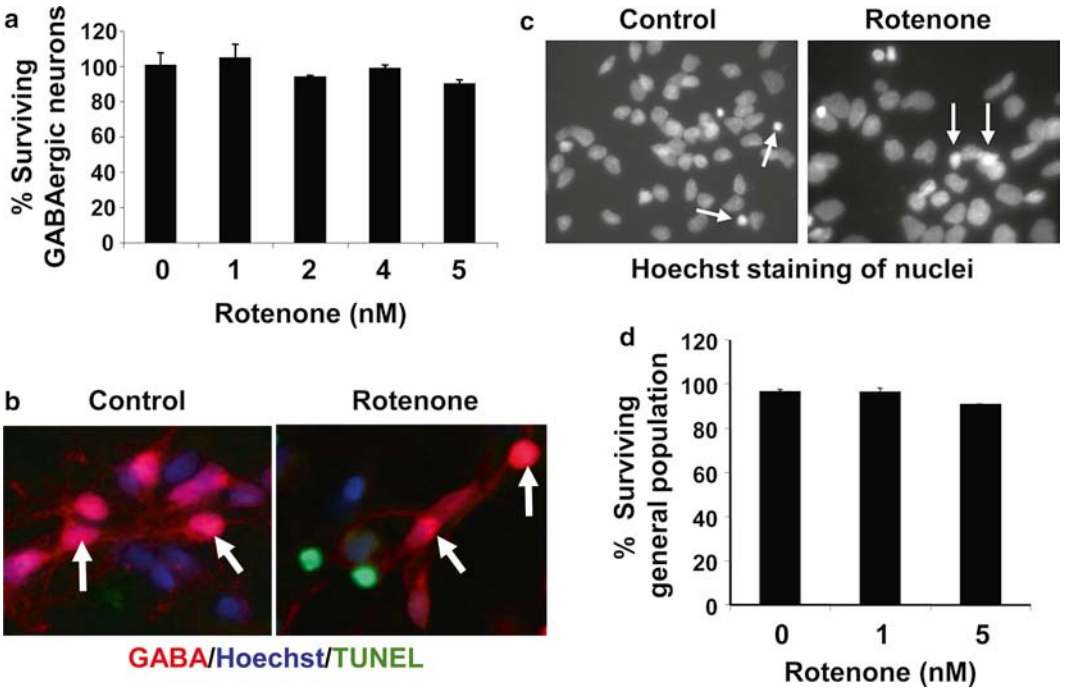

Fig. 3. Rotenone did not induce cell loss of GABAergic neurons or the general population. Primary ventral mesencephalic cultures were prepared from E14 rats, and treated with 0–5 nM rotenone for 24 h. (**a**) Cell were immunostained for GABA, and the number of GABA+ cells in ten randomly selected fields was counted. Vehicle control represents 100% survival. (**b**) Representative photomicrographs of cells treated with rotenone or vehicle control for 24 h and stained for TUNEL, GABA, and Hoechst. *Arrows* point to GABA+ neurons that are negative for TUNEL. (**c**) Representative photomicrographs of nuclear morphology of the entire population, revealed by Hoechst staining. Cells were treated with rotenone as in (**a**). *Arrows* point to condensed or fragmented nuclei, characteristic of apoptosis. (**d**) Rotenone treatment did not affect survival of the general population based on nuclear morphological changes revealed by Hoechst staining as in (**c**). Data are representative of two (panel A) or three (panel D) independent experiments with duplicate determinations. At least 2,000 cells were counted for each condition. (Reproduced from ref. 6 with permission from the Society for Neuroscience).

3. Permeabilize cells in 1× PBS containing 0.1% Triton X-100 and 0.1% sodium citrate at 4°C for 2 min.
4. Wash cells two times with 1× PBS 10 min each.
5. Incubate with 20 µL per coverslip of a TUNEL labeling mixture containing 0.5 U/µL of TdT, 1× of provided 5× buffer and 0.2 nmol biotin-11-dUTP at 37°C for 45 min.
6. Wash two times with 1× PBS containing 1% BSA.
7. Incubate with 50 µl per coverslip of 3 µg/mL streptavidin-FITC in 1× PBS with 1% BSA at RT for 30 min in the dark.
8. Wash two times with 1× PBS.
9. Either mount coverslips onto slides or incubate in blocking solution for 1 h to continue protocol for ICC at step 5.
10. TUNEL staining is monitored using fluorescence microscopy. See Figs. 2 and 3 for examples.

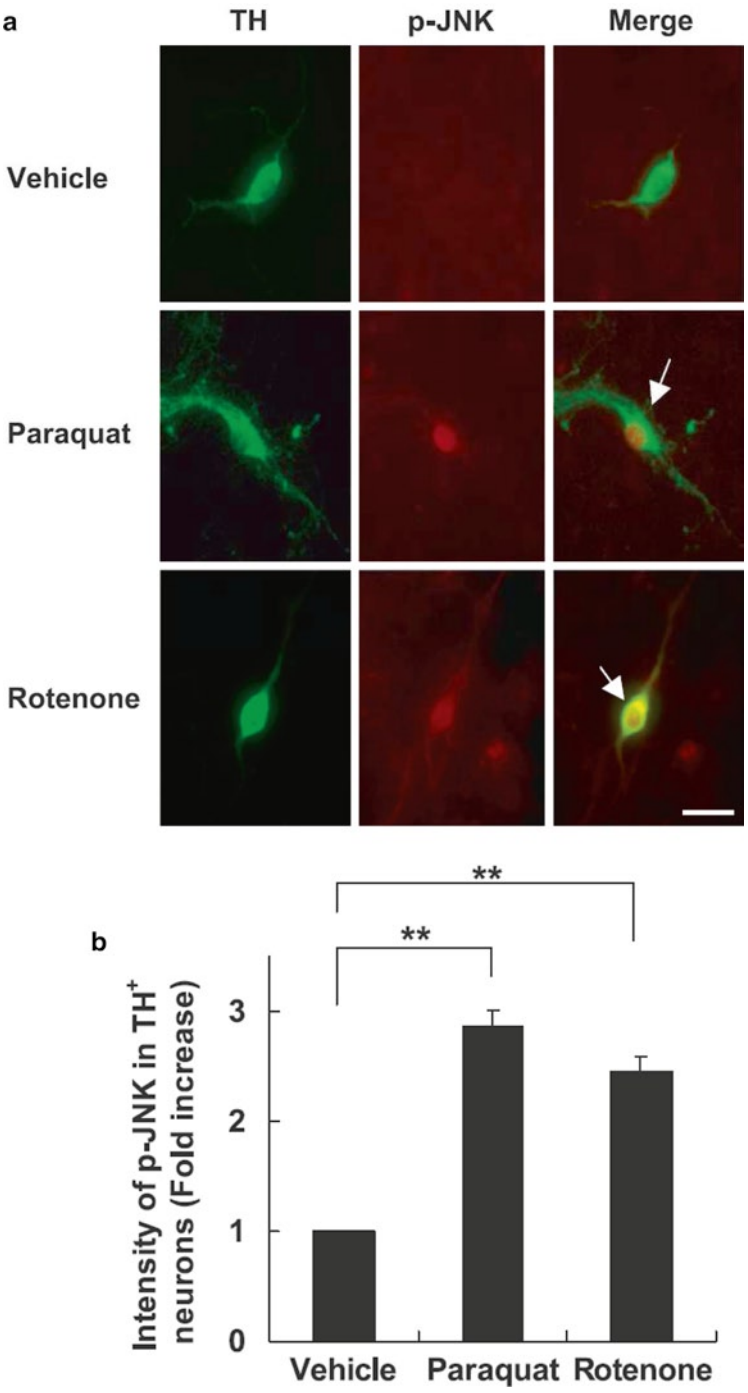

Fig. 4. Treatment of paraquat and rotenone induces JNK phosphorylation in dopaminergic neurons. Primary ventral mesencephalic cultures were prepared from E14 mice and treated with paraquat, rotenone, or vehicle control. (**a**) Paraquat (40 μM, 8 h) and rotenone (5 nM, 8 h) induce JNK phosphorylation in dopaminergic neurons. Images are representative photomicrographs of cells immunostained for tyrosine hydroxylase (TH), a marker for dopaminergic neurons, and for phosphorylated JNK (p-JNK). *Arrows* point to dopaminergic neurons with JNK phosphorylation, indicative of JNK activation. *Scale bar*: 20 μm. (**b**) Quantification of data in panel A for JNK phosphorylation in TH^+ dopaminergic neurons. $^{**}p < 0.01$. (Reproduced from ref. 3 with permission from Lippincott, Williams and Wilkins).

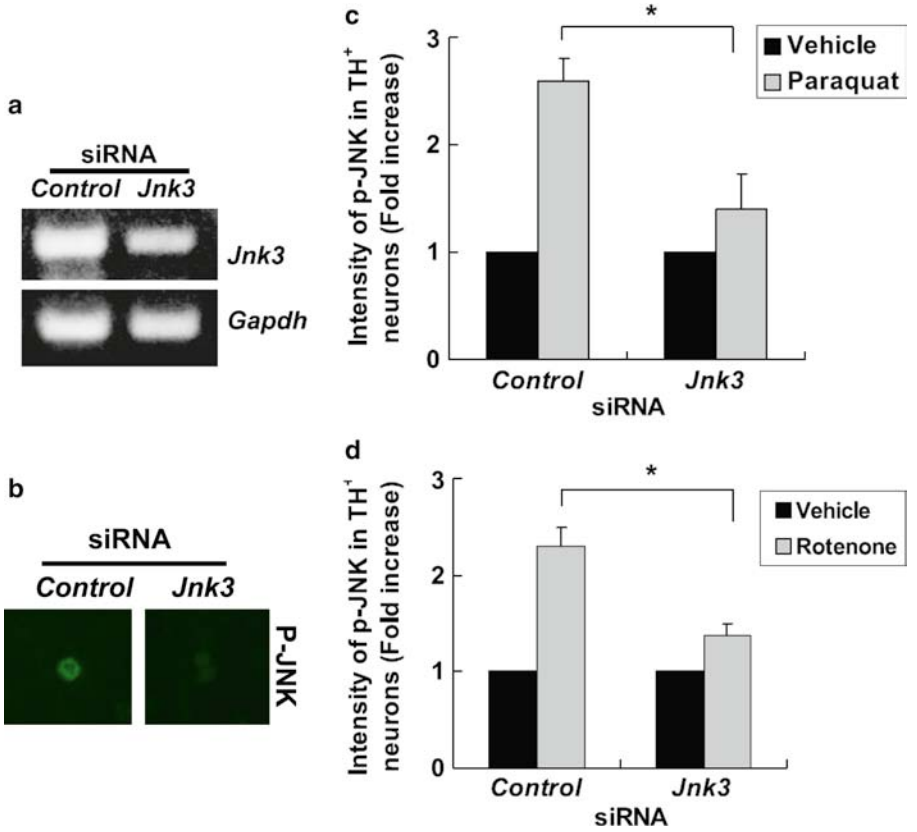

Fig. 5. JNK3 is the major isoform of JNKs in dopaminergic neurons activated by paraquat or rotenone. (**a**) Transfection of siRNA specific for *Jnk3* reduces *Jnk3* mRNA levels. Primary cultured E14 mesencephalic neurons were transfected with *Jnk3* siRNA or control siRNA. Total mRNA was purified 24 h after transfection and analyzed by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. (**b**) Images of representative cells transfected with Jnk3 siRNA or control siRNA and immunostained for phosphorylated JNK (p-JNK). (**c**, **d**) The Jnk3 siRNA reduced JNK activation induced by paraquat (**c**) or rotenone (**d**) in dopaminergic neurons. Twenty-four hours after transfection of siRNA, E14 mesencephalic cultures were treated with 40 μM paraquat, 5 nM rotenone, or their respective vehicle controls for another 8 h. Cells were fixed and stained for TH and p-JNK. The intensity of JNK phosphorylation in TH^+ dopaminergic neurons was quantified. *$p < 0.05$. (Reproduced from ref. 3 with permission from Lippincott, Williams and Wilkins).

3.5. Transfection of JNK siRNAs into Primary Cultured Neurons

1. Plate E14 Sprague–Dawley rat mesencephalic primary neurons on 24-well plates at a density of 300,000 cells/well in 300 μL medium.
2. Add 300 μL of pre-warmed medium on the next day.
3. Incubate cells for 2 more days.
4. Dilute 4 μL Enhancer R in Buffer EC-R (final volume 100μL). Add 2.5 μg RNA and mix by vortexing for 10 s.
5. Incubate at room temperature (15–25°C) for 5 min.
6. Add 8 μL TransMessenger Transfection Reagent to the mixture and mix by vortexing for 10 s.
7. Incubate the mixture for 10 min at room temperature.

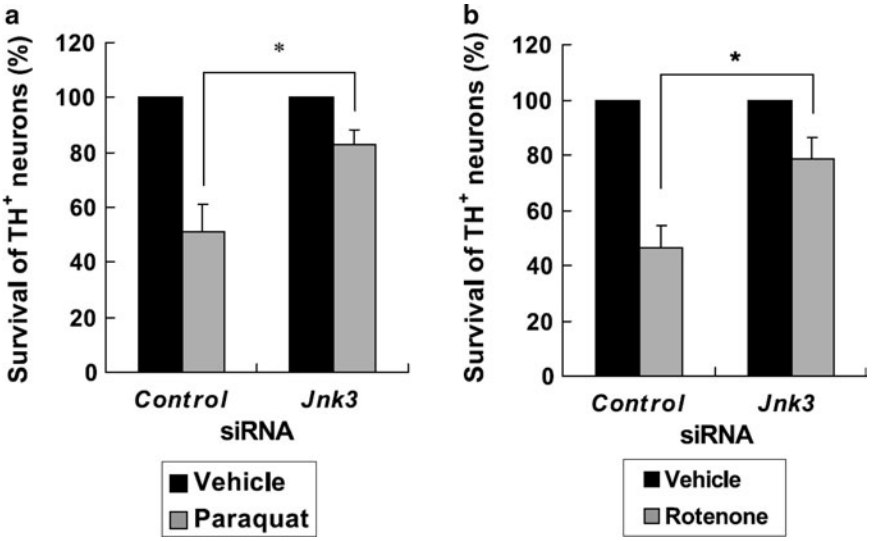

Fig. 6. siRNA silencing of JNK3 protects dopaminergic neurons from paraquat and rotenone toxicity. Twenty-four hours after transfection of siRNA, primary cultured E14 mesencephalic neurons were treated with 40 μM paraquat (**a**), 5 nM rotenone (**b**), or their respective vehicle control for another 24 h. The cells were fixed and stained for TH. The number of TH^+ neurons was normalized to vehicle control-treated groups. *$p < 0.05$. (Reproduced from ref. 3 with permission from Lippincott, Williams and Wilkins).

8. Remove 300 μL of medium from the cells and add 1 mL of pre-warmed DMEM.
9. Repeat step 8 and remove 1 mL of medium.
10. Add 400 μL of DMEM to the mixture from step 7 and mix by pipetting in and out twice.
11. Add 300 μL of mixture to each well.
12. Incubate for 4 h.
13. Remove 300 μL of medium and add 300 μl pre-warmed N2 medium.
14. After 24 h, remove 300 μL of medium and add 300 μL pre-warmed N2 medium.
15. After another 24 h, treat cells with drugs as desired. See Figs. 5 and 6 for an example.
16. (Optional) If the siRNA has no label, eGFP expression vector could be cotransfected to identify transfected cells (4:1 for siRNA:eGFP).

4. Notes

1. All water is filtered through Millipore filtration system (Milli-Q Plus PF, Millipore, Billerica, MA) and is tested to have a resistance of 18.2 MΩ cm. In addition, all water

used for tissue culture purposes is autoclaved at 121°C for 40 min.

2. It is important to include controls for background immunofluorescence of primary and secondary antibodies. Therefore, some slides should be set aside and stained with only primary antibody or only secondary antibody.
3. Avoid air bubbles when mounting coverslips as this may interfere with imaging. Apply drop of mounting media to slide and ensure no bubbles appear. Then carefully place coverslip on top of mounting media. If needed, very gently apply pressure to coverslip to guide any remaining air bubbles to the edge.

Acknowledgments

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Chapter 20

Measurement of Proteasomal Dysfunction in Cell Models of Dopaminergic Degeneration

Faneng Sun, Anumantha G. Kanthasamy, and Arthi Kanthasamy

Abstract

Parkinson's disease (PD) is the second most common neurodegenerative diseases, which occurs in both inheritable and sporadic forms. The interplay of the genetic mutations and environmental exposure to disease risk factors contributes to the pathogenic events leading to the demise of dopaminergic neurons in PD. Proteasome is one of the major proteolytic machinery responsible for degrading unwanted and damaged intracellular proteins. Emerging evidence implicates the incomplete proteolysis by ubiquitin–proteasome system (UPS) in PD pathogenesis. Proteasome inhibition recapitulates some of the key features of PD in vivo and in vitro. Varieties of dopaminergic neurotoxins emerge to inhibit proteasomal function. Given that some PD-related gene mutations impair proteolytic function of UPS, it has been well-accepted that both genetic and environmental factors may conspire to compromise the UPS in the initiation and progression of the disease. The enzymatic assays for the proteasomal activities with fluorogenic substrates and western blot analysis of ubiquitinated proteins provide an entry point to determine UPS function in the process of dopaminergic degeneration.

Key words: Dopaminergic neuronal model, Ubiquitin, Proteasome inhibitor, Protein aggregation, Oxidative stress, Parkinson's disease

1. Introduction

Proteolytic destruction of the unwanted, misfolded, or damaged proteins is critical for the maintenance of normal cellular homeostasis and cell viability. UPS and lysosome are the two principal proteolysis machinery essential for degrading toxic proteins and thereby providing important protein quality control. Proteins destined to be eliminated by UPS are firstly labeled with lysine 48-linked polyubiquitin chains, thus recognized by the 19S regulatory particle, and subsequently degraded by the 20S core particle of the 26S proteasome. The mammalian 20S proteasome

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is made of two outer α-rings and two inner β-rings with seven homologous β subunits (β1–β7). The different β subunits harbor specific catalytic activities for proteolysis, with post-glutamyl peptide hydrolase site being associated with β1 subunit, trypsin-like activity with β2 subunit, and chymotrypsin-like activity with β5 subunit (1, 2). The polyubiquitination is a multistep, highly regulated process involving a serial of enzymes (1, 3), a ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3). The deubiquitination enzymes such as Uch-L1 ensure the availability of free ubiquitin by releasing and recycling it from the polyubiquitin chains. Failure in the efficient clearance of the polyubiquitinated proteins by UPS conceivably leads to their intracellular accumulation and aggregation into inclusion bodies, and eventually results in neuronal death.

Several lines of evidence have converged to implicate the deregulation of UPS in PD pathogenesis, including reduced enzymatic activities of proteasome in PD substantia nigra (4, 5), enrichment of ubiquitin in PD characteristic protein aggregation Lewy bodies, mutation of several PD genes in UPS pathway (2), formation of Lewy body-like proteinaceous aggregation in substantia nigra neurons of Rpt2/S4 (19S regulatory subunit of 26S proteasome) conditional knockout mice (6). A variety of dopaminergic neurotoxins including mitochondrial inhibitors, pesticides, irons, metals, as well as dopamine and its metabolites, may impair the UPS function in PD pathogenesis (2). We recently showed that the two types of polyubiquitination play distinct roles in regulating the neuronal viability upon proteasomal inhibition (7), suggesting that polyubiquitination may be a fundamental determinant for the dopaminergic neurodegeneration process in PD.

2. Materials

2.1. Cell Culture and Lysate Preparation

1. Cell culture medium and supplements: RPMI 1640 medium with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 U penicillin, and 50 μg/ml streptomycin (Invitrogen).
2. 1× Trypsin/EDTA solution (Invitrogen).
3. Test substance: Dieldrin (Sigma) dissolved in DMSO and freshly prepared before use.
4. 5.0 mM stock solution of MG-132 (Calbiochem) dissolved in DMSO and kept at −80°C.
5. 30% Hydrogen peroxide stock, stored at 4°C. Work solutions for 3, 4, and 5 are made by diluting in sterile PBS.
6. Phosphate-buffered saline (PBS) 10×: 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, and 20 mM KH_2PO_4, pH 7.4.

7. 1× work solution of PBS is prepared by diluting 1 volume of the 10× PBS stock with 9 volumes of water.
8. 1 mM (100×) Digitonin in DMSO, stored at room temperature.
9. Lysis buffer for proteasomal activity assays: pH 7.4, 50 mM Tris–HCl, 1 mM EDTA, 10 mM EGTA, and 10 μM digitonin (Fisher Scientific).
10. Lysis buffer for the western blot: PBS containing 0.5% Triton X-100 and protease inhibitors. Buffer is kept at 4°C and made fresh once a month (see Note 1).

2.2. Fluorogenic Substrates for Proteasomal Peptidase Activity

1. Chymotrypsin-like activity substrate: 1.5 mM Suc-LLVY-AMC (Calbiochem), dissolved in DMSO (stock solution); aliquot and store at −20°C.
2. Trypsin-like activity substrate: 2.0 mM Boc-LRR-AMC (Boston Biochem), dissolved in DMSO (stock solution); aliquot and store at −20°C.
3. Peptidyl-glutamyl peptide hydrolase (PGPH) substrate: 2 mM Z-LLE-AMC (Boston Biochem), dissolved in DMSO (stock solution); aliquot and store at −20°C.

2.3. SDS–Polyacrylamide Gel Electrophoresis

1. Commercial premade 30% acrylamide solutions (Bio-Rad).
2. Separating buffer: 1.5 M Tris–HCl, pH 8.8, for preparing resolving gel; keep at room temperature.
3. Stacking buffer: 1 M Tris–HCl, pH 6.8, for making stacking gel; keep at room temperature.
4. *N*,*N*,*N*′,*N*′-tetramethylethylene diamine (TEMED), stored at 4°C.
5. 10% w/v ammonium persulfate (APS) in water; aliquot and store at −20°C.
6. Water-saturated isobutanol. Make 50% isobutanol by mixing with equal volume of water. After the separation of isobutanol at top and water at the bottom, the upper isobutanol is ready to use; store at room temperature.
7. Running buffer: 10× premixed electrophoresis buffer (Bio-Rad). After dilution with water, the final work solution contains 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3; store at room temperature.

2.4. Western Blotting for Ubiquitinated Proteins

1. 10× Transfer buffer, 10× premixed transfer buffer (Bio-Rad).
2. Work solution (1×) transfer buffer: Mix 1 volume of 10× transfer buffer, 3 volumes of methanol, and 6 volumes of water to obtain the final work solution: 25 mM Tris, 192 mM glycine, and 30% methanol, pH 8.3. Store at room temperature and chill before use for better transfer. Up to 0.05% SDS may be added for transfer of proteins of high molecular weights.

3. Nitrocellulose blotting membrane with the pore size 0.2 μm (Bio-Rad).
4. Tris-buffered saline (TBS) and Tween (TBS/T): 10× stock TBS and 10% Tween 20 were purchased from Bio-Rad. To make 1,000 ml TBS/T work solution (pH 7.4, 25 mM Tris/Tris–HCl, 137 mM NaCl, 2.7 mM KCl, and 0.1% Tween 20), mix well 100 ml 10× TBS stock solution, 10 ml 10% Tween 20 solution and 890 ml water, store at room temperature.
5. Blocking solution: 5% (w/v) nonfat milk in TBS/T, mix well and use fresh.
6. Primary antibody against ubiquitin (Dako North America), diluted in TBS/T buffer.
7. Wash buffer: TBS/T.
8. Secondary antibody: Anti-rabbit IgG conjugated to horseradish peroxidase (GE Healthcare).
9. Enhanced chemiluminescent (ECL) reagents (Amersham Pharmacia Biotech).

2.5. Blot Stripping and Reprobing for β-Actin

1. Reprobe buffer for membrane stripping (Thermo Fisher Scientific). Other home made stripping buffers, which commonly contains SDS and reducing agents (DTT or 2-mercaptoethanol) also work well; warming buffer in advance improves stripping (30–70°C).
2. Wash buffer: TBS/T.
3. Primary antibody: Anti β-actin (Sigma).

2.6. Confocal Immunofluorescence for Ubiquitin and α-synuclein

1. Microscope cover slips (Circles, thickness 0.13–0.17 mm, size: 12 mm; Fisher).
2. Fixation solution, 4% paraformaldehyde work solution (w/v): Dissolve paraformaldehyde in PBS (4 g into 100 ml), heating to facilitate dissolving, then cool to temperature, filter with Whatman filter paper and adjust pH to 7.4.
3. Permeabilization solution, 0.1% Triton X-100 in PBS: make 20% Triton X-100 stock (v/v) in PBS, then further dilute (1:200) in PBS to make final 0.1% Triton X-100 in PBS. Keep both solutions at room temperature.
4. Blocking solution: 5% goat serum and 0.1% Triton X-100 in PBS. This solution is used for primary and secondary antibody incubation.
5. Primary antibodies: alpha-synuclein monoclonal antibody (mouse, Santa Cruz Biotechnology) and ubiquitin (rabbit polyclonal antibody, Dako), diluted in the blocking solution for use.

6. Secondary antibodies: Cy3-conjugated goat anti-mouse and Alexa 488-conjugated goat anti-rabbit secondary antibodies, diluted in the blocking solution for use.
7. Nuclei co-staining solution: dissolve Hoechst 33342 into DMSO to make 1 mg/ML stock (100×, −20°C storage, protected from light). Prepare fresh work solution each time by diluting in PBS (1:100, 10 μg/ml).
8. Prolong antifade mounting medium from Invitrogen.

3. Methods

Impairment in UPS function is manifested by the reduction of peptidase activities and the resultant accumulation of the polyubiquitinated proteins, which are otherwise degraded by UPS. Multiple types of proteolytic capacities of 20S proteasome have been experimentally established according to its intrinsic catalytic activities for the synthetic peptides, with the major three types of peptidase activities being the chymotrypsin-like (C-L), trypsin-like (T-L), post-glutamyl peptide hydrolytic-like (PGPH) activities. The proteolytic release of the fluorescent molecules (such as AMC) conjugated to the specific peptide by 20S proteasome provides a straightforward determination for the proteasomal activities, as the fluorogenic AMC can be readily quantified in a spectrofluorometer. The different peptidase activities, which are associated with different subunits, may be differentially affected by various proteasomal inhibitors (8) or neurotoxins. Furthermore, due to the cross-reaction of proteases, the peptidyl substrates may not be highly specific to one protease. As an example, the C-L proteasomal substrate Suc-LLVY-AMC is also frequently used as one of the calpain substrates (9). Therefore, assays with multiple proteasomal substrates provide more reliable and acceptable determinations of the proteasomal activities.

As a consequence of perturbation in the degradative function of proteasome, the polyubiquitinated proteins, which are otherwise degraded, may accumulate. Since ubiquitin monomer is a small protein with 76 amino acids, it is readily separated and distinguished from the polyubiquitin attached to the substrates on SDS–PAGE. Immunoblotting with ubiquitin antibody allows the detection of the intracellular polyubiquitinated proteins, thereby providing convenience in further determining UPS function.

3.1. Cell Culture

The immortalized rat mesencephalic dopaminergic cell line (N27 cells) is cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U of penicillin, and 50 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. For proteasomal activity assays, about 0.2×10^6 cells/wells

are seeded in 6-well plates. For western blot analysis, 2×10^6 cells are grown in T75 flasks. After in culture for 20–24 h, N27 cells at about 70% confluence were subjected to the indicated treatments.

3.2. Cell Lysate Preparation and Proteasomal Activity Assays

1. The cells cultured in 6-well plates receive the indicated treatments.
2. To collect the cells, suck out the culture medium completely, and add 400 μL Trypsin/EDTA to each well, and incubate at the cell culture incubator for 5 min (see Note 2), then add 800 μL normal growth medium with FBS to stop the trypsin, collect and pellet the cells at $106 \times g$ for 5 min at 4°C.
3. Wash cells once by resuspending them in ice-cold PBS and then pellet cells at $200 \times g$ for 5 min at 4°C.
4. Lyse cells by resuspending in the lysis buffer (approximately 350 μL) (see Notes 3 and 4) by vortexing. Incubate the cell suspension at room temperature for 15 min.
5. Centrifuge the samples at $16{,}000 \times g$ for 5 min at room temperature and collect the supernatant.
6. Add 95 μL cell lysates and 5 μL fluorogenic substrate stock solutions (see Note 5) [Suc-LLVY-AMC (final 75 μM), Boc-LRR-AMC (final 100 μM), or Z-LLE-AMC (final 100 μM)] in one well of a 96-well plate, mix well and avoid creating air bubbles (see Note 6). Set up at least one well of blank reaction for each substrate by mix 95 μL lysis buffer and 5 μL substrate (see Note 7).
7. Incubate the 96-well plate for 30 min at 37°C in the dark (see Note 8).
8. Take reading for AMC fluorescence using a fluorescence plate reader, with excitation at 380 nm and emission at 460 nm (Molecular Devices).
9. Take 10 μL cell lysate from each sample for determining protein concentration using the Bradford protein assay.
10. The peptidase activity of the proteasome (fluorescence units per mg protein) is expressed as the percentage over the vehicle-treated control group. Figure 1 is an example of change in the chymotrypsin-like (C-L) proteasomal activity in cells exposed to dieldrin, H_2O_2, or MG-132.

3.3. Preparation of Cell Lysates for Western Blot

1. The cells cultured in T75 flasks receive the indicated treatments.
2. To collect the cells, suck out the culture medium completely, and add 3 ml Trypsin/EDTA to each plate, and incubate at the cell culture incubator for 5 min (see Note 2), add 6 ml of normal growth medium. Collect and pellet cells. Wash cells once with ice-cold PBS.

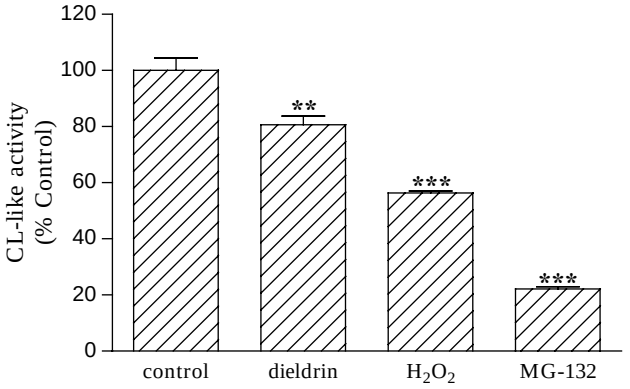

Fig. 1. The effect of dieldrin, H_2O_2, or MG-132 on the chymotrypsin-like (C-L) peptidase activity of proteasome in N27 cells. N27 cells were treated with dieldrin 60 μM, H_2O_2 200 μM, or MG-132 2.5 μM for 40 min. The C-L proteasomal activity was assayed with fluorogenic substrate Suc-LLVY-AMC. The enzymatic activity (fluorescence unit per milligram proteins) was expressed as the percentage over the vehicle-treated control cells. $N = 6$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. control.

3. Resuspend the cells with PBS-containing protease inhibitors and 0.5% Triton X-100 and keep on ice for 15 min.
4. Centrifuge the lysates at 16,000 × *g* for 40 min at 4°C to obtain the supernatant.
5. Determine protein concentration for all samples.
6. Add equal amounts of proteins for each sample (20–40 μg total proteins) (see Note 9) into 1.5-ml microcentrifuge tube, and adjust the volume with the PBS lysis buffer in step 3 to make all the sample volumes the same.
7. Add equal volume of 2× SDS loading buffer to each sample, mix well, heat samples in boiling water or a heating block at 100°C for 5 min.
8. Quickly spin the samples in microcentrifuge (up to 10 s), and the samples are ready for SDS–PAGE separation.

3.4. SDS–Polyacrylamide Gel Electrophoresis

1. The procedure described here assumes the use of Bio-Rad minigel system. Other electrophoresis systems can also be used. If glass plates are used to make SDS–PAGE, make sure that they are clean and intact. Disposable plastic cassettes with three sides sealed provide a better option for pouring the SDS–PAGE.
2. To make 1.5-mm thick 15% separating gel, mix well 2.2 ml dH_2O, 2.6 ml separating buffer, 5.0 ml 30% acrylamide solution, 100 μl 10% SDS, 100 μl 10% APS, and 4.0 μl TEMED, and immediately pour the mixed solution into glass or plastic gel cassette, leaving about 1.5 cm space on top for stacking gel later on.

3. Gently add 500 µl water-saturated isobutanol on top of the gel.
4. After polymerization of the separating gel is complete (generally 15–25 min), pour off the upper layer of isobutanol and rinse the gel twice with dH_2O, then remove the residual dH_2O with Kimwipes.
5. To prepare 4% stacking gel, mix 2.26 ml dH_2O, 380 µl stacking buffer, 400 µl 30% Acrylamide gel solution, 30 µl 10% SDS, 30 µl 10% APS, and 3.0 µl TEMED, and immediately pipette the mixed gel solution directly onto the top of the separating gel until flash with top edge of cassette.
6. Insert the comb into the gel gently; wait for the stacking gel to polymerize (15–20 min).
7. Put gel in electrode assembly, then place electrode with gel into tank. Fill the inside of the electrode assembly with 1× running buffer full, and at least half full for the outside of the electrode assembly.
8. Remove the comb and the gel is ready for loading samples and the protein marker.
9. Running gel at 100 V until the sample dye reach the end of the gel.

3.5. Western Blot

1. The procedure described is based on the use of Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad); other transfer systems can also be used.
2. Upon the completion of gel running, dissemble and rinse the gel in 1× transfer buffer for 10 min.
3. Soak the sponge, filter papers, and nitrocellulose membrane with 1× transfer buffer for 5 min, then assemble the gel/blot sandwich in the gel holder cassette in this order: black side of cassette (cathode)–three sponges–one filter paper–gel–membrane–one filter paper–three sponge–red side of the cassette (anode). Make sure that no air bubbles are trapped in between the gel and membrane.
4. Transfer at 120 V for 90 min with prechilled 1× transfer buffer, including cooling unit with ice inside the tank.
5. Upon the completion of the transfer, take out membrane, rinse once with 1× TBS/T buffer, and then incubate with blocking solution for 1 h at room temperature on a rocking shaker.
6. Incubate the membrane with the primary ubiquitin antibody (1:500) in TBS/T for 1 h at room temperature or overnight at 4°C on a rocking shaker.
7. Remove the primary antibody solution and wash the membrane twice (15 min each) with TBS/T on a rocking shaker.

8. Incubate membrane with freshly prepared horseradish peroxidase-conjugated secondary antibody in TBS/T (1:2,000) for 45 min at room temperature on a rocking shaker.
9. Discard the secondary antibody solution and follow by 3 × 10 min washings of the membrane with TBS/T on a rocking shaker.
10. Prepare ECL detection solution by mixing equal volumes of the reagent A and B (2 ml for each), and apply the detection solution onto the membrane, ensuring the entire membrane is evenly and completely covered by the solution for about 1 min.
11. To develop, place the membrane in between two sheet protectors (transparency propylene sheet). The membrane covered with sheet protector is placed in the X film cassette, and an X film placed on top of it. Expose the X film and/or develop the film for various durations until the satisfactory detection of signal is achieved (generally one to a few minutes for both exposure and developing), as exemplified in Fig. 2a, b.

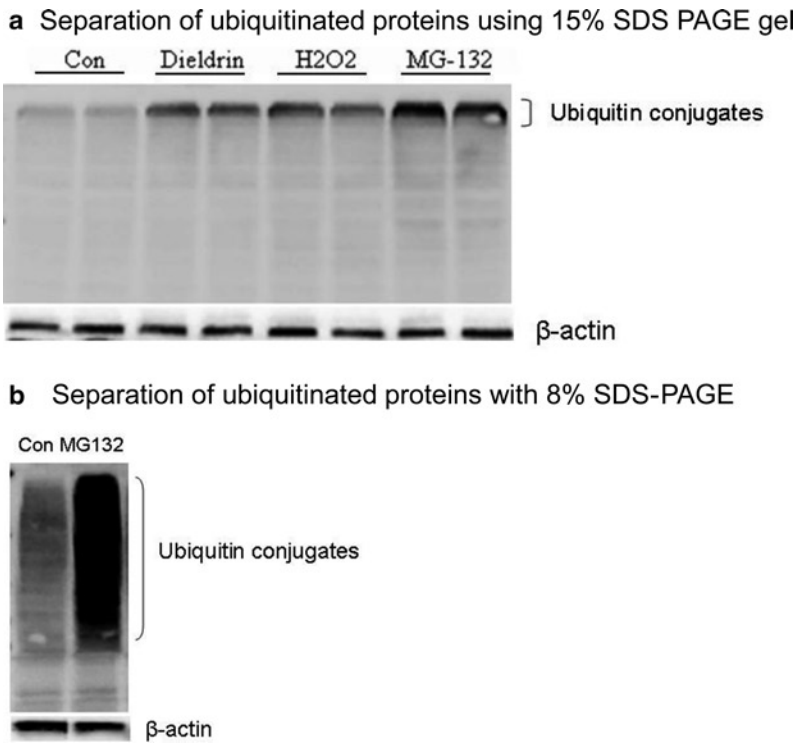

Fig. 2. Western blot analysis of the elevation of ubiquitinated proteins. (**a**) N27 cells were treated with neurotoxins dieldrin (60 μM), H_2O_2 (200 μM) or proteasomal inhibitor MG-132 (2.5 μM) for 40 min. The lysates prepared from the treated cells were processed for SDS–PAGE in 15% acrylamide gel, and subsequent western blot analysis of the ubiquitinated proteins. Duplicate samples were used for each treatment. (**b**) N27 cells were treated with vehicle or MG-132 for 40 min, then processed for assay of ubiquitinated proteins using Western blot as described in (**a**) except that 8% acrylamide gel is used for SDS–PAGE. The blots are stripped and reprobed with antibody recognizing β-actin, which served as the loading control.

12. Low percentage such as 8% gel may achieve better separation of the ubiquitinated proteins, if the ubiquitinated protein accumulates significantly such as in the case of MG-132 treatment (Fig. 2b). The advantage of high percentage gel (15%) is to concentrate ubiquitinated proteins on the top, thereby making it readily detectable even the minimal elevation of ubiquitinated proteins (see Note 9).

3.6. Stripping and Reprobing Blots for β-Actin

1. After a satisfactory detection of signal for the polyubiquitinated proteins, the blot is then stripped of the signal derived from the ubiquitin antibody and reprobed with an antibody recognizing β-actin. The β-actin serves as a loading control here, which confirms that all samples start with equal amounts of protein in the SDS–PAGE and western blot analysis.
2. Incubate the membrane with the reprobed buffer with vigorous shaking for 30 min at room temperature.
3. Rinse the blot twice with TBS/T once the stripping is complete, and then reblock with 5% nonfat milk in TBS/T.
4. The membrane is then ready for the reprobing with anti β-actin (1:5,000 in TBS/T), by following the same procedure described above for washing, secondary antibody, ECL detection, etc. An example result of β-actin immunoblotting is included in Fig. 2.

3.7. Confocal Immunofluorescence for Ubiquitin and α-Synuclein and Their Colocalization in Protein Aggregates

1. Preparing coverslips for culture. Soak coverslips in 70% nitric acid for 1 h, and then wash coverslips with water 30 times, and repeat three times. Then dip the coverslip in 100% ethanol, and leave it on Kimwipes in culture hood, with UV light to sterilize coverslips for 30 min. The dry and sterile coverslips are ready for use.
2. Place coverslips in culture wells of 24-well plates and incubate with poly-L-lysine (0.1 mg/ml) overnight at room temperature, and then wash coverslips with sterile PBS three times and leave to dry in cell culture hood.
3. Seed about 20,000 cells per coverslip in culture well; 20–24 h later subject cells to the indicated treatment paradigm.
4. Rinse once with room temperature PBS, then discard the PBS, and incubate cells with fixation solution (4% paraformaldehyde in PBS) at room temperature for 10 min.
5. Remove the fixation solution, and rinse the cells on coverslips twice for washing.
6. Incubate cells with permeabilization solution (PBS with 0.1% Triton X-100) for 5 min at room temperature.
7. To minimize the nonspecific binding, the samples are incubated with the blocking solution (5% goat serum and 0.1% Triton X-100 in PBS) for 1 h at room temperature.

8. Discard the blocking solution and incubate the samples with the primary antibodies (ubiqutin, 1:100 and alpha-synuclein 1:500) in blocking solution at 4°C overnight.
9. Remove the primary antibody solution, washing three times for 5 min with PBS. The samples are protected from the light for all the steps described below.
10. Incubate samples with secondary antibodies (Cy3-conjugated goat anti-mouse and Alexa 488-conjugated goat anti-rabbit secondary antibodies, 1:600 dilution for both) in the blocking solution for 30 min at room temperature.
11. Remove the secondary antibodies, add Hoechst 33342 (10 μg/ml), incubate for 3 min at room temperature.
12. Discard the Hoechst 33342 solution, and follow with 5 min washings with PBS for three times. Samples now are ready to be mounted.
13. First add a drop of Prolong antifade mounting medium on a slide, then hold coverslip vertically and dip once in water to remove salt. Then wipe the bottom edge of coverslip with Kimwipe to remove residual water, then invert the coverslip and place on the top of mounting medium on the slip. Apply nail polish along the edge of the coverslip to seal the samples.
14. The samples are ready for confocal analysis once the nail polish dries. The fluorescence staining lasts for up to 1 month when stored in the dark at 4°C.
15. The slides are viewed under fluorescence microscopy (locate the cells) and then analyzed under the confocal microscopy.

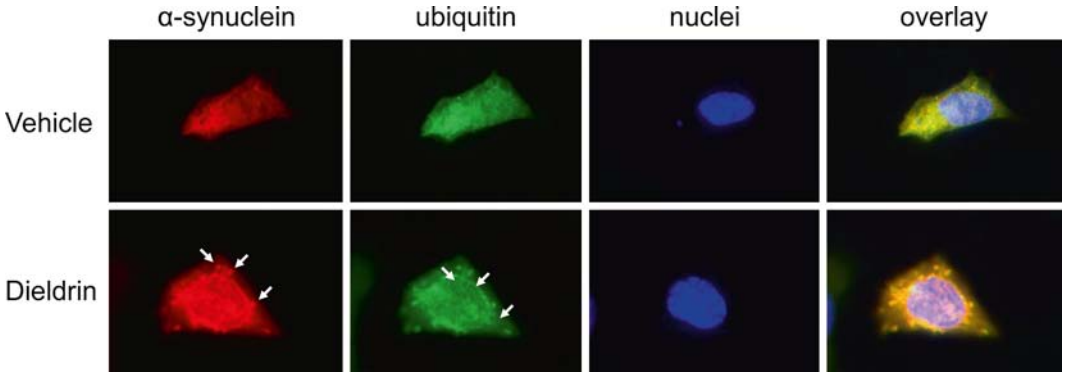

Fig. 3. Immunofluorescence analysis of alpha-synuclein aggregates colocalized with ubiquitin. N27 cells overexpressing human alpha-synuclein were treated with 30 μM dieldrin for 24 h or treated with vehicle as control. Following fixation, double immunostaining was conducted with antibodies recognizing alpha-synuclein (mouse) and ubiquitin (rabbit). Cy3-conjugated anti-mouse (alpha-synuclein) and Alexa-488-conjugated anti-rabbit (ubiquitin) were used for the visualization of alpha-synuclein and ubiquitin, respectively. The images were analyzed with confocal microscopy. *Arrows* point to alpha-synuclein and ubiquitin-positive aggregates.

Excitation at 550 and 495 nm for the Cy3 fluorescence (red emission) and Alex 488 (green emission) allows the visualization of α-synuclein and ubiquitin, respectively. Metamorph Software can be used to overlay and analyze the fluorescence images. Images of ubiquitin alpha-synuclein and nuclei are included in Fig. 3.

4. Notes

1. Detergent Triton X-100 in the lysis buffer is very necessary for lysing the cells completely and solubilizing the polyubiquitinated proteins. Without detergent, homogenizing cells just with sonication significantly reduces the level of the ubiquitinated proteins in homogenate and may result in failure in the detection.
2. Check under light microscopy to ensure that the majority of the cells are detached. Otherwise, extend the trypsin/EDTA incubation.
3. The buffer used here (see 2.9) appears to lyse cells more efficiently than the buffer (10 mM HEPES, 5 mM $MgCl_2$, 10 mM KCl, 1% sucrose, and 0.1% CHAPS) used in our earlier publication (10–12) and yields better consistence for the assay. Digitonin (100×, 10 mM in DMSO) is made separately, and added to the buffer and mixed well before use.
4. Since each sample is used for assay with three different types of substrates: 95 µl per each × 3 and 10 µl for protein assay ($95 \times 3 + 10 = 295$ µl); 350 µl lysis buffer per sample is an ideal volume.
5. Suc-LLVY-AMC, Boc-LRR-AMC, and Z-LLE-AMC are the fluorogenic substrates for C-L, T-L, and PGPH proteasomal activities, respectively.
6. Mix the reaction gently and thoroughly, being careful not to introduce air bubbles in the well. Air bubbles may cause an inaccurate reading from the spectrofluorometer.
7. For each substrate, a blank reaction has to be set up by thoroughly mixing the 95 µL lysis buffer with 5.0 µL fluorogenic substrate, as the blank reading is used to establish the baseline for spectrofluorometric determinations of all the samples assayed with the same substrate.
8. The 96-well plate may be wrapped with aluminum foil to protect samples from light.
9. Generally, 20–40 µg protein of cell lysate is the ideal amount for this analysis.

Acknowledgments

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Chapter 21

Cell Signaling and Neurotoxicity: Protein Kinase C In Vitro and In Vivo

Prasada Rao S. Kodavanti

Abstract

There is a growing concern about the effects of chemicals on the developing nervous system. Chemical exposure at critical periods of development can be associated with effects ranging from subtle to profound on the structure and/or function of the nervous system. Understanding critical biological molecular targets, which underlie chemical-induced neurotoxicity, will provide a scientific basis for risk assessment. Cell signaling molecules such as protein kinase C (PKC) have been shown to play critical roles in motor activity, development of the nervous system, and in learning and memory. PKC also has been shown to be associated with several neurological disorders including Alzheimer's disease, status epilepticus, and cerebellar ataxia. In the literature, there is abundant information linking PKC to cognitive function, long-term potentiation, or brain structural changes. Here, we show the relationship between changes in PKC (as assayed using radioactive material or by western blots) and the neurotoxic effects caused by environmental chemicals in vitro and in vivo.

Key words: Protein kinase C, Calcium signaling, Learning and memory, Neurotoxicity, Nervous system development

1. Introduction

Cell signaling is a mechanism by which extracellular signals are transferred to the cytosol and nucleus of the cell. Although several intracellular signaling pathways have been identified to be important in the nervous system (1), G-protein-coupled receptor activation, and their downstream events such as activation of protein kinase C (PKC), play a significant role in the development and function of the nervous system. The PKC family of serine/threonine phosphorylating enzymes consists of ten isoforms divided into three subfamilies based on their enzyme properties. The conventional PKC (cPKC) isoforms (α, βI, βII, and γ) require

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Ca^{2+} and diacylglycerol (DAG) for their activation. The novel PKC (nPKC) isoforms (δ, ε, η, θ, and μ) require only DAG, and the atypical (aPKC) isoforms (ι and ζ) require neither Ca^{2+} nor DAG for activation (2). Following an increase in Ca^{2+}, PKC translocates from the cytosol to binding domains on the internal surface of the cell membrane. Subsequently, DAG or other lipid activators bind and induce a conformational change, leading to activation (3). Upon activation, PKC binds receptors for activated c-kinase (RACK), which confer the selective functions and downstream interactions of each of the isozymes (4, 5).

PKC is involved in a number of cellular functions including nervous system function, development, and cancer. Changes in cell morphology (6), cell growth (7), and gene expression (8) have been reported. It has also been implicated in the regulation of spatial learning (9) and in neuroprotection (10). As the activation and signaling mechanism of PKC is better understood, its role in cancer proliferation becomes apparent. Tumor-promoting phorbol esters, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), can activate PKC in the absence of DAG and increase its affinity to Ca^{2+} and phospholipids (11). Consequently, the alteration of PKC expression has been involved in breast (12), lung (13), colon (14), and liver (15) tumor progression. Understanding the PKC signaling pathway will bring insight into disease manifestation, progression, and future therapies.

We have shown in neuronal cultures that PKC translocation preceded cell death induced by several persistent chemicals, including polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers ((16, 17); Fig. 1). In adult animals, following repeated administration of PCBs, total PKC activity was significantly decreased in the cerebellum, while membrane-bound PKC activity was elevated, indicating translocation and activation of PKC at the cell membrane ((18); Fig. 2). These changes in cerebellar PKC activity were associated with decreases in horizontal motor activity (18). In addition, exposure to persistent chemicals at critical periods of development that led to changes in different PKC isoforms ((19); Fig. 3) has been associated with deficits in learning and memory in the adult age (20).

In this chapter, we describe how to determine PKC translocation via measuring ^{3}H-phorbol ester binding (21), activity via [^{32}P]-ATP incorporation into PKC substrates such as neurogranin (22), and protein levels via western blot (23). ^{3}H-phorbol ester binding is mostly applied to neuronal cultures to understand the in vitro effects of chemicals on PKC translocation from cytosol to the membrane. [^{32}P]-ATP incorporation into the PKC-specific substrate provides information on the activity of PKC in the fractions (cytosol or membrane) assayed. While ^{3}H-phorbol ester binding and [^{32}P]-ATP incorporation indicate PKC activation and translocation, it does not provide information about the role of specific PKC isozymes. Through western blotting, we are able

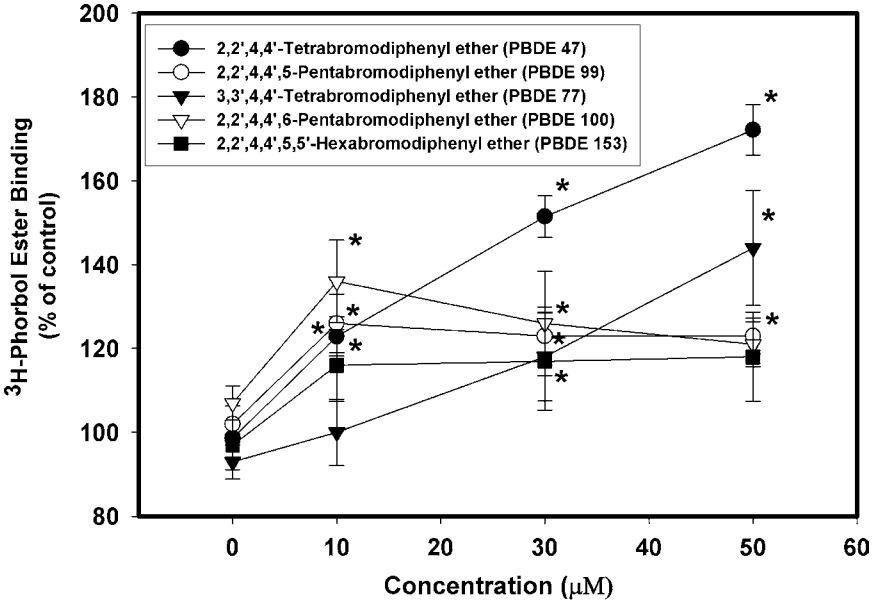

Fig. 1. Concentration-dependent effects of PBDE congeners on ^{3}H-phorbol ester binding in cerebellar granule neurons. The selected PBDE congeners, differing in position and number of bromines, caused PKC translocation (as indicated by increased ^{3}H-phorbol ester binding) at concentrations where no significant cytotoxicity was observed. Longer exposures and higher concentrations of some congeners did result in cytotoxicity, indicating PKC translocation as a preceding event for cell death (17). The effect of PBDE congeners decreased with increasing number of bromines, probably due to low solubility or increasing hydrophobicity. (Reproduced from ref. 17 with permission from Oxford University Press).

to determine the translocation of PKC from the cytosol to the plasma membrane required for activation by monitoring the changes in protein level in the different compartments and to identify levels of specific PKC isozymes via the use of isozyme-specific antibodies.

2. Materials

2.1. ^{3}H-Phorbol Ester Binding in Cerebellar Granule Neurons

1. Locke's Buffer: 154 mM sodium chloride (NaCl), 5.6 mM potassium chloride (KCl), 3.6 mM sodium bicarbonate ($NaHCO_3$), 2.3 mM of calcium chloride ($CaCl_2$), 5.6 mM D-glucose (5.6 mM), and 5 mM *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) in double-distilled water (see Note 1) and adjust pH to 7.4. Store in refrigerator.
2. Locke's Buffer + 0.1% BSA (Locke's-BSA): Prepare 0.1% BSA in Locke's buffer on the day of the assay by dissolving 0.2 g of fat-free bovine serum albumin (BSA) in 200 ml of Locke's buffer (see Note 2).
3. 10 mM Glutamate: Prepare 10 ml solution by dissolving 0.0169 g of Glutamate (F.W. 169.11) in 10 ml of Locke's buffer.

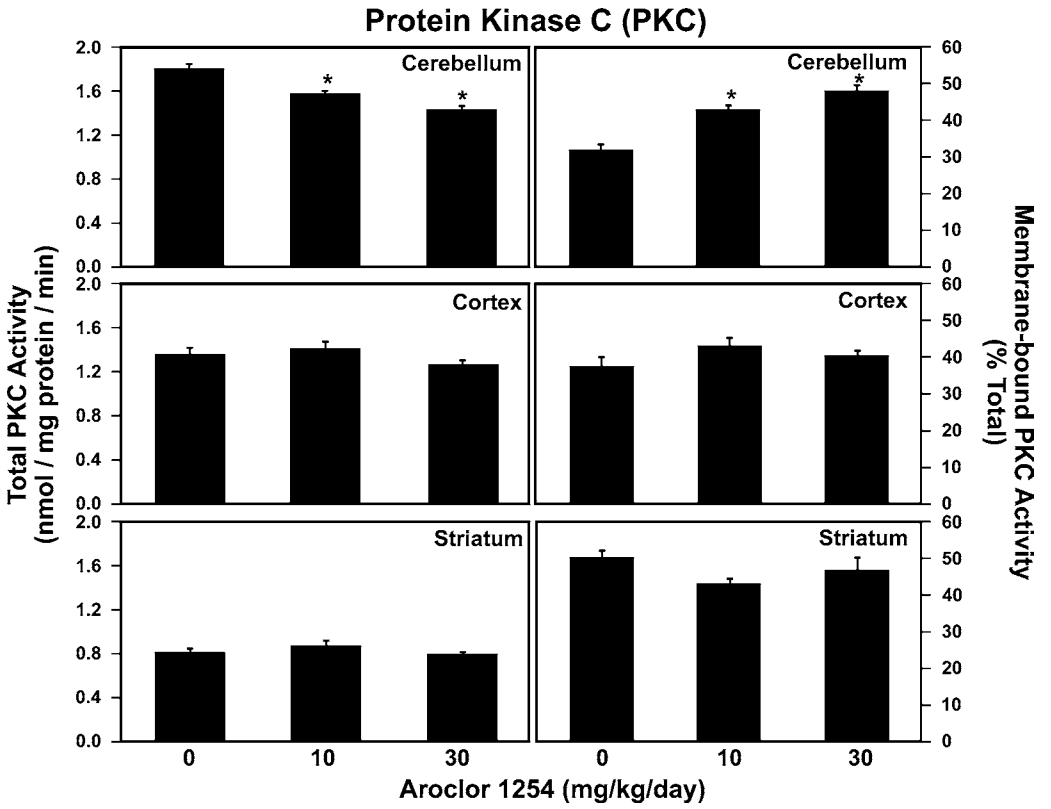

Fig. 2. PKC activity in cerebellum, frontal cortex, and striatum of rats following exposure to Aroclor 1254 (a commercial polychlorinated biphenyl mixture) for 4 weeks. In the cerebellum, total PKC activity was decreased significantly while membrane-bound PKC activity was elevated with increasing dose. Total or membrane-bound PKC activities were not altered in cortex or striatum. The changes in cerebellar PKC activity were associated with decreases in horizontal motor activity. (Reproduced from ref. 18 with permission from Elsevier Science).

Divide into small aliquots of 0.1 ml and store in freezer. Use this as positive control (see Note 3).

4. 1 mM *N*-methyl-D-aspartic acid (NMDA): Prepare 10 ml solution by dissolving 0.00147 g of NMDA (F.W. 147.13) in 10 ml of Locke's buffer. Divide into small aliquots of 0.1 ml and store in freezer. Use this as positive control.
5. Phorbol 12-myristate 13-acetate (PMA, Sigma): Prepare a stock solution of 100 mg/ml in DMSO. Dilute 1,000 times in Locke's buffer to yield a 0.1 mg/ml working solution. Store stock solution in freezer.
6. 4-β-[^{3}H]phorbol 12, 13 dibutyrate: The specific activity varies with lot (see Note 4). Final concentration should be 1 nM in the assay buffer with 0.1 μCi/ml. For this, dilute the solution obtained from the manufacturer in Locke's buffer. Store the stock radioactive chemical in freezer.
7. 0.1 M Sodium hydroxide (NaOH): Prepare 500 ml solution and store at room temperature.

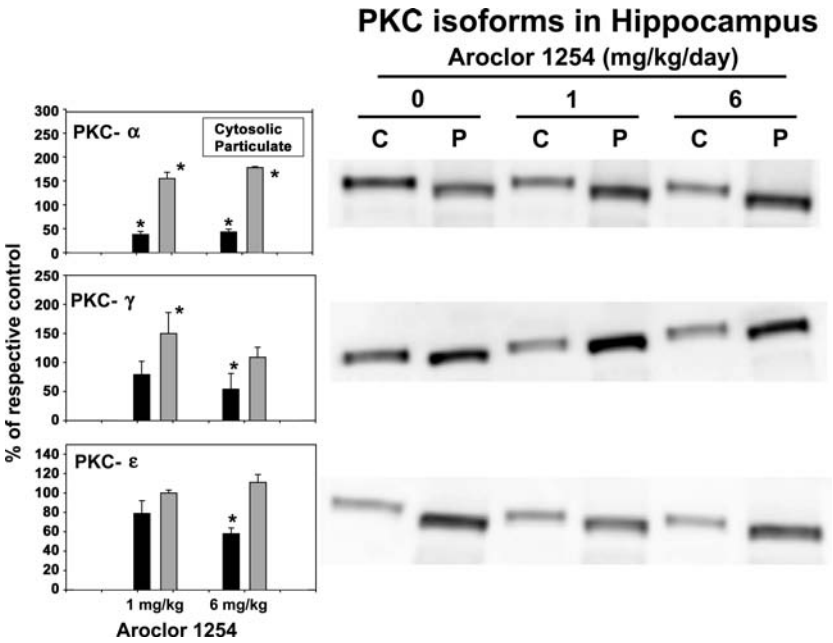

Fig. 3. Changes in cytosolic (C, *filled bar*) and membrane (particulate; P, *gray bar*) fractions of PKC isoforms in the hippocampus at postnatal day (PND) 14 following developmental exposure to Aroclor 1254 (a commercial polychlorinated biphenyl mixture). The quantitative information as percentage of control for the respective isozymes are shown on the *left*, while the corresponding western blots are shown on the *right* side of each of the *bar graphs*. PCB exposure decreased the cytosolic PKC-α and increased the particulate PKC-α in both 1 and 6 mg/kg dose groups. PCB exposure tended to decrease cytosolic PKC-γ and PKC-ε in the 6 mg/kg dose group, while no changes are seen in particulate fractions. (Reproduced from ref. 19 with permission from Elsevier Science).

2.2. Incorporation of [^{32}P]-ATP into Neurogranin

1. Homogenization Buffer: 20 mM Tris base, 2 mM ethylenediamine tetraacetic acid (EDTA), 2 mM ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 50 mM β-mercaptoethanol, and 0.32 M sucrose. Prepare 500 ml solution, bring the pH to 7.5, and store in the refrigerator. On the day of use, add 10 µg/ml Leupeptin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM sodium fluoride (NaF) to 50 ml buffer.
2. 0.2% Triton X-100: Prepare 50 ml solution by dissolving 100 µl of Triton X-100 in 50 ml buffer.
3. Stock solutions:
   (a) 66.4 µM Neurogranin$_{(28–43)}$ (NG): Dissolve 1 mg NG in 10 ml water. Separate into 1.2 ml aliquots and store at –80°C.
   (b) 1 mg/ml Phosphatidylserine/dioctanoylglycerol (PS/DAG): Make up 10 mg/ml of each in chloroform. Aliquot 100 µl (1 mg)/vial on ice. Cap under nitrogen and store in at –80°C.
4. Stimulating Assay Buffer: Prepare 8 ml solution enough for 50 samples containing 20 mM Tris base, 5 mM $MgCl_2 \cdot 6H_2O$, 66.4 µM NG substrate, and 0.5 mM $CaCl_2 \cdot 2H_2O$.

5. Basal Buffer (if needed): 20 mM Tris base, 5 mM $MgCl_2 \cdot 6H_2O$, 66.4 μM NG substrate, and 1.5 mM EGTA in double-distilled water.

2.3. Western Blot Analysis to Identify Different PKC Isozymes

2.3.1. Cell Fractionation

1. Buffer A: 20 mM Tris–HCl, 0.25 M sucrose, 2 mM EDTA, and 2 mM EGTA in double-distilled water and adjust pH to 7.5. Prepare 500 mL solution. On the day of use, add leupeptin (10 μg/ml), 0.5 mM PMSF, 1 mM NaF, and pepstatin (10 μg/ml).
2. Buffer B: 20 mM Tris–HCl, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, and 1% Nonidet P-40 in distilled water. Prepare 500 ml solution and adjust pH to 7.5. On the day of use, add leupeptin (10 μg/ml), 0.5 mM PMSF, 1 mM NaF, and pepstatin (10 μg/ml).

2.3.2. Gel Electrophoresis and Immunoblotting

1. Sample Buffer: Mix 50 μl of 2-β-mercaptoethanol (Sigma) with 950 μl Laemmli sample buffer (Bio-Rad). Hold at 4°C on ice.
2. Electrophoresis Running Buffer: Mix 100 ml of 10× running buffer concentrate (Bio-Rad) with 900 ml of distilled water.
3. Electrophoresis Transfer Buffer: Mix 100 ml of 10× transfer buffer concentrate (Bio-Rad) with 700 ml of distilled water and then 200 ml of methanol. Keep on ice to chill to 4°C.
4. Tris-buffered saline (TBS): Dissolve 9.0 g of NaCl, 0.9 g of Tris base, and 6.63 g of Tris–HCl (Sigma) in about 950 ml of distilled water. Bring volume to 1,000 ml with distilled water.
5. Tween-20 (0.1%)–TBS (T-TBS): Prepare 1,000 ml of 0.1% Tween-20 (Sigma) in TBS.
6. Blocking Buffer: Dissolve 25 g of nonfat dry milk (Bio-Rad) in 500 ml of T-TBS.
7. Novex Ready Gel 4–10% or 8–12% (The choice of gel depends on the molecular weights of the proteins to be separated: the former for separation of larger proteins and the latter, smaller proteins).
8. Molecular Weight Marker (Amersham), ready for use.
9. Thin filter paper (Bio-Rad). Cut to 7.2 × 6.6 cm.
10. Nitrocellulose membranes (0.2 μm, Bio-Rad). Cut to 7.2 × 6.6 cm.
11. Ponceau S solution (Sigma): Use 0.1% Ponceau S solution (w/v) in 5% acetic acid.
12. Sodium dodecyl sulfate (SDS, Bio-Rad, Hercules, CA).
13. Enhanced Chemiluminescent (ECL) reagent (Amersham-Pharmacia).

14. Antibodies (primary and secondary): Isoform-specific monoclonal antibodies for alpha, gamma, and epsilon are obtained from Transduction Lab, Lexington, KY. Standardization is necessary for the use of these antibodies.

3. Methods

3.1. Phorbol Ester Binding in Cerebellar Granule Neurons

1. Warm up the Locke's buffer to room temperature.
2. Observe the neurons under a microscope for dendritic network to verify the neurons grew normally (see Note 5).
3. Aspirate the cell culture medium and wash by immediately adding 1.0 ml of warm Locke's buffer. Repeat the washing procedure.
4. Allow the cells to incubate for 15 min at room temperature (30–37°C).
5. Aspirate the Locke's buffer and add 1.0 ml of radiolabeled phorbol premixed with test substance.
6. Incubate the cells for 15 min at room temperature.
7. Wash the cells three times with 1.0 ml of ice cold (4°C) Locke's buffer.
8. Lyse the cells in 1.0 ml of NaOH.
9. Remove 0.2 ml for protein determination (Bradford method, (24)) and count 0.7 ml in a liquid scintillation counter using 10 ml of Ultima Gold scintillant.
10. Use appropriate blanks (see Note 6). Present activity as femtomoles per millligram protein/15 min.

3.2. Incorporation of [^{32}P]-ATP into Neurogranin

3.2.1. Isolation of Cell Fractions

1. Homogenize tissue in 10 volumes of cold homogenization buffer using a teflon/glass homogenizer (i.e., a sample of frontal cortex weighing approximately 400 mg should be homogenized in 4 ml buffer) (see Note 7).
2. Centrifuge at 100,000 × g at 5°C for 30 min in a Ti50 rotor or a similar one using an ultracentrifuge.
3. Save supernatant in a plastic vial with cap. This is the cytosolic PKC fraction.
4. Resuspend the pellet in homogenization buffer with 0.2% Triton X-100 (100 µl TX-100/50 ml of buffer) and sonicate for 30 s on ice.
5. Incubate on ice for 30 min.
6. Add TX-100 to a final concentration of 0.2% to the cytosolic fraction (8 µl of 25% TX-100/ml cytosol).

7. Centrifuge at 100,000 × *g* for 30 min. Save the supernatant in 3-ml plastic tube with cap. This is the membrane PKC fraction.
8. Measure protein by the Bradford method (23). Keep these PKC fractions on ice until assayed.

3.2.2. PKC Assay Procedure

1. Prepare stimulating assay buffer.
2. Prepare PS/DAG by adding 2.5 ml water to a vial from the freezer and sonicate the solution.
3. Thaw radioactive [^{32}P]-ATP. Prepare fresh cold ATP (0.2 mM).
4. Calculate volume of [^{32}P]-ATP needed for 45 µCi. 45 µCi equals 10^8 cpm which is enough for 100 samples (10^6 cpm/sample).
5. Add volume calculated to a 20 ml scintillation vial and add 2.5 ml of cold ATP (0.2 mM).
6. Dilute protein samples to 0.4 mg protein/ml (20 µg/50 µl) using homogenization buffer with 0.2% TX-100. For purified PKC, bring up stock (500 ng) in a total of 1.25 ml homogenization buffer (=20 ng/50 µl, enough for 25 samples).
7. Add 150 µl assay buffer, 50 µl PKC protein, and 25 µl PS/DAG or 150 µl assay buffer, 50 µl PKC protein, 25 µl PS/DAG, and 25 µl test compound (10× concentration) to the tubes.
8. Preincubate for 5 min in 25°C water bath. Add 25 µl/sample of [^{32}P]-ATP solution to begin assay (5 nmol).
9. Stop after 10 min by adding 1 ml/sample of 25% TCA and placing on ice.
10. Rinse 0.45 µm membrane filters with 1 ml of 25% TCA, then filter sample.
11. Rinse sample tube with 1 ml 25%TCA and then wash filters with 2 × 2 ml 25%TCA.
12. Place filters in 7 ml scintillation vial, add 5 ml Ultima Gold, wait 20 min, shake, and count.
13. Count a 25 µl aliquot of radiolabled ATP solution to determine total dpm added/sample.

3.2.3. Calculation of PKC Activity

1. Using counts from step 13 above, determine dpm/nmol ATP (specific activity). dpm/25 µl/sample ÷ 5 nmol ATP/25 µl/sample = dpm/nmol ATP.
2. From each value, subtract the basal dpm from the stimulated dpm.
3. dpm ^{32}P ÷ dpm/nmol ATP = nmol ^{32}P.
4. nmol ^{32}P ÷ 5 min ÷ 0.020 mg protein = nmol ^{32}P/min/mg protein.

3.3. Western Blot Analysis to Identify Different PKC Isozymes

3.3.1. Cell Fractionation

1. Scrape the cells off in 2 ml buffer A, sonicate briefly on ice for 30 s at 12 W power, and transfer into labeled microcentrifuge tubes.
2. Centrifuge the tubes at 100,000×*g* for 1 h at 4°C. Carefully remove the supernatant (cytosolic fraction) without disturbing the pellet and store in a plastic vial.
3. Resuspend the pellet in buffer B and allow it to incubate on ice for 30 min. Centrifuge the tubes at 15,000×*g* for 30 min.
4. Carefully remove the supernatant (membrane fraction) without disturbing the pellet and store in a plastic vial.
5. Dilute the samples at a ratio of 1:1 with sample buffer in 1.5 ml microcentrifuge tubes. After mixing with sample buffer, samples may be kept frozen at –20°C.
6. To denature the proteins, heat the samples at 95–100°C for 5 min.
7. Cool samples to room temperature and quick spin the sample tubes for ~1 min to bring down condensation.

3.3.2. SDS–PAGE

1. These instructions assume the use of Novex mini-Gel Box (Invitrogen) and Bio-Rad mini transfer system. Remove the gel from the package and remove the combs from the gel very gently.
2. Place the gel in the gel holder, clamp the gel holder in the gel clamp, and then place the gel clamp into the reservoir tank. Fill the upper chamber with 1× running buffer.
3. Gently rinse the gel lanes with 1× running buffer to eliminate any unpolymerized acrylamide.
4. Carefully load each sample into a designated well of the gel using gel loading pipette tips. In the same fashion, load 5–10 µl of prestained molecular weight standard to its designated well.
5. Fill the lower chamber with 1× running buffer to a level at the middle of the gel.
6. Connect the electrodes of the gel apparatus with the receptacles on the power supply and run at 100 V.
7. Run for approximately 2 h until the dye front approaches the bottom of gel. During the run, monitor the buffer level of the top chamber and to ensure that it does not become dry.
8. At the end of the run, turn off the power supply, remove the electrode assembly from the chamber and remove the gel holder.

3.3.3. Gel Transfer

1. Remove the gel from the gel clamp and carefully cut open the plastic plates that hold the gel. Put the gel into a tray which contains 1× transfer buffer.

2. Pre-wet the sponges, filter paper, and nitrocellulose membrane in transfer buffer.
3. Place the transfer holder flat on the bench and assemble the sandwich by stacking the parts in the following order: mini-sponge, filter paper, nitrocellulose membrane, gel, filter paper, and sponge. Carefully close the transfer holder.
4. Place the holder in the transfer box in the correct orientation. Place the transfer icebox (with ice inside) besides the gel holder.
5. Place the electrode cover on the top and connect the electrodes with the power supply. Run the apparatus at constant voltage 100 V for 1 h.
6. After 1 h, turn off the power supply.

3.3.4. Blotting

1. Remove the holder from the buffer tank and carefully open the holder.
2. With forceps, very carefully remove the nitrocellulose membrane and place the membrane in a clean tray.
3. Add a small amount of Ponceau S solution to cover the entire surface area of the membrane. Gently shake the tray for a few minutes.
4. Pour off the Ponceau S solution and wash the membrane several times with distilled water. Proteins transferred onto the membrane should be visible now.
5. Pour off the distilled water and add enough blocking buffer to cover the membrane.
6. Place the tray on a rocking platform and gently rock for 1 h at room temperature. Pour off the blocking buffer.
7. Add primary antibody diluted at a ratio of 1:1,000 in blocking buffer to the blocked membrane (see Note 8). Gently rock overnight at 4°C.
8. Pour off the antibody-containing solution and wash the membrane in T-TBS by gently rocking for 10 min at room temperature. Repeat the washing once and pour off the washing solution.
9. Add secondary antibody diluted at a ratio of 1:2,500 in blocking buffer. Incubate for 2 h at room temperature by gently rocking on the platform. Pour off the antibody and wash the membrane with T-TBS by gently rocking for 10 min at room temperature. Repeat the washing procedure two more times. Take the membrane to a darkroom.

3.3.5. ECL Detection

1. Mix 5 ml ECL Reagent A with 4 ml ECL Reagent B.
2. Completely pour off the T-TBS and add the mixed ECL reagents to the membrane. After exactly 1 min, completely pour off the ECL reagents.

3. With forceps, place the membrane over a clean paper towel, carefully touching the edge of the membrane to drain off residual ECL reagents.
4. Again with forceps, place the membrane in the center of a piece of Saran wrap and fold the wrap to cover the membrane. Place the wrapped membrane in the center of the film cassette and secure it with labeling tape.
5. Turn off the regular lights in the darkroom. Place a sheet of film over the membrane and close the cassette.
6. After desired periods of time (1–10 min), remove the film from the cassette and place it in the automatic developing machine.
7. Return developed film to cassette briefly to mark molecular weight marker bands and membrane edges on the film for future reference in identifying the proteins of interest.
8. The gels (if not transferred to films) or films can be scanned for the quantification of density of the band. All values should be normalized to their respective loading control value such as GAPDH (see Note 9).
9. Repeat membrane hybridization protocol for control protein (e.g., GAPDH).

4. Notes

1. All solutions must be prepared in double-distilled water.
2. For ^{3}H-phorbol ester binding, prepare Locke's + BSA solution on the day of the assay.
3. Use proper positive controls in all assays to improve confidence in the results.
4. For radioactive assays, the specific activity of radioactive chemical varies with lot. Therefore, calculate specific activity for each batch of isotope.
5. When using cell cultures, make sure that the cells look healthy before conducting the assay.
6. For radioactive assays, the blank values should not be more than 15–20% of controls.
7. Homogenization buffer, homogenizers and centrifuge tubes should be kept on ice throughout the protocol.
8. Western blot antibody dilution levels should be determined empirically for each antibody. Dilutions quoted are for antibodies used in this lab. Similarly, wash stringency (salt concentration, temperature, etc.) needs to be individually determined.

9. For western blot assay, loading controls should be run for all gels and the sample values should be normalized to the loading control values (e.g., GAPDH) before subjecting them for statistical analysis.

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Chapter 22

Cell Signaling and Neurotoxicity: ^{3}H-Arachidonic Acid Release (Phospholipase A_2) in Cerebellar Granule Neurons

Prasada Rao S. Kodavanti

Abstract

Cell signaling is a complex process which controls basic cellular activities and coordinates actions to maintain normal cellular homeostasis. Alterations in signaling processes have been associated with neurological diseases, such as Alzheimer's and cerebellar ataxia, as well as cancer, autoimmunitiy, and diabetes. Recent evidence also indicates a role for signaling molecules in the adverse effects associated with the exposure to environmental chemicals. One of these signaling molecules is arachidonic acid (AA). AA is abundant in the membrane phospholipids of the brain, where its release has been shown to be involved in synaptic plasticity processes, such as long-term potentiation. AA release is primarily produced by the activation of phospholipases, most commonly by phospholipase A_2 (PLA_2). The release of ^{3}H-AA is often used as a measure of PLA_2 activity in cell culture studies. In our laboratory, we have demonstrated the relationship between the stimulation of ^{3}H-AA release by persistent chemicals, such as polychlorinated biphenyls and polybrominated diphenyl ethers, and the associated cytotoxicity following in vitro exposure. Understanding the role of the AA signaling pathway in chemically-induced effects on the nervous system will provide specific mode of action information that can be used in assessing the compound risk.

Key words: Phospholipase A_2, Arachidonic acid release, Neurotoxicity, Cell signaling, Persistent chemicals, Cytotoxicity

1. Introduction

Arachidonic acid (AA, 20:4 $n-6$), an essential omega-6 fatty acid, is a major component of cell membranes and is abundant in the mammalian brain. De novo synthesis of this polyunsaturated fatty acid is not possible in mammals and must be supplied via

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diet or by elongation and desaturation of its precursor, linoleic acid (1). AA release can be initiated by phospholipase C (PLC), which cleaves the soluble headgroup from membrane phospholipids and generates inositol phosphate and diacylglycerol (DAG). DAG lipase then acts to release AA. Phospholipase D (PLD) also initiates AA release by hydrolyzing the choline headgroup of phosphatidylcholine, leaving phosphatidic acid anchored in the membrane. Phosphatidic acid can be converted to DAG by phosphatidic acid phosphohydrolase and further processed into AA (2). However, the major pathway for AA release involves hydrolyzation of the polyunsaturated fatty acid from the *sn*-2 position of membrane phospholipids by a phospholipase A_2 (PLA_2) enzyme (3). There exists many different families of PLA_2, including the secretory PLA_2, cytosolic calcium-dependent PLA_2, and calcium-independent PLA_2. Each unique family confers different enzymatic properties, which lead to differential effects in the cell.

AA has been shown to play a role in long-term potentiation (4) and long-term depression (5) in the mammalian brain. These markers of synaptic plasticity demonstrate AA's role in memory formation and information storage. AA also affects the cell signaling pathway as a retrograde messenger, causing the release of calcium from microsomal and mitochondrial stores (6), alterations in neurotransmitter release and uptake (7), and stimulation of protein kinase C (8). Additionally, AA has been shown to provide a neuroprotective role in glutamate-induced neurotoxicity and hydrogen peroxide-induced oxidative stress (9, 10). Dysregulation of AA metabolism has been implicated in a number of neurological disorders, including epilepsy (11), Parkinson's disease (12), Alzheimer's disease (13), and schizophrenia (14). We have shown in in vitro neuronal cultures that ^{3}H-AA release preceded cell death by persistent chemicals, such as polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs). The effects on ^{3}H-AA release were significant at concentrations as low as 10 μg/mL and required only 10–30 min of exposure (15; Figs. 1 and 2) while cytotoxicity was observed at much higher concentrations (>16–33 μg/mL) and required longer exposure time (>120 min) (16).

In this chapter, we describe ^{3}H-AA release measurement following the incorporation of [^{3}H]-AA into cerebellar granule cells. During the exposure to pharmacological agents or environmental chemicals, cells release [^{3}H]-AA into the media, where it is measured by liquid scintillation counting and percent [^{3}H]-AA release calculated. The described method is according to the procedure modified from Lazarewicz et al. (17) and Tithof et al. (18). The details of this method are available in our previous publication (15).

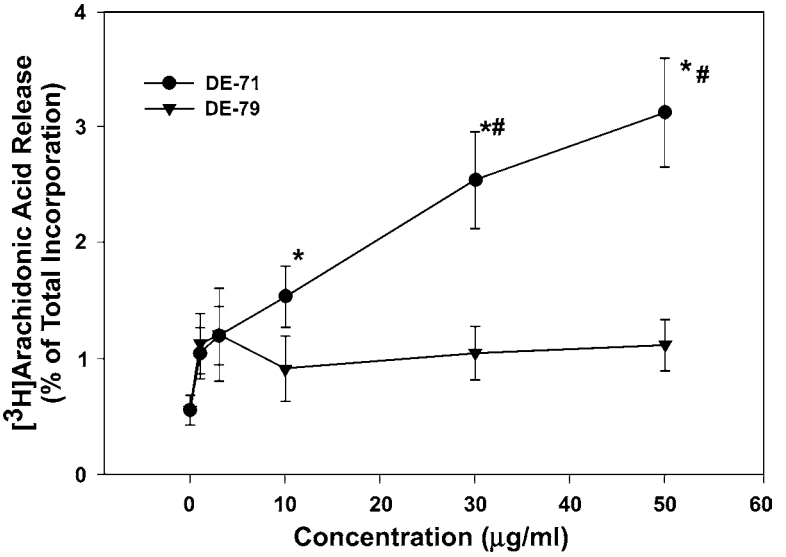

Fig. 1. Concentration-dependent effects of pentrabrominated diphenyl ether (PBDE) mixtures (DE-71 and DE-79) on ^{3}H-arachidonic acid release in cerebellar granule neurons. DE-71, which is a PBDE mixture, stimulated AA release while DE-79, which is an octabrominated diphneyl ether mixture, did not. The lack of effect of DE-79 could be due to its low solubility when compared to DE-71. The effect of DE-71 on AA release preceded DE-71-induced cytotoxicity. (Reproduced from ref. 15 with permission from Oxford University Press).

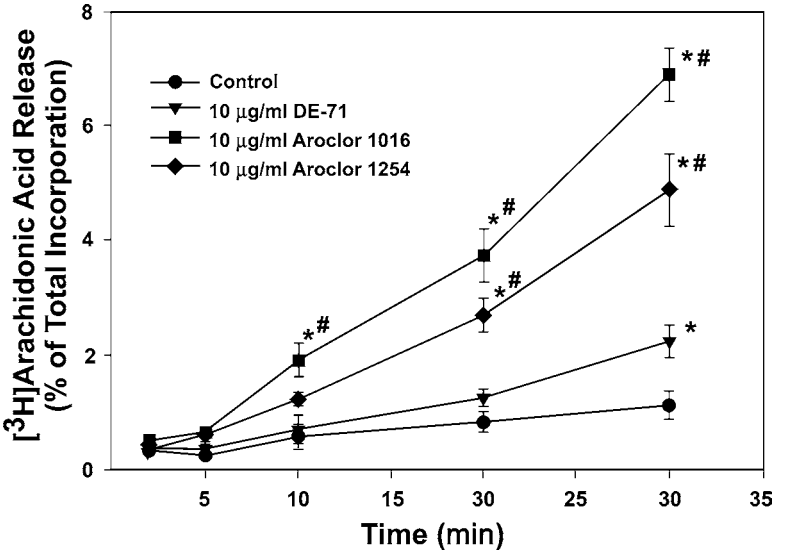

Fig. 2. Time course of ^{3}H-arachidonic acid release in cerebellar granule neurons by pentrabrominated diphenyl ether (PBDE) or polychlorinated biphenyl (PCB) mixtures. DE-71, which is a PBDE mixture, as well as Aroclor 1016 and Aroclor 1254, which are PCB mixtures, stimulated AA release in a time-dependent manner. The effect of PCB mixtures was much greater when compared to that of PBDEs, and this correlated well with their cytotoxic potency. (Reproduced from ref. 15 with permission from Oxford University Press).

2. Materials

2.1. Cerebellar Granule Neuronal Cultures

1. Stock Poly-L-Lysine (10 mg/mL): Prepare 50 mL solution by dissolving 500 mg of Poly-L-Lysine hydrobromide in sterile-distilled water (see Note 1). Divide into 0.5 mL aliquots and store in freezer. On the day of use, add 0.5 mL of this solution to 100 mL of sterile-distilled water to get the final concentration of 50 μg/mL (see Note 2).
2. KRB Buffer: 9 mM Tris–Base (MW 121.1), 10 mM glucose (FW 180.16), 127 mM NaCl (FW 58.4), 5.5 mM KCl (FW 74.6), and 9 mM Na_2HPO_4 (FW 141.16). Adjust the pH to 7.4. Add 4 mM $MgSO_4$ (FW 246.47) to the above solution, adjust the pH to 7.4, and sterilize by filtering through 0.22-μ filter unit. Store in refrigerator.
3. Dulbecco's Modified Eagle's Medium (DMEM): For 1 L of medium, dissolve 13.4 g of DMEM powder (contains 25 mM glucose and 4 mM glutamine) (see Note 3), 25 mM KCl, and 4.5 mM $NaHCO_3$ in 850 mL distilled water. Add 2 mL of Media Mix (see below). Add 100 mL of heat-inactivated fetal calf serum. The media tends to be basic when made, so adjust the pH to 7.35 with 1N HCl (or 1N NaOH depending on the initial pH value). Bring up the volume to 1 L and sterilize by filtering through 0.22-μ filter unit. (Prepare the required amount of DMEM 1 day before cell prep).

   For convenience, make a mixture of insulin, transferrin, and GABA (Media Mix). Weigh 100 mg of each and dissolve in 40 mL water (suspension). Divide this into 1 and 2 mL aliquots and store at –80°C. This solution contains 2.5 mg/mL each of insulin, transferrin, and GABA. To make 1 L of DMEM, use 2 mL.
4. 50 mM Cytosine arabinoside: Prepare 50 mM solution by dissolving 14 mg cytosine arabinoside (MW 279.7) in 1-mL KRB buffer. Take 150 μL of 50 mM solution and bring the volume to 10 mL with KRB buffer. Divide into 2–3 mL aliquots and store at –80°C. On the day of use, thaw this solution and add 10 μL to 1.0 or 1.5 mL and 20 μL to 3.0 mL of media (10 μL in 1.5 mL = 5 μM final concentration).
5. Trypsin–DNase solution (TD; 25 mg/mL trypsin and 5 mg/mL DNase): Dissolve 500 mg trypsin and 100 mg DNase in 20 mL KRB buffer; divide them into 1 mL aliquots and store at –80°C. Since this solution is not sterile, it must be filtered prior to adding with a 0.22-μm syringe filter.

2.2. [^{3}H] Arachidonic Acid Release

1. Locke's Buffer: 154 mM of sodium chloride (NaCl), 5.6 mM of potassium chloride (KCl), 3.6 mM of sodium bicarbonate ($NaHCO_3$), 2.3 mM of calcium chloride ($CaCl_2$), 5.6 mM of

D-glucose, and 5 mM of *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) in deionized water. Adjust the pH to 7.4 and store refrigerated at 4°C until used.

2. Locke's Buffer + 0.2% BSA (Locke's-BSA): Add 0.4 g of fat-free BSA in 200 mL of Locke's Buffer.
3. [^{3}H] AA: The radioactive material obtained from New England Nuclear Life Science products is 100 μCi/mL in ethanol with a specific activity of 180–240 Ci/mmol. Dilute this in Locke's + BSA buffer to yield 1 μCi/mL. Use this solution for loading the cells (see Note 4).
4. 10 mM Glutamate: Add 0.0169 g of Glutamate (F.W.169.11) in 10 mL of Locke's-BSA buffer. Use 50–100 μM of glutamate as a positive control.
5. Sodium hydroxide (NaOH): 0.5N NaOH (FW 40.0) in double-distilled water.
6. Ultima Gold scintillation fluid (PerkinElmer, Shelton, CT): Use this as is.

3. Methods

^{3}H-AA release can be measured in any cells. In this chapter, we have focused on cerebellar granule neurons (CGN) cultured at 7 days in vitro. The method described for CGN cultures is a modified version of Gallo et al. (19), and the details of this procedure can be found in our previous publication (20). Use all sterile materials and sterile procedures for culturing CGN or any cultures of choice.

3.1. Preparation of CGN

1. Coat 12-well culture plates obtained from Costar with polylysine (50 μg/mL).
2. Obtain the cerebella from 7-day-old pups and digest them with trypsin–DNase solution under sterile conditions. Triturate 6–8 times with a sterile 10-mL seriological pipet and allow to settle for 2 min.
3. Pass the supernatant through 100-μm Nitex screen. Add an additional 10-mL DMEM to the remaining tissue, triturate as before, and pass through the 100-μm Nitex screen.
4. Centrifuge the solution at 100 × *g* (1,000 rpm) for 10 min, suspend the cell pellet in DMEM, triturate, and pass the cell suspension through 30-μm Nitex screen.
5. Bring the cell suspension to the desired volume with DMEM and count the cells in hemocytometer.
6. Plate the cerebellar granule cells in 12-well plates (1×10^6/well in 1 mL DMEM: F-10 + 10% FBS). Grow them at 37°C

at 5% CO_2 incubator for 6 days without changing the media (see Note 5).

3.2. Labeling of CGN with [^{3}H] Arachidonic Acid

1. Observe the neurons under a microscope for dendritic network and to make sure the neurons grew normally (see Note 6).
2. At 6 days in vitro, add 1 μCi [^{3}H]AA per well to label the cell cultures.
3. Incubate these cell cultures at 37°C in 5% water jacketed CO_2 incubator for 16–20 h.

3.3. [^{3}H] Arachidonic Acid Release from CGN

1. Allow the Locke's buffer to warm to room temperature. Aspirate the cell culture media from ^{3}H AA-labeled cells and immediately add 1.0 mL warm Locke's buffer. Keep these cells to sit for 10 min at room temperature.
2. Aspirate the Locke's Buffer and immediately add 1.0 mL of warm Locke's Buffer + 0.2% BSA (see Note 7). Allow the cells to sit for 10 min at room temperature. Repeat the washing procedure one more time. Now, the labeled cells are ready for experiment.
3. Aspirate the Locke's Buffer + 0.2% BSA from the cells and add 1.0 mL of Locke's Buffer + 0.2% BSA premixed with the test substance or glutamate as positive control (see Note 8). Incubate for 20 min at room temperature.
4. Immediately after 20 min exposure, remove an aliquot of the media and count in a liquid scintillation counter using 10 mL of Ultima Gold scintillant. Time-course experiments can be conducted by taking samples at different intervals.
5. To determine the total cellular incorporation of [^{3}H]AA, lyse an equal number of cells in 1.0 mL of 0.5N NaOH. Remove the solution and count an aliquot in a liquid scintillation counter using 10 mL of Ultima Gold scintillant.
6. The data is represented as % of the total ^{3}H-AA incorporation, obtained by dividing the ^{3}H-AA in media by the total ^{3}H-AA in the lysed cells. Use appropriate blanks (see Note 9).

4. Notes

1. All solutions must be prepared in double-distilled water.
2. One can save time when making poly-L-lysine for cell cultures, by preparing a stock solution, dividing it into aliquots appropriate for one culture, and storing at –80°C.
3. Please note that the DMEM as ordered from GIBCO (Life Technologies) contains 5 mM KCl, approximately 1 mM $MgSO_4$ (anhydrous), and 25 mM D-glucose.

4. For radioactive assays, the specific activity of radioactive chemical varies a lot. Therefore, calculate the specific activity for each batch of isotope.
5. Cultures should be observed daily for proper dendritic growth patterns, attachment efficiency, and possible contamination. Cessation of growth of glial cells following cytosine arabinoside administration is also monitored to determine homogeneity of the cultures. Cultures suspected of being contaminated by bacteria or fungi must be removed and disposed of.
6. When using cell cultures, make sure that the cells look healthy before conducting the assay. Assays can be conducted after 6 days in culture.
7. For ^{3}H-AA release from cells, prepare Locke's + BSA solution on the day of the assay.
8. Use proper positive controls, such as glutamate, in the assay to improve confidence in the results.
9. For radioactive assays, the blank values should not be more than 10% of the controls.

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Part IV

Other End-Points

Chapter 23

Quantitative Assessment of Neurite Outgrowth in PC12 Cells

Joshua A. Harrill and William R. Mundy

Abstract

In vitro test methods can provide a rapid approach for the screening of large numbers of chemicals for their potential to produce toxicity. In order to identify potential developmental neurotoxicants, assessment of critical neurodevelopmental processes, such as neuronal differentiation and growth has been proposed. PC12 cells have been widely used to study the neurotrophic factor-induced signaling pathways that control differentiation, and as in vitro models to detect the effect of chemicals on neurite outgrowth. Upon exposure to nerve growth factor (NGF), PC12 cells cease to proliferate, extend multiple neurites, and acquire the properties of sympathetic neurons. Measurement of the number and length of neurites during exposure to NGF provides a quantitative assessment of neuronal differentiation and growth. Differentiation and neurite outgrowth can be measured using simple contrast microscopy in live cells, or using automated imaging systems in cells prepared with immunocytochemistry.

Key words: PC12 cells, Neurite growth, Photomicroscopy, Immunofluorescence, High-content analysis

1. Introduction

While in vitro models are used routinely for understanding the mechanism-of-action of neurotoxicants (1), use of neuronal cell cultures as tools for hazard identification and risk assessment is becoming a more common practice (2). The use of neuronal cell cultures for assessing the potential of chemicals to perturb the developing nervous system is based on the premise that the cellular processes critical for normal brain development in vivo (e.g., proliferation of neural progenitors, differentiation into neurons and glia, growth of dendrites and axons) can be recapitulated in vitro (3). In particular, the initiation and extension of dendrites and axons (collectively termed "neurite outgrowth") has received much attention, and chemical effects on this process

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have been examined in many different in vitro neuronal models (4). Common metrics used to quantify neurite outgrowth in these studies include the number of neurite bearing cells and neurite length.

The pheochromocytoma PC12 cell line has been used in both neurobiological (5) and neurotoxicological (4) studies as a model of neurite outgrowth. PC12 cells differentiate in response to nerve growth factor (NGF) with a dramatic change in phenotype, acquiring biochemical and morphological characteristics of sympathetic neurons. Upon exposure to NGF, PC12 cells growing in culture cease to proliferate, extend multiple neurites and become electrically excitable (6). The elaboration of neurites occurs within 24–48 h of NGF exposure and continues for up to a week (7). We describe two methods to quantify the number of neurite bearing cells and neurite length in PC12 cells. The first method is based on the imaging of live cells using phase contrast or interference contrast microscopy, followed by manual tracing of neurite length. The second method uses a high-content image analysis system that automates the acquisition and analysis of neurite outgrowth in cells that have been immunocytochemically labeled with a fluorescent tag (8).

2. Materials

2.1. Cell Culture

1. Neuroscreen-1™ clone of PC12 cells (Thermo Scientific, Inc., Waltham, MA) (see Note 1).
2. RPMI 1640 Medium (Lonza BioWhittaker, Walkersville, MD) supplemented with L-glutamine, Penicillin/Streptomycin (Lonza BioWhittaker, Walkersville, MD), 10% equine serum (Hyclone Thermo Scientific, Waltham, MA), and 5% fetal bovine serum (Hyclone Thermo Scientific, Waltham, MA). The L-glutamine and penicillin/streptomycin mixture are stored as 10 mL aliquots at –20°C. On the day of use, thaw and add to 1 L of RPMI medium. Store the supplemented medium at 4°C until use.
3. Trypsin–Versene (EDTA) solution (Lonza BioWhittaker, Walkersville, MD).
4. Human-recombinant Nerve Growth Factor beta (NGF, Sigma-Aldrich, St. Louis, MO) is received as a 0.1 mg lyophilized powder. The powder is diluted with 500 µL of 0.1% BSA in phosphate buffered saline (PBS), aliquoted at 50 µL and stored at –80°C until time of use. On the day of use, NGF-supplemented medium (100 ng/mL) is prepared by adding a 50 µL aliquot to 100 mL of RPMI medium.
5. BD BioCoat® Collagen Type IV, 75-cm^2 culture flask with vented cap (BD Biosciences, Inc., Franklin Lakes, NJ).

6. BD Biocoat® Collagen Type IV, 96-well polystyrene plates (BD Biosciences).
7. BD Biocoat® Collagen Type IV, 12-well polystyrene plates (BD Biosciences).

2.2. Immunocytochemistry

1. PBS 10× stock (Invitrogen, Inc., Carlsbad, CA). Prepare 1× working solution by dilution of one part 10× with nine parts water. Store 1× and 10× solutions at 4°C.
2. Immunocytochemical staining buffer (ISB): Prepare 10× stock containing 9 mM $CaCl_2$, 26.8 mM KCl, 14.7 mM KH_2PO_4, 4.9 mM $MgCl_2$, 1.4 mM NaCl, 80.6 mM Na_2HPO_4, 0.1% Saponin, 5% BSA, and 0.5% NaN_3. Prepare a 1× working solution by dilution of one part 10× with nine parts water. Store 1× and 10× solutions at 4°C.
3. 20% paraformaldehyde solution (Electron Microscopy Sciences, Hatfield, PA).
4. Sucrose solution. Prepare a solution of 5% sucrose by dilution of 5 g sucrose in 100 mL of 1× PBS.
5. Bisbenzimide H 33258 (Hoechst) fluorochrome solution: Dissolve 3 mg Bisbenzimide H 33258 trihydrochloride in 1 mL of water.
6. Fixative: For each mL of fixative solution, combine 600 µL of 5% sucrose solution, 400 µL of 20% paraformaldehyde and 1 µL of Bisbenzimide H 33258 solution.
7. Mouse neuronal β_{III}-tubulin IgG antibody and DyLight 488 conjugated goat anti-mouse IgG antibody (Cellomics Neurite Outgrowth HitKit, Thermo Scientific, Inc.) (see Note 2).
8. MicroAmp™ optical adhesive film (Applied Biosystems, Inc., Foster City, CA).

3. Methods

While PC12 cells reliably differentiate upon exposure to NGF, the extent of neurite outgrowth is dependent on the density of cells plated, growth substrate, and culture medium constituents (including serum and growth factors). Therefore, to obtain reproducible results, it is important to be consistent in the materials used and conditions under which the cells are grown. Quantitative assessment of neurite outgrowth provides a calibrated measure of some aspect of neurite length (e.g., length of the longest neurite, total neurite length per cell, average neurite length). A more complete assessment of neurite outgrowth can include quantification of the number of neurites per cell and the number of branch points. Manual assessment of the length of the longest neurite

emanating from a cell can be performed on images of live cells taken under modulation contrast illumination on an inverted microscope linked to a digital camera. Because the cells are not stained, they can be imaged repeatedly over time. In order to facilitate more rapid assessment of neurite outgrowth, systems have been developed with automation of both image capture and image analysis (9). Automated systems include mechanized stage movement for positioning, autofocusing and exposure adjustment for image capture, and software for image analysis, image and associated metadata storage, and data presentation. Cells are fixed and labeled with an antibody staining the cell body and neurites (e.g., β-tubulin), and counterstained with a nuclear dye (e.g., Hoechst). Data on various parameters of neurite outgrowth (e.g., total neurite length, neurite count, branching) can be presented on a per cell basis or calculated as a well average.

3.1. Preparation of Cells for Neurite Outgrowth

1. NS-1 cells growing in 75-cm² culture flasks are collected when approaching confluence by removing the RPMI growth medium and adding 3 mL of Trypsin/Versene solution. After 5 min, 10 mL of RPMI medium is added to the flask and cells are collected in a 15-mL conical centrifuge tube.
2. The cells are centrifuged at 300×*g* for 10 min. Discard the supernatant and resuspend the cells in 10 mL RPMI medium. An aliquot is taken for cell counting to determine the total number of cells. Adjust the volume of RPMI medium to achieve a final concentration of 20,000 cells/mL (see Note 3).
3. For manual assessment of neurite outgrowth in living cells, 30,000 cells in 1.5 mL RPMI medium are plated into each well of a type IV collagen-coated 12-well plate. For automated assessment of neurite outgrowth after immunocytochemical staining, 2,000 cells in 100 µL RPMI medium are distributed into each well of a type IV collagen-coated 96-well plate. Place the cells into a cell culture incubator at 37°C for 2 h to allow for adherence to the collagen substrate.
4. While the cells are in the incubator, all materials for the initiation of cell differentiation and neurite outgrowth are prepared. RPMI medium containing NGF is warmed to 37°C. To examine inhibitory effects on differentiation and neurite outgrowth, test chemicals are diluted into RPMI medium containing NGF. To determine whether differentiation and neurite outgrowth can be stimulated, chemicals are diluted into RPMI medium without serum. Enough solution is prepared to provide for 1.5 mL per well in a 12-well plate, or 100 µL per well in a 96-well plate. Each well provides a single experimental data point.
5. Differentiation is initiated by aspirating the RPMI medium from the wells and adding the appropriate volume of RPMI

medium containing NGF to each well (1.5 mL for 12-well plates, 100 μL for 96-well plates). Control wells (not differentiated) receive RPMI medium without NGF.

6. The cells are placed back into the cell culture incubator at 37°C until time of measurements. For manual neurite tracing, cells can serially be imaged at different time points and placed back in the incubator. Automated measurements of immunolabeled, fixed cells require sampling over multiple plates.

3.2. Immunocytochemistry

1. Warm fixative solution to 37°C.
2. Remove the 96-well plates containing the cells to be immunolabeled from the incubator.
3. To each well, directly add a volume of warm fixative solution equal to the amount of medium in the well (typically 100 μL). This direct fixation method effectively preserves the fine cytoarchitecture of neurite-bearing cells. Be careful not to overfill the wells if >100 μL of medium was used for culturing the cells.
4. Allow fixation for 20 min at room temperature, protected from light.
5. During fixation, prepare 1:800 solution of β_{III}-tubulin primary antibody in ISB (see Note 4).
6. Remove the fixative solution/culture medium from the wells using gentle aspiration.
7. Fill the wells with 100 μL of ISB and then drain each well with gentle aspiration (see Note 5). Repeat two more times for a total of three washes.
8. Dispense 50 μL of β_{III}-tubulin primary antibody solution into each well and incubate for 1 h at room temperature, protected from light.
9. During primary antibody incubation, prepare a 1:500 solution of DyLight 488 goat anti-mouse secondary antibody in ISB.
10. Aspirate the primary antibody solution and wash the plate three times with ISB as described in step 11.
11. Dispense 50 μL of DyLight 488 secondary antibody solution into each well and incubate for 1 h at room temperature, protected from light.
12. Wash the plate two times with 100 μL of ISB followed by two washes with PBS. Do not remove the final PBS wash from the immunolabeled wells.
13. Seal the plate with optical adhesive film.
14. Wrap plate in tin foil (to protect from light) and store at 4°C. When ready to scan, remove the plate from 4°C and allow plate to warm to room temperature.

3.3. Computer-Assisted Assessment of Neurite Outgrowth

1. The 12-well plates containing live cells are removed from the incubator and placed on the stage of an inverted microscope, and the cells are viewed using modulation contrast optics with a 10× objective (see Note 6). Cells are located in the middle portion of the well, and two random fields are photographed in each well.
2. A calibration measure is obtained by photographing a calibrated stage micrometer under the same conditions.
3. For the assessment of cell differentiation, digital images are examined using any standard viewing software that can accurately display the bit depth of the image (e.g., Microsoft Office Document Imaging). The number of differentiated cells is determined by visual examination of the field and counting cells that have at least one neurite with a length equal to or greater than the cell body diameter. The counts are combined for both fields from a single well and expressed as the number of differentiated cells as a proportion of total cells counted.
4. Neurite length is determined by manually tracing the length of the longest neurite on a differentiated cell using the NIH Image J software (see Note 7). For calibration of scale, open the stage micrometer image in Image J (File Open). Select the Line button from the toolbar, right click and select Straight Lines. Draw a line over a known distance in microns.
5. Select Analyze/Set Scale. Record the "Distance in Pixels" number and associated known distance in microns. These numbers can now be used to Set Scale when you open an image captured using the 10× objective with the same microscope and camera settings.
6. For quantification of neurite length, open the file of an image with cells and neurites. Select Analyze/Set Scale. Fill in "Distance in Pixels" and "Known Distance" with values obtained above. Select OK.
7. Select the Line button from the toolbar, right click and select Freehand Lines. Move the cursor to the beginning of the identified longest neurite on a cell (at the point where it emerges from the cell body). Hold down mouse button and trace the neurite, releasing the mouse button when you are at the end of the neurite.
8. Select Analyze/Measure then Analyze/Label. A Results window will open with the measured distance in microns. Continue tracing neurites for each cell in the image for which the longest neurite can be identified (see Note 8).

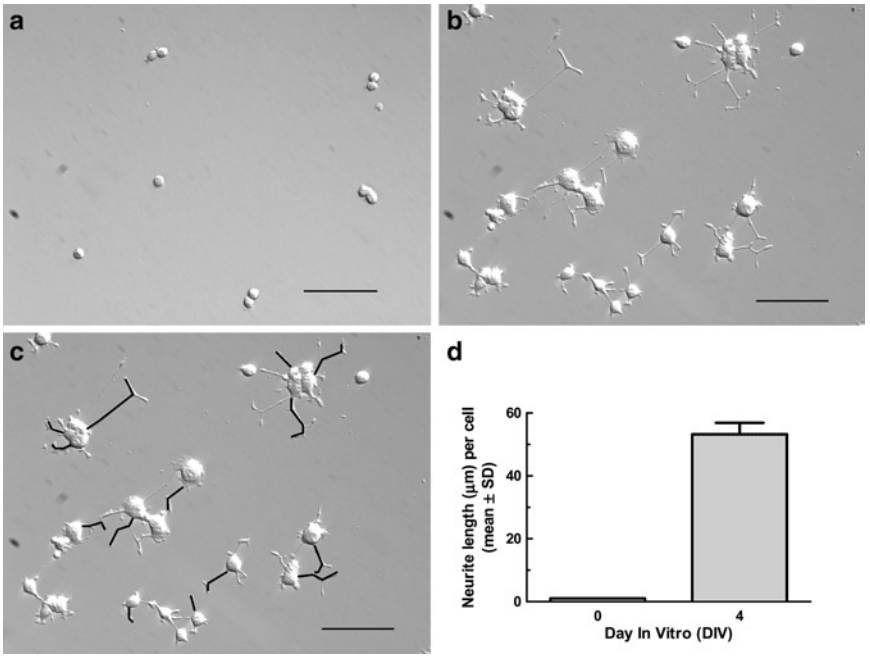

Fig. 1. Manual assessment of neurite outgrowth in PC12 cells. The NS-1 clone of PC12 cells was plated at 30,000 cells per well in a 12-well collagen-coated tissue culture plate and exposed to 100 ng/mL NGF for 4 days. Modulation contrast images of living cells were obtained on an inverted Nikon Eclipse TE200 using a 10× objective (*scale bar* = 100 microns). (**a**) Cells not receiving NGF do not differentiate and do not exhibit neurites (processes with a length greater than 1 cell diameter). (**b**) Cells treated with NGF for 4 days exhibit an increase in cell body size and multiple neurites. (**c**) The longest neurite was identified for individual cells and traced using the NIH Image J software. (**d**) Effect of NGF on neurite length. The length of the longest neurite was measured in cells prior to NGF treatment (DIV 0) or after 4 days of NGF treatment (DIV 4). Data was obtained from every cell with an identifiable neurite from two fields in each well (approximately 25 cells per field) and averaged. Data shown is the mean ± SD from three wells ($n = 3$).

9. When all cells in an image have been analyzed, save the data in the Results window by choosing File/Save As. The data will be saved as an Excel spreadsheet.
10. Steps 4–9 must be repeated for each new image that is analyzed.
11. Length (in microns) of the longest neurite is averaged for all cells in both fields within a single well. An example of the collected data for differentiated and nondifferentiated cells is given in Fig. 1.

3.4. Automated Assessment of Neurite Outgrowth: Set-Up

1. The following instructions are based on the use of Cellomics ArrayScan V^{TI} HCS Reader and assume familiarity with the vHCS:Scan software package and the Neuronal Profiling BioApplication. The method describes automated measurement of neurite outgrowth in differentiated cells in 96-well

plates stained using the immunocytochemical procedure described above. The assay parameters cited in this method are associated with an optimized image analysis protocol (_Cellomics_AS_1.0x_NP.V3.5_NS1_10x_p5.1.3) for measuring neurite outgrowth in differentiated PC12 cells. The notes associated with this method provide guidance for the adjustment of default settings and assay parameters for adapting the protocol to measure neurite outgrowth in other neuronal cells types (i.e., other cell lines, primary neuronal cultures, etc.). **Bolded** text refers to a menu or heading in the Neuronal Profiling BioApplication. *Italicized* text refers to an option or value input by the end user (see Note 9).

2. Turn on the ArrayScan V^{TI} HCS Reader, open the vHCS:Scan Software and log in.
3. Set the workspace to the **Create Protocol** window using the large icons in the upper left-hand corner of the screen.
4. Select *NeuronalProfiling* from the **Assay Algorithm** drop-down menu.
5. Set the **Image Acquisition** parameters: Set the **Camera Configuration** to an option that is compatible with the ArrayScan system in use from the associated drop-down menu. Select the *10*× imaging **Objective**. Set **Acquisition Camera Mode** to *Standard*. Set **Autofocus Camera Mode** to *Autofocus*. Check the box next to **Intra-well Autofocus Interval** and set the number of fields to *2*.
6. Set the protocol **Scan Limits** (see Note 10): Check the box beside **Max Fields for Well** and set value to *10*. Check the box beside **Min Objects for Well** and set value to *250*. Check the box beside **Max Sparse Fields for Well** and set value to *10*. Set **Min Objects for Field** to *1*.
7. Under **Assay**, set **#Channels** to *2* and set the **Focus Channel** to *1*.
8. Set the **Assay Parameters**: Determine the format of neurite outgrowth measurements to be reported either in pixels or micrometers. For the **Use Micrometers** parameter, enter *0* to report lengths in pixels or *1* to report lengths in μm (see Note 11). Check the box next to **Hide Advanced Parameters** (see Note 12). 24 parameters and their corresponding values are now displayed in the **Assay Parameters** menu. Set the Assay Parameters to the values listed in Table 1.
9. Select Channel *1* under **Channel Specific Parameters** and set Channel 1 specific parameters. Enter *Nucleus* in the **Label** text box. Select *XF93 – Hoechst* from the **Dye** drop-down menu. Select *AutoExpose* from the **Exposure Type** drop-down menu. Set the **Initial Exp Time (sec)** to *0.01*. Select *1* from the **Disk-Based Channel Remap** drop-down menu.

Table 1
Assay parameters for NS-1 neurite outgrowth tracing

Parameter type[a]	Parameter	Value
Qualitative	UseReferenceWells	0
	NucTypeCh1	0
	CellBodyandNeuriteTypeCh2	0
Background	BackgroundCorrectionCh1	100
	BackgroundCorrectionCh2	100
Exclusion	RejectBorderNucsCh1	0
	RejectBorderCellBodiesCh2	0
	RejectMultiplyTracedNeuritesCh2	0
Display	NeuritePointDisplayModeCh2	0
Nucleus morphometrics	NucSegmentationCh1	5
	NucSmoothFactorCh1	0
Cell body morphometrics	MinCellBodyNucOverlapCh2	10
	CellBodySmoothFactorCh2	0
	CellBodyDemarcationCh2	3
	CellBodySegmentationCh2	7
	UseNucforCellBodySegmentationCh2	1
	CellBodyMaskModifierCh2	0
Neurite morphometrics	NeuriteSmoothFactorCh2	1
	NeuriteIdentificationModifierCh2	–0.97
	NeuriteDetectRadiusCh2	3
	NeuriteDetectMethodCh2	1
	NeuriteDirectionCh2	3
	NeuritePointResolutionCh2	1
	UseCellBodyZOIForNeuriteTracingCh2	0

[a]"Parameter types" are not standard Cellomics Neuronal Profiling nomenclature. Defined for use as descriptors in this chapter

10. Open the **AutoExpose Options**… menu and set Channel 1 autoexposure parameters. Enter the coordinates of well to autoexpose in the **List of wells to Autoexpose** text box (see Note 13). In the **List of fields to Autoexpose** text box, enter a numerical values corresponding to the fields desired for autoexposure time calculations. Select *Peak Target(Percentile)* from the **Method Name** drop-down menu. Numerical values of *25*, *2*, *0.5*, *8*, and *8* should be entered in the **Target**, **Tolerance**, **Skip Percent**, **Max Exposure Time**, and **Max Iterations** text boxes, respectively. Exit the **AutoExposure Options**… menu by clicking **OK**.

11. Under the **Object Identification** heading select *Fixed Threshold* from the **Method** drop-down menu and enter *250* in the **Value** text box.
12. Under the **Image Display Options** heading check the boxes next to **Include This Channel in Composite**, **ValidNucleus**, and **RejectedNucleus**. Be sure that the color listed beside the **Include This Channel in Composite**, **ValidNucleus**, and **RejectedNucleus** options are blue, blue and orange, respectively.
13. Select Channel *2* under **Channel Specific Parameters**.
14. Set the Channel 2 specific parameters: Enter *Neuron* in the **Label** text box. Select *XF93 – FITC* from the **Dye** drop-down menu. Select *AutoExpose* from the **Exposure Type** drop-down menu. Set the **Initial Exp Time (sec)** to *0.3*. Select *2* from the **Disk-Based Channel Remap** drop-down menu.
15. Open the **AutoExpose Options**… menu and set Channel 2 autoexposure parameters: Well coordinates and numerical values for **List of wells to AutoExpose** and **List of fields to AutoExpose** are already populated from previous steps. Select *Peak Target (Percentile)* from the **Method Name** drop-down menu. Numerical values of *30*, *2*, *0.5*, *8*, and *8* should be entered in the **Target**, **Tolerance**, **Skip Percent**, **Max Exposure Time** and **Max Iterations** text boxes, respectively. Exit the **AutoExposure Options**… menu by clicking **OK**.
16. Under the **Object Identification** heading select *IsodataThreshold* from the **Method** drop-down menu and enter *–0.6* in the **Value** text box.
17. Under the **Image Display Options** heading check the boxes next to **Include This Channel in Composite**, **Neurite, Neurite_, Neurite__, SelectedCellBody, RejectedCellBody**, and **NeuritePoint**. Be sure that the color listed beside the **Include This Channel in Composite**, **Neurite, Neurite_, Neurite__, SelectedCellBody, Rejected CellBody**, and **NeuritePoint** options are green, green, purple, cyan, blue, red, and gold, respectively.
18. Set display to the **Protocol Interactive** window using the large icons in the top left corner of the screen. Automatic system calibration may occur at this time.

3.5. Automated Assessment of Neurite Outgrowth: Optimization

1. Select the appropriate form factor template from the drop-down menu in the upper tool bar of the display. The form factor should match the type of 96-well titer plate used in the experiment (see Note 14).
2. Load a 96-well plate containing immunocytochemically labeled cells into the ArrayScan V^{TI}. The commands for

extension (**Load Plate**) and retraction (**Retract Plate**) of the instrument stage are located in the upper tool bar.

3. Select a control well on the 96-well plate map.
4. Autofocus the image by clicking the **Autofocus** button. Channel 1 (Hoechst-labeled nuclei) is the default autofocus channel.
5. Autoexpose the Channel 1 image by clicking the **Auto Expose** button under the **Exposure** heading (Fig. 2a, b).
6. Select Channel 2 under the **Channel Parameters** heading.

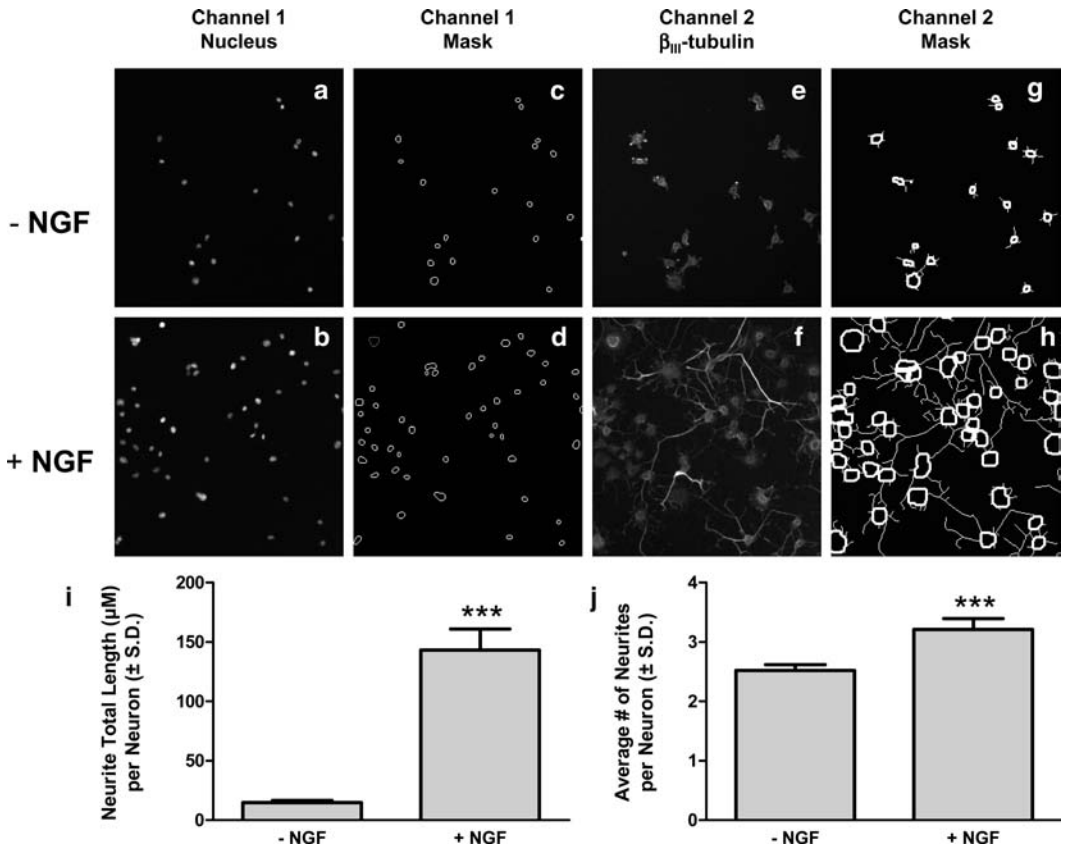

Fig. 2. Automated assessment of neurite outgrowth in PC12 cells. The NS-1 clone of PC12 cells was plated at 2,000 cells per well in a 96-well collagen-coated tissue culture plate. To induce differentiation cells were exposed to 100 ng/mL NGF for 4 days. Nondifferentiated cells were not exposed to NGF. Cells were labeled with a β_{III}-tubulin primary/DyLight 488 secondary antibody combination and Hoechst 33428 nuclear counterstain. Images corresponding to nondifferentiated (–NGF) and differentiated (+NGF) PC12 cells are shown on the top (**a**, **c**, **e**, **g**) and bottom (**b**, **d**, **f**, **h**) rows of panels, respectively. (**a** and **b**) Channel 1; Hoechst-labeled nuclei. (**c** and **d**) Nuclear masks in channel 1. (**e** and **f**) Channel 2; β_{III}-tubulin labeling of neurites and cell bodies. (**g** and **h**) Cell body and neurite masks in Channel 2. (**i** and **j**) Quantitative measures of the average number of neurites per neuron (**i**) and total neurite length per neuron (**j**) from automated image analysis. Data were collected from at least 100 cells per well across 6-wells. Data are well level means ± standard deviation (SD).

7. Autoexpose the Channel 2 image by clicking the **Auto Expose** button (Fig. 2c, d). The matched Channel 1 and Channel 2 images now comprise an image set. A pseudocolored composite image can now by viewed by toggling the **RGB** button in the lower-left hand portion of the image display window.
8. Return to the Channel 1 image by selecting *1* under the **Channel Parameters** heading.
9. Click the **Identify Objects** button. Nuclei are now bordered by a white mask (Fig. 2e, f).
10. Examine the traced nuclei and evaluate the quality of the nuclear masks. Masks should tightly border the area of Hoechst-positive staining for each nucleus. If the nuclei are not tightly masked, adjust the numeric value under the **Object Identification** heading by using the associated slider bar or entering a number manually. Decreasing the value yields a looser mask while increasing the value yields a tighter mask. Set a value that accurately traces the border of the nuclei and reliably separates bright objects from background (see Note 15).
11. Determine which bright objects (i.e., nuclei) correspond to the cells-of-interest for morphometric analysis. Toggle between the channel 1 image and pseudocolored composite image, noting the position of nuclei that correspond to cells which should be included in the morphometric analysis.
12. Set **Object Selection Parameters** for nuclei corresponding to the cell-type-of-interest: Click the **Identify Objects** button. In the image display window, select some nuclei corresponding to cells-of-interest by clicking on them (the nucleus masks will turn red). Manual highlighting of nuclei populates a set of geometric and pixel intensity measurements under the **Object Selection Statistics (Channel 1)** heading. Minima and maxima for the selected population of nuclei are displayed. Transfer the minimum and maximum values to the **Object Selection Parameters (Protocol)** sheet. Objects which fall within the specified range for all the parameters are now selected by the algorithm. Objects falling outside of any of the specific ranges are rejected. Click the **Run Algorithm** button. Selected nuclei are masked with a blue ring, and rejected nuclei are masked with an orange ring. If any nuclei corresponding to cells that need to be included in downstream morphometric analysis are rejected (orange masks), continue to **Identify Objects**, highlight the rejected nuclei, and adjust the **Object Selection Parameters** until all (or most) of the desired nuclei are selected (blue masks). Likewise, if bright Channel 1 objects that are undesirable for inclusion in morphometric analysis are selected (blue masks), limit the

Object Selection Parameters until they are rejected (orange masks) (see Note 16).

13. Return to the Channel 2 image by selecting *2* under the **Channel Parameters** heading.
14. Assess the accuracy of Channel 2 object identification: Click the **Identify Object** button. Cell bodies and neurites are now overlaid with white traces (Fig. 2g, h). Masks should overlay all visible cell bodies or neurites in the channel 2 image. If the trace provided by Channel 2 object identification is inaccurate, adjust the numerical value (*IsodataThresholding* offset) under the **Object Identification Method**. If the neurites and cell bodies present in the image are not overlaid with a trace, make the offset value more negative. If the trace overlays objects that are not present in the image (such as neurites erroneously extended past their true termination point), make the offset value more positive.
15. Assess the accuracy of Cell Body masking: Access the **Image Display Options** menu by clicking the color palette button under the **Channel Parameters** heading. Uncheck all **Neurite** options as well as **SelectedCellBody**, **RejectedCellBody** and **NeuritePoint** options and close the menu. Click the **Run Algorithm** button. Toggle between the masked and unmasked Channel 2 image and assess whether cell body masks accurately outline the borders of cell bodies present in the image. If necessary, adjust the "cell body morphometrics" values (see Table 1) in the **Assay Parameters** menu until desired cell body masks are obtained. If cell body masks desired for inclusion in the analysis are masked in red (i.e., rejected), use the **Identify Objects** function to highlight incorrectly rejected cell bodies and adjust the values under the **Object Selection Parameters** (see Note 17).
16. Assess the accuracy of Neurite masking: Access the **Image Display Options** menu by clicking the color palette button under the **Channel Parameters** heading. Check all **Neurite** options as well as the **NeuritePoint** option and close the menu. Click the **Run Algorithm** button. Toggle between the masked and unmasked Channel 2 image and assess whether neurite masks accurately overlay neurites present in the image. If necessary, adjust the "neurite morphometrics" values (see Table 1) in the **Assay Parameters** menu until desired neurite traces are achieved (see Note18). Neurite selection can also be implemented by adjusting values under the **Object Selection Parameters** heading.
17. Select a positive-control treated well and repeat steps 4–16. After optimization, the protocol should accurately mask, trace, and measure neurite outgrowth in both control and

positive-control treated cultures. This iterative optimization process may require sampling of multiple fields and adjustment of the algorithm multiple times to make automated tracing as accurate as possible across different treatment conditions.

18. Save the protocol once optimization is complete (see Note19).

3.6. Automated Assessment of Neurite Outgrowth: Scanning

1. Set display to the **Scan Plate** window using the large icons in the top left corner of the screen.
2. Name the plate to be scanned by filling in the **Plate ID (User Defined)** and **Plate Name** text boxes.
3. Click the **Scan Area Selection** icon and highlight the wells to be scanned.
4. Click the **Starting Field Selection** icon and select the field in which to begin image acquisition.
5. Enter relevant experimental information in the **Scan Comments** window.
6. Activate the **Store Images** icon.
7. Scan the plate (green "play button" icon).
8. An example of average neurite count per neuron and total neurite length per neuron measurements are presented in Fig. 2i, j.

4. Notes

1. We have found that the NS-1 clone of PC12 cells adheres well to collagen-coated tissue culture plates, do not clump excessively, and reliably differentiate upon the treatment with NGF. PC12 cells from other sources have also been used routinely to assess neurite outgrowth.
2. The primary and secondary antibodies listed have provided excellent results for immunocytochemical staining. There are many other commercial sources for primary β_{III}-tubulin and secondary fluorophore-labeled antibodies that can be successfully employed for immunostaining.
3. Preliminary experiments should be performed to optimize the cell density for both manual and automated measurements of neurite outgrowth. A cell density that maximizes cell viability but allows for identification of individual cells and accurate tracing of neurites back to the cell body of origin is desired.
4. The primary and secondary antibody dilutions required for high-quality immunocytochemical labeling may vary with

cell-type and cell culture protocol. It is recommended that a range of antibody dilutions be tested to find the dilution which provides the best signal (with minimal background staining) under the conditions used.

5. Mechanical shearing of neurites from cell bodies and wash out of cells from the plating surface is commonly encountered with immunocytochemical labeling of cultures grown in 96-well plate format. To minimize these mechanical stressors, use gentle aspiration to remove solutions from the wells while holding the plate tilted at an angle. Avoid contacting the bottom of the well with the tip of the aspiration manifold. When manually dispensing solutions into the wells, contact the tip of the pipettor with the side of the well wall and allow the solution to wick down the side. Use of an automated pipettor or robotic liquid handler at low speed is recommended for reducing the variation in immunolabeling quality from plate to plate.
6. Other forms of contrast microscopy (phase contrast, differential interference contrast) may be used.
7. NIH Image J software is available as a free download from http://rsbweb.nih.gov/ij/download.html. Other image analysis software packages that allow for calibrated linear measurements may be used.
8. A neurite should be identified as coming from a particular cell and the entire length of the neurite visible in the field. This may not be possible for all cells in an image.
9. For detailed descriptions of terminology and assay parameters associated with this method, the reader is referred to the Thermo Scientific Cellomics ArrayScan VTI HCS Reader User Guide and the Cellomics® Neuronal Profiling V3.5 BioApplication Guide. It should be noted that this is one of the several commercial high-content imaging platforms available. Analysis workflow, terminology, and resulting data outputs vary between platforms.
10. Scan Limits are set to control the number of cells (**Min Objects for Well**) and/or the number of fields sampled within each well (**Max Fields for Well**). In addition, fields and wells that do not contain a sufficient number of cells for neurite outgrowth measurements can be identified (**Min Objects for Field** and **Max Sparse Fields for Well**). These parameters can be adjusted to suit the needs of the user.
11. The binary value entered for the **UseMicrometers** parameter can impact downstream protocol optimization. Changing the **UseMicrometers** parameter in a preoptimized protocol requires the adjustment of some **Object Selection** parameters that are based on unit length or unit area measurements.

The data presented in this chapter reports neurite lengths in um (**UseMicrometers** = *1*).

12. Advanced assay parameters are used to define and quantify specific events based on cell morphometry and to separate different cell populations that display distinct features. The reader is referred to the Thermo Scientific Cellomics Neuronal Profiling v3.5 BioApplication Guide for further information.
13. Exposure times in each fluorescent channel are determined by presampling of reference wells. Exposure times are calculated in a manner that utilizes a pre-determined percentage of the dynamic range (0–4,096) of the 12 bit CCD-camera (**Target** parameter in **AutoExposure** options). Reference well sets may include untreated or vehicle-treated control wells and/or wells in which cells have been exposed to a positive control compound. In applications where the fluorescent intensity of the immunolabeled objects may either increase OR decrease with treatment, it is recommended that a combination of control and positive-control treated wells be used to set exposure times. In addition, pixel saturation (i.e., maximizing the dynamic range of the camera) should be avoided in order to avoid imposing an artificial ceiling on the fluorescent intensity values recorded by the instrument. This ensures that both increase and decrease in intensity can be measured with the calculated exposure times in each fluorescent channel. For neurite outgrowth, it is recommended that the dynamic range of the camera (**Target** parameter) be set between 25 and 50%.
14. Form factors are data files that contain a set of pre-defined coordinates for movement of the ArrayScan V^{TI} automated stage. Form factors for most brands of commonly used 96-well titer plates are available from ThermoFisher Scientific, Inc. Positional calibration for a particular plate type should be performed prior to the experiment.
15. Nuclei in an immunolabeled cell culture can sometimes cluster together. This can result in channel 1 **Object Identification** masks that trace around multiple nuclei. If this occurs, adjust the **NucSegmentationCh1** parameter in the **Assay Parameters** menu. Two options are available for the segmenting nuclei: (1) Peak Intensity (negative values) and (2) Geometric (positive values). Use of the Geometric method is recommended for most neuronal cell types, given the regular, rounded appearance of neuronal nuclei. If nuclei are too clustered for accurate segmentation, consider adjusting the cell culture method by decreasing the number of cells plated per well or use manual dispersion methods, such as needle aspiration, at the time of plating.

16. The described functions can be used to exclude small Hoechst-positive objects (i.e., cellular debris), large noncellular debris and, in some cases, nonneuronal cell types found in mixed cultures. In some primary mixed neural cultures, glial nuclei are larger and stain less intensely than neuronal nuclei, facilitating separation of different cell populations by size and intensity.
17. Cell body masks may also register as rejected due to rejection of the nucleus in the channel 1 image. Adjustment of channel 1 **Object Selection** parameters may be required to resolve this issue. Rejected nuclei are also projected into the channel 1 image. In some cases, rejected nuclei (e.g., a dense, apoptotic nucleus with no cytoplasmic staining) can be in close proximity to a neurite extending from another cell. The image analysis algorithm may incorrectly identify this as cell body cytoplasm belonging to the rejected nucleus and cast a cell body mask around them that is labeled as rejected. This can be especially problematic in mixed neural cultures with a large percentage of nonneuronal nuclei. Adjustment of the **MinCellBodyNucOverlapCh2** may resolve this issue. This parameter defines a minimum percentage of the nucleus perimeter that must be bordered by a fluorescent signal (i.e., labeled cytoplasm) in channel 2 in order for a cell body mask to be cast. Increasing this value will inform the algorithm to cast cell body masks only around nuclei that are surrounded by a larger percent of fluorescent signal, such as in the case of nuclei truly contained with a labeled cell body.
18. Optimization for accurate neurite tracing is an iterative process that may involve adjustment of several "neurite morphometric" parameters. Images with overly complex neurite fields may not be amenable to accurate automated (or manual) neurite tracing. In this case, adjustment of culture procedures, including the cell density, may be necessary.
19. Assay optimization should be performed at the onset of any series of experiments measuring morphological features of cultured neurons. In the case where a number of experiments are to be performed with independent cultures of the same cell type within the course of a larger study, it is desirable that the same image analysis algorithm be used across experiments to facilitate comparison between experiments. Assay optimization need not be performed for every independent culture. However, assay performance should be monitored throughout the course of study to assure traces are accurate and reproducible. In automated image analysis, archived images can readily be reanalyzed with different assay protocols.

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Chapter 24

Measurements of Astrocyte Proliferation

Marina Guizzetti, Terrance J. Kavanagh, and Lucio G. Costa

Abstract

Astrocytes, the most abundant cell type in the brain, proliferate during brain development. While it is generally accepted that mature astrocytes do not proliferate, neural stem cells, which have characteristics of astrocytes, retain the ability of self-renewal. Furthermore, astrocytes can regain their proliferative properties under pathological situations, such as reactive astrogliosis, a consequence of brain injury and brain tumors. Measurements of astrocyte proliferation can thus be used in investigations of physiological and pathological processes occurring in the developing and the adult brain. This chapter describes two methods for the determination of astrocyte proliferation: the incorporation of a radioactive nucleotide [^{3}H]thymidine into DNA, which occurs during the process of DNA synthesis preceding cell division, and the flow cytometric analysis of cell cycle progression through the different phases of the cell cycle by BrDu/Hoechst and ethidium bromide labeling.

Key words: Astrocytes, Cell proliferation, DNA synthesis, Cell cycle progression, Flow cytometer, Hoechst, Bromodeoxyuridine, [^{3}H]thymidine

1. Introduction

Astrocytes have been implicated in many physiological and pathological functions. Proliferation of astrocytes and neural progenitor cells is an essential event during brain development. Chemicals which affect proliferation of astrocytes may have a profound impact on brain development, and the inhibition of astrocyte proliferation may lead to microencephaly, as astrocytes are the most abundant cells in the brain. The measurement of astrocyte proliferation may, therefore, be applied to the investigation of developmental neurotoxic effects of specific agents. Astrocytes do not normally proliferate in the mature brain, however, they can reenter the cell cycle after brain injury (reactive astrogliosis), or in the

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case of tumors. In addition, self-renewing neural stem cells from the subventricular zone of the lateral ventricles and from the subgranular zone of the dentate gyrus are considered "special" astrocytes expressing the astrocytic marker Glial Fibrillary Acidic Protein (GFAP) and exhibiting astrocytic and radial glial properties (1). We have implicated the inhibition of astrocyte proliferation in the developmental effects of ethanol (2) and organophosphate insecticides (3), and the acceleration of astrocytoma cell proliferation in the effects of lead (4) and electromagnetic fields (5) as tumor promoters.

This chapter describes two methods to measure astrocyte proliferation. The first method quantifies DNA synthesis as an index of cell proliferation; this type of analysis provides a rapid and sensitive method to screen chemicals for their effects on DNA synthesis, although it does not provide a direct measurement of cell proliferation. Astrocytes are treated with selected agents and then labeled with the radioactive nucleoside [^{3}H]thymidine, which is incorporated into DNA during the synthesis (S) phase of the cell cycle. At the end of the incubation, DNA is precipitated and radioactivity is quantified (6). The second method described is a flow cytometer-based method, which allows for the simultaneous assessment of the number of cells in the different phases of the cell cycle for up to three successive cell cycles (7–9). The phases of the cell cycle are: Gap_0 (G_0): quiescence; Gap_1 (G_1): in which cells increase the synthesis of enzymes and prepare for DNA synthesis; DNA synthesis (S); Gap_2 (G_2): during which cells continue to grow and the various components involved in mitosis, such as microtubules, are synthesized; Mitosis (M): the process of cell division. Upon completion of the mitotic phase, two daughter cells are generated from the original cell. Cells are labeled with the thymidine analog 5-bromodeoxyuridine (BrdU). After cell treatments with the agents of interest, cells are incubated in the presence of Hoechst, a blue fluorescent DNA dye whose fluorescence is quenched in cells that have incorporated BrDu into DNA during the S phase of the cell cycle. Before flow cytometer analysis, the red fluorescent DNA dye ethidium bromide (EB), whose fluorescence is not quenched by BrdU, is added to the samples (10). Thus, by examining the intensity of red and blue fluorescence, one can simultaneously determine the cell cycle phase of each cell and the number of cell divisions it has undergone.

2. Materials

2.1. Cell Culture

1. 10,000 U/mL sterile penicillin and 10,000 µg/mL sterile streptomycin (Invitrogen), stored as 5 mL aliquots at –20°C.

2. Fetal Bovine Serum (FBS), heat-inactivated for 30 min at 56°C in a water bath under gentle shacking (the bottle needs to be almost completely covered by preheated water); stored at –20°C as 55 mL aliquots.
3. 10% fatty acid-free, cell culture-tested, Bovine Serum Albumin (BSA): Dissolve 5 g BSA in 50 mL of sterile water. Filter through 0.2-μm syringe filters (Corning) to sterilize the solution, and store 5 mL aliquots at –20°C.
4. Low-glucose Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with Penicillin/Streptomycin (100 U/mL and 100 μg/mL final concentrations, respectively) and 10% FBS (DMEM/10%FBS). Store the complete medium at 4°C for up to 4 weeks.
5. Low-glucose DMEM supplemented with Penicillin/Streptomycin and 0.1% fatty acid-free BSA (DMEM/0.1%BSA).
6. Sterile Ca^{2+}-, Mg^{2+}-free Hanks' Balanced Salt Solution (HBSS-CMF).
7. Sterile 10× Ca^{2+}-, Mg^{2+}-free Phosphate-Buffered Saline (PBS), in sterile deionized water, stored at room temperature.
8. 2.5% trypsin solution: Dilute 50 mL 2.5% trypsin in 450 mL sterile PBS. Make 5 mL and store 25 mL aliquots at –20°C. Make sure to label each vial with the trypsin expiration date, and avoid using expired tryspin as it becomes less effective and requires longer times of incubation that can damage tissue and cells.
9. Multiwell 24-well plates and 75-cm^2 vented-cap flasks (Becton-Dickinson, Franklin Lakes, NJ) coated for 10 min with 40 μg/mL poly-D-lysine (molecular weight range: 150,00–300,000; Sigma), followed by washing in sterile water. Stock solutions are prepared by dissolving 500 mg poly-D-lysine in 50 mL of sterile water (10 mg/mL). The final dilution (40 μg/mL) is prepared by adding 2 mL of 10 mg/mL poly-D-lysine to 498 mL of sterile water. This solution is sterilized by filtration using 500-mL bottle top filters (Nalgene, Rochester, NY).
10. Cell strainer with 100 μM nylon mesh (Becton Dickinson).

2.2. [3H]thymidine Incorporation

1. [*Methyl*-^{3}H]thymidine (PerkinElmer Life Sciences).
2. 10% Trichloroacetic Acid (TCA) (Sigma): Dissolve 1 kg of TCA in 250 mL of water; after the acid is completely dissolved, transfer the solution to a graduate cylinder and bring the volume to 1 L.
3. 0.5% TCA: Dilute 10% TCA in water (2.6 mL 10% TCA in 497.5 mL water).

4. 1N Sodium Hydroxide (NaOH): Dissolve 40 g NaOH in 1 L water.
5. EcoLume scintillation fluid (MP Biochemicals, Solon, OH) and 7-mL polyethylene scintillation vials with screw cap (VWR).

2.3. Flow Cytometry Analysis

1. 15 mM 5BrdU: Dissolve 46 mg BrdU in 10 mL water. BrdU is photosensitive; do not expose to light; freeze 1 mL aliquots in black Eppendorf tubes and store at −20°C for up to 2 weeks.
2. 10 mg/mL ethidium bromide staining solution (Molecular Probe-Invitrogen): On the day of the experiment, dilute ethidium bromide 1:20 in nanopure water for a stock solution of 500 μg/mL. Protect the solution from light until use. Ethidium bromide is an animal carcinogen. Use extreme care while handling.
3. Hoechst buffer: Dissolve 8.53 g NaCl (0.146 M final concentration) and 12.11 g Tris base (0.1 M final concentration) in 700 mL of nanopure water. Adjust the pH to 7.4 with HCl. Add to the solution: 1 mL of 500 mM $MgCl_2$ (0.5 mM final concentration); 2 g BSA (0.2% w/v final concentration); 1 mL Igepal CA-630 (Sigma) (0.1% v/v final concentration); 5.9 mg Hoechst 33258 (Molecular Probes-Invitrogen). Bring the solution up to 1 L with nanopure water and store for up to 1 month in the dark at 4°C.

3. Methods

This chapter describes two methods to measure astrocyte proliferation. Astrocytes are synchronized in the G_0 phase of the cell cycle (quiescence) by 48 h serum-deprivation before their stimulation with mitogens or expected mitogens (see Note 1). In the first method, astrocytes are treated with selected agents for 24 h; 6 h before the end of the incubation, [^{3}H]thymidine is added to the cultures. Cells are then fixed, DNA is precipitated, and [^{3}H] thymidine incorporated into DNA is counted. This method offers a sensitive, simple, and relatively inexpensive method to measure DNA synthesis, an early step in cell proliferation (2–6, 11).

The second method offers a more direct measurement of cell proliferation. Flow cytometric analysis of cell cycle progression by BrdU/Hoechst and ethidium bromide labeling also provides quantitative information on the number of cells in each phase of the cell cycle (G_0/G_1, S, G_2/M) in up to three independent cell cycles. Glial cells are incubated with BrdU, a thymidine analog that is incorporated into DNA during DNA synthesis, for 24 or 48 h and are then exposed to Hoechst 33258, a fluorescent DNA

stain that binds preferentially to AT base pairs, and ethidium bromide, a DNA intercalating fluorochrome. Because Hoechst fluorescence is quenched by BrdU, cells that have undergone DNA replication during BrdU incubation can be distinguished from nonproliferating cells by their diminished Hoechst fluorescence. Ethidium bromide fluorescence is not quenched by BrdU and is proportional to the amount of DNA present in the cell. Its fluorescence is minimal when cells are in the G_0/G_1 phase, increases during the S phase, and is maximal in the G_2 phase. The use of BrdU/Hoechst and ethidium bromide in combination allows for the separation of $G_0/G_1/S$ and G_2/M phases of the cell cycle for three complete cell cycles (2, 7–10).

3.1. Preparation of Astrocyte Primary Cultures

1. Primary astrocyte cultures are prepared from cerebral cortices of 21-day-old rat fetuses. The fetuses are decapitated and cerebral hemispheres are dissected free of meninges and mechanically dissociated in HBSS-CMF.
2. The cells are dispersed by treatment with 20 mL 0.25% trypsin for 10 min at 37°C; trypsin is inactivated by the addition of an equal volume of DMEM/10% FBS; the cell suspension is centrifuged at $200 \times g$ for 10 min.
3. Discard the supernatant and resuspend cells in 20 mL of DMEM/10% FBS, vortex energetically for 1 min to kill the neurons, and centrifuge at $200 \times g$ for 10 min.
4. Discard the supernatant, resuspend cells in 20 mL DMEM/10% FBS medium, and centrifuge at $200 \times g$ for 10 min. This step is repeated two more times.
5. Resuspend cells in 20 mL DMEM/10% FBS and filter to remove aggregates through a 100 μm-pore size nylon cell strainer.
6. Count cells and plate them in 75-cm² flasks precoated with poly-D-lysine at the density of 4×10^6 cells/flask in DMEM/10% FBS; incubate at 37°C in a humidified atmosphere of 5% CO_2/95% air.
7. The day after, to remove the layer of nonadherent cells growing on top of the flat monolayer, shake flasks manually, and wash twice with PBS; fresh DMEM/10% FBS medium is replaced. Cells are grown for 9 days changing the medium every 2–3 days.
8. Remove the medium, wash cells twice in PBS-CMF, and dislodge the adherent astrocytes by the addition of 2 mL trypsin (0.25%) for 10 min at 37°C in a humidified atmosphere of 5% CO_2/95% air; neutralize trypsin with an equal volume of DMEM/10% FBS, and centrifuge the cell suspension at $200 \times g$ for 10 min; discard the medium and resuspend the pellet in DMEM/10% FBS; plate cells in 24-wells plates at the density 5×10^5 cells/well.

3.2. [^{3}H]thymidine Incorporation

1. Cells plated in 24-well plates are maintained in DMEM/10%FBS; the medium is changed the day after plating. On the third day after plating, cells are washed twice in PBS to completely remove FBS and 1 mL/well DMEM/0.1% BSA is added to the cultures for 48 h.
2. Treatments are prepared in DMEM/0.1 BSA and added to cultures for a total of 24 h. Each treatment should be carried out in triplicate. Eighteen hours after the beginning of the incubation, 10 μL [^{3}H]thymidine (100 μCi/mL), diluted in PBS, is added to each well to reach the final concentration of 1 μCi/well and cells are incubated for an additional 6 h.
3. Stop incubations by placing the plates on ice and washing them twice with ice-cold PBS. The medium and the washes are disposed as radioactive wastes.
4. Cells are fixed by adding 1 mL/well 100% methanol for 10 min; methanol is then removed.
5. Precipitate DNA by adding 1 mL/well 10% TCA for 10 min followed by the removal and addition of 1 mL/well 10% TCA for 5 min and removal. Add 1 mL/well 0.5% TCA to the monolayer for 5 min and then remove it. This step reduces the concentration of residual TCA, which may prevent the solubilization of the monolayer (see step 6) while keeping the DNA precipitated.
6. Solubilize the monolayer by the addition of 500 μL 1N NaOH. Incubate plates at room temperature under gentle agitation for 30 min. Transfer 250 μL of each sample into a 7-mL scintillation vial; add 5 mL of scintillation fluid, cap each vial, vortex, and count radioactivity using a liquid scintillation counter (see Notes 2 and 3).

3.3. Cell Cycle Analysis of Glial Cells by BrdU/Hoechst Flow Cytometry

1. Cells plated in 24-well plates are maintained in DMEM/10%FBS; the medium is changed the day after plating. On the third day after plating, cells are washed twice in PBS to completely remove FBS and 1 mL/well DMEM/0.1% BSA is added to the cultures for 48 h.
2. Replace the medium with 1 mL of fresh serum-free medium in the absence or in the presence of chemicals to be tested for their proliferative or antiproliferative effects. Each treatment should be carried out in triplicate.
3. At the end of the treatment, add 10 μL of 15 mM BrdU (150 μM final concentration) to each well (see Note 4). Incubate for 48 h.
4. Remove the medium and harvest the cells from each well by adding 200 μL/well trypsin (0.25%); incubate the plate for 5 min at 37°C in a humidified atmosphere of 5% CO_2/95% air. Neutralize the trypsin by adding 200 μL DMEM/10%FBS

and pipet repeatedly up and down to obtain a single-cell suspension (see Note 5).

5. Transfer the cells into 1.5-mL tubes and centrifuge them for 10 min at 1,000 × *g*, 4°C. Remove the supernatant by aspiration and resuspend the cells in 500 µL/tube Hoechst buffer (see Note 6). Cells resuspended in Hoechst buffer must be analyzed by flow cytometry within 8 h; if samples cannot be analyzed in this time frame, resuspend cells in Hoechst buffer supplemented with 10% DMSO (v/v) and store the cells at –20°C for up to 1 week.
6. Transfer the samples to 12 × 75-mm polystyrene tubes. Add 5 µL ethidium bromide (stock solution: 10 mg/mL) to reach a final concentration of 5 µg/mL. Mix each sample thoroughly; the samples at this stage need to be analyzed by flow cytometry immediately.
7. Flow cytometry analysis of the samples prepared as described above is carried out by exciting both Hoechst and ethidium bromide at 352–362 nm (argon-ion laser) or at 365 nm (mercury lamp). Collect Hoechst emission with a 420–490 nm band-pass filter and ethidium bromide emission a 610-nm long pass filter. Each sample should contain 10,000 cells or more in order to obtain a representative distribution of cells across three different cell cycles.
8. The MPLUS AV program (Phoenix Flow Systems, San Diego, CA) or equivalent flow cytometry software program is used for data analysis. Cell cycle compartments are analyzed by plotting the Hoechst-associated fluorescence (abscissa) against ethidium bromide-associated fluorescence (ordinate) (9) (see Note 7).

4. Notes

1. The described methods are optimized for the measurement of cell proliferation in astrocytes synchronized in the G_0 phase of the cell cycle and then stimulated by mitogens. After 48 h in serum-free medium 92–95% of astrocytes are quiescent (see Fig. 1a and Table 1). However, a protracted period in serum-free medium may induce cell death; if other CNS cell types are used, optimization of conditions for reaching quiescence without cell death should be carried out. It is possible to reach synchronization of certain cells by incubating them in low serum concentrations (0.1% FBS) or by the addition of synchronizing agents (i.e., lovastatin and vincristine have been previously used to synchronize cells in the G_1 phase of the cell cycle). It is also possible to measure cell proliferation

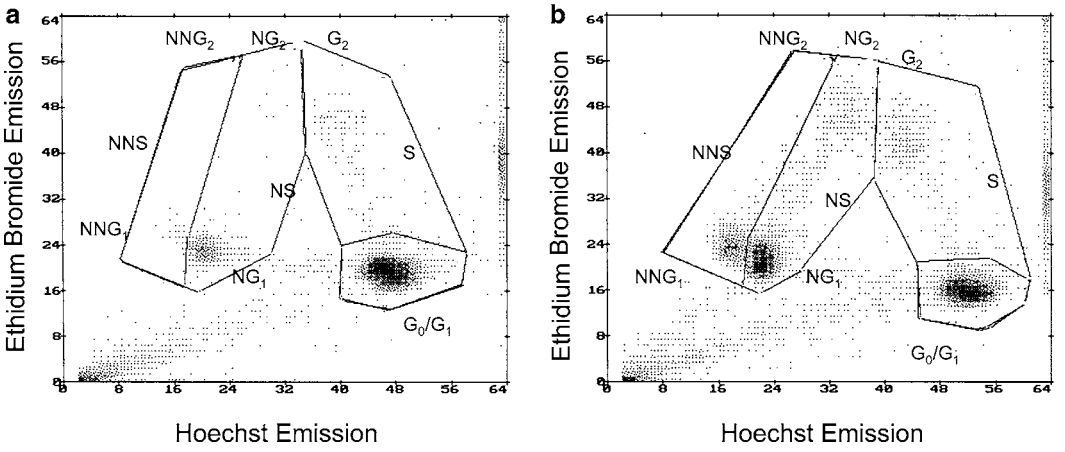

Fig. 1. Flow cytometry plot for bivariate cell cycle analysis. Quiescent rat astrocytes were maintained for 48 h in serum-free medium (**a**) or were stimulated with 10% FBS (**b**) in the presence of BrdU. On the abscissa: Hoechst fluorescence emission. On the ordinate: ethidium bromide emission. G_1: cells in Gap1 phase; S: cells in DNA synthesis phase; G_2: cells in Gap_2/mitosis phases; NG_1, NS, NG_2: cells that have completed the first mitosis and are in the second cell cycle (N = new); NNG_1, NNS, NNG_2: cells that have completed two cell divisions and are now in the third cell cycle.

Table 1
Distribution of cells in the cell cycles

	First cell cycle G_0/G_1 phases	First cell cycle S/M/G_2 phases	Cells in second cell cycle	Cells in third cell cycle
Serum-free	94.13%	1.96%	3.78	0.12
10% FBS	65.63	3.90	28.00	2.46

Data are expressed as percentage of cells initially present in the cultures. The raw data relative to cells in the second and third cell cycles have been adjusted to account for the number of divisions undergone by the cells (see description in the text)

in asynchronous cells, when the experimental design allows for it.

2. Scintillation vials containing the sample and the liquid scintillation fluid should be mixed with a vortex at high speed for at least 30 s/vial and let to rest in the dark overnight before counting in a liquid scintillation counter. In order to make sure that the counts are stable, count the same samples at least twice at intervals of 24 h. If the radioactivity readings increase with time, count the samples again until they stabilize. [^{3}H] has a half-life of more than 12 years; therefore, vials containing [^{3}H]thymidine can be stored for a long time without losing radioactivity.
3. It is important to verify that the monolayer is fully dissolved by NaOH before transferring the samples to scintillation vials. The bottom of each well should be totally transparent and clear. If cells are still visible, shake the plates for a long time or

store them overnight at 4°C after wrapping them tight in Parafilm to prevent evaporation.

4. BrdU is photosensitive and cannot be exposed to direct light. Turn off the light in the tissue-culture hood used for the treatment before adding BrdU and wrap the plates in aluminum foil before transferring them to the incubator; keep them protected from light exposure during the 48 h incubation period. Exposure of cells that have incorporated BrdU to light results in DNA strand breaks. From this point on, samples should be protected from light.
5. Primary astrocyte cultures have strong cell–cell and cell–vessel adhesion; it is, therefore, important to ensure that trypsinization is complete in order to obtain a good cell yield and avoid cell clumping that can result in spurious flow cytometric data and clog the machine. It is advisable to check that cells are well dissociated by looking at the cell suspension after trypsinization under a phase-contrast microscope. Should clumping occur, it is advisable to pass the cell suspension through a 26- or 28-gauge syringe needle before flow cytometric analysis or to increase the time of trypsinization (e.g., 10 min). It should also be ascertained that the trypsin used is not expired, as old trypsin has decreased proteolytic activity.
6. The Hoechst dye that has been used for this procedure is not cell permeable, and therefore necessitates the presence in the buffer of a detergent (Igepal CA-630). In the case that Hoechst-associated fluorescence is not detected, it is possible that the dye does not efficiently enter the cells. To increase the entering of Hoechst into the cells, it is possible to slightly increase the concentration of the detergent in the buffer or to increase the time of incubation of samples in the Hoechst buffer before flow cytometry analysis. It is also possible to substitute the cell-permeable Hoechst 33342 for the nonpermeable Hoechst 33258.
7. During DNA synthesis (S-phase), the nuclear DNA content increases; this can be observed as an increase in ethidium bromide fluorescence and a decrease in Hoechst-associated fluorescence due to the incorporation of BrdU into DNA which quenches Hoechst fluorescence. Cells in the G_2/M phase of the cell cycle, when the DNA is completely duplicated and the cells prepare to undergo cell division, are visualized at the maximal ethidium bromide fluorescence (because both G_2 and M phases have double DNA content, they cannot be distinguished with this flow cytometric technique). When the cells divide, the content of DNA decreases visualized as a drop in ethidium bromide fluorescence; as cells that have undergone a full division during the incubation time have incorporated more BrdU, they display also a decreased Hoechst fluorescence. The newly duplicated cells in the

G_1 phase of the cycle are, therefore, distinguished from the nonproliferation cells by a shift to the left in the abscissa (Hoechst fluorescence). Using this approach, the G_0/G_1, S and G_2/M phases of three successive cell cycles can be quantified by two parameter flow cytograms. The number of proliferating cells is obtained by dividing the number of cells in the second or third cycle by two and four, respectively, because in these cases the cell number is the result of one mitosis (second cell cycle) or two mitoses (third cell cycle) occurring from the beginning of BrdU incubation. Figure 1 shows the distribution of cells in the different phases of the cell cycle across three cell cycles after bivariate (Hoechst/ethidium bromide) analysis of quiescent astrocytes incubated for 48 h with serum-free medium (a) or stimulated in the presence of 10% serum (b). Table 1 represents the quantification of flow cytometry plots in Fig. 1 and shows the number of cells in each cell cycle. The vast majority of the cells kept in serum-free medium (94.13%) are in the G_0 phase; after serum-stimulation, however, there is an evident decrease in quiescent cells and an increase in cells in the S/G_2 phase of the first cell cycle as well as in cells which have already completed one or two rounds of cell division.

Acknowledgments

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Chapter 25

Quantification of Synaptic Structure Formation in Cocultures of Astrocytes and Hippocampal Neurons

Pamela J. Roqué, Marina Guizzetti, Gennaro Giordano, and Lucio G. Costa

Abstract

The ability to quantify changes of synaptic structure, whether associated with the formation of synapse in early development or the degeneration of synapses in adult life in an in vitro culture system, is important for understanding the underlying mechanisms. Astrocytes play a vital role in neuronal development and functioning, including synapse formation and stabilization. The method described in this chapter allows for the determination of the modulation by astrocytes of synaptic structure formation in hippocampal neurons. Using a sandwich coculture system, highly pure, hippocampal neurons are grown in culture for 14 days on glass coverslips, after which they are inverted, without contact, over separately cultured astrocytes or pretreated astrocytes for 24 h. Neuronal immunocytochemical staining of the presynaptic marker, synaptophysin, and the postsynaptic marker, PSD-95, is used to assess the localization of synaptic proteins into pre and postsynaptic structures. Deconvolved, confocal images are used to determine a mean puncta intensity threshold for use in rendering the surface of the synaptic structures using three-dimensional object analysis software. Once rendered in three-dimensional space, automatic quantification of the number of pre- and postsynaptic specializations and the number of those structures that overlap is obtained, allowing the ability to compare how different treatments may modulate the formation of synapses. Because synapses not only consist of distinct pre- and postsynaptic specializations, but are also defined by their apposition, the determination and study of synapse formation can only benefit by methods that use all of the available data to assess the actual structure.

Key words: Astrocytes, Confocal microscopy, Deconvolution, Three-dimensional analysis, Immunocytochemistry, Postsynaptic density protein-95, Synaptophysin, Hippocampal neurons

1. Introduction

The disruption of normal synaptic formation and function is often seen as an endpoint in neurological diseases. This is true for neurodevelopmental diseases, like autism or fetal alcohol syndrome (1, 2), as well as for many neurodegenerative diseases, like

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Alzheimer's and Parkinson's (3). Whether the process is synapse formation or synapse degradation, for the field of neurotoxicology, the ability to quantify changes in structural synaptic formation after the treatment with a toxicant may not only provide the well-needed information about the extent of effect, but may also provide clues to the underlying mechanisms.

Synaptogenesis is a complex, highly regulated process, involving numerous signaling molecules and ultimately resulting in not only the trafficking of distinct components to pre- and postsynaptic sites, but also the precise matching of those assembled structures to form a synapse (4).

The idea that neurons are the sole mediators of this process is changing rapidly, as recent findings are making it clear that they are not acting alone. Astrocytes, the most abundant cells in the brain, have been long thought of as merely supporting cells, but increasing evidence shows that with their fine processes in close spatial proximity to the synapse, astrocytes are important contributors to neuronal development and function (5, 6). Not only do astrocytes play a role in neuritogenesis and neuronal differentiation, but also the incubation of both retinal ganglion cells and hippocampal neurons with astrocytes causes a dramatic increase in synapse formation (7–9), suggesting that astrocyte-derived factors are an important part of this process. In fact, both astrocyte-released thrombospondin-1, an extracellular matrix protein, and cholesterol have been shown to play a role in this effect (10, 11). Due to their increasingly interesting role in promoting synapse formation and function, the ability to measure changes in synapse formation in a system, where the release of factors by astrocytes can be isolated and manipulated, is an important one.

The method presented here uses a coculture system, where astrocytes and neurons are cultured separately and introduced for a short time only, without contact, at a late time point so that the effect of astrocytes on synapse formation can be studied. Because neurons are never in direct contact with astrocytes, each culture system can be manipulated experimentally with treatment compounds without exposure to the other. Using this system, one can investigate how the soluble factors released by astrocytes promote the formation of synaptic structures in neurons and how the treatment of astroctyes mediates their effect (see Fig. 1).

There are numerous primary culturing methods that employ coculturing of neurons and astrocytes using a sandwich coculture (12–14). Most use astrocytes early on to aid in neuronal survival and development. Because the interest here is to determine the role of astrocytes in synaptogenesis, it becomes important to have a coculture system where the neurons are relatively pure and have been grown without the influence of astrocytes. Additionally, because the endpoint of interest is the study of synaptic formation, the neurons must be grown in a culture system that permits

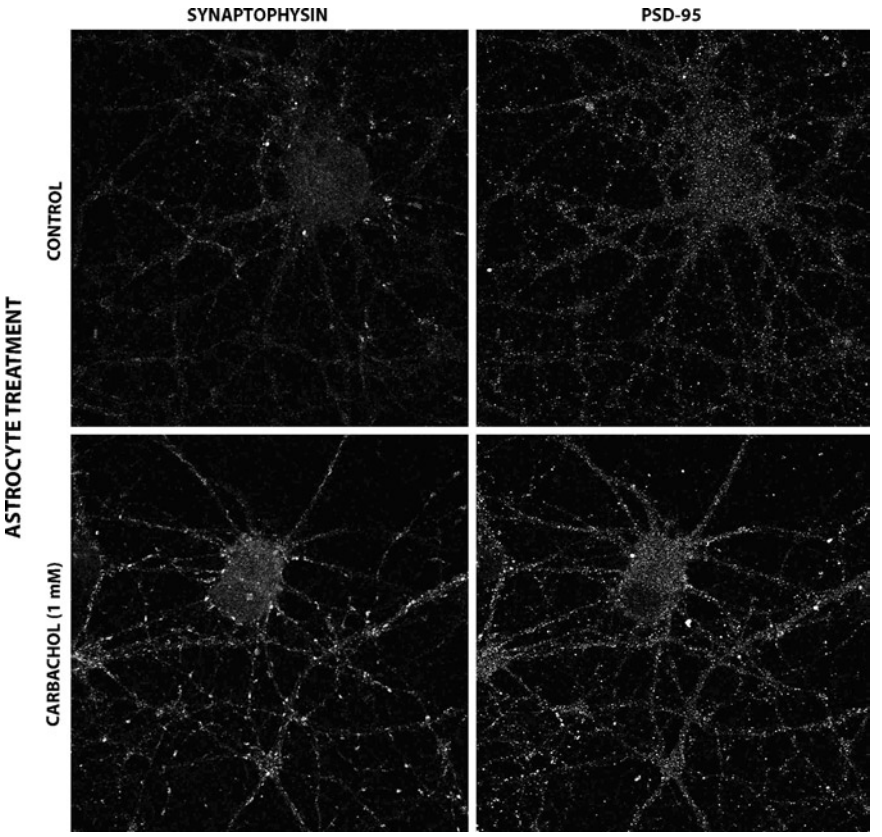

Fig. 1. Representative images of the effect of carbachol-treated astrocytes on the localization of pre- and postsynaptic proteins. Shown are representative single-slice deconvolved images of hippocampal neurons (14DIC) after the coculture for 24 h with carbachol (1 mM)-treated astrocytes. Treated astrocytes show an increase in localization of the pre- and postsynaptic proteins, synaptophysin, and PSD-95 after immunocytochemical staining, as evidenced by the increase in puncta intensity.

the development of extensive networks and allows them to reach a maturation stage where the process of synapse formation has begun, necessitating a long-term culture. While the sandwich culture described here incorporates many aspects of those previously developed systems, these culturing needs necessitate some modification.

This optimized method presented here produces a long-term growth environment for highly pure, primary hippocampal neurons that differentiate and develop a neuronal network and synaptic structures without the influence of astrocytes. It is one that is grown at a density high enough to support neuronal survival, but low enough to allow for the imaging of single cells for quantification.

To identify synaptic structures, immunocytochemistry is used to label the presynaptic protein synaptophysin and the postsynaptic protein, postsynaptic density-95 (PSD-95). Because of their

unique roles in their respective synaptic terminals, both synaptophysin, a vesicular protein that clusters at the presynaptic terminal (15), and PSD-95, an important scaffolding protein which anchors excitatory neurotransmitter receptors at the synaptic cleft (16), are useful in imaging studies and have been used extensively in the study of synapses (17, 18). With the proteins labeled, laser scanning confocal microscopy is used to identify the presence of these proteins in the neurons. Because confocal imaging enables the collection of signal in three dimensions, this information is collected throughout the depth of the neuronal extension, providing information not only about the location of proteins throughout the neuronal extensions, but also about the extent of localization into pre- or postsynaptic structures or puncta, with a higher concentration of protein yielding a more intense fluorescent signal. Additionally, since a defining quality of synapses is the apposition of pre- and postsynaptic structures, these images allow for the determination of where they overlap, and so yield quantitative information about synapse formation.

Inherent in the process of microscopy, when light waves move through various media, it bends and scatters, resulting in a slight blurring of the actual signal. To improve the resolution and contrast and reduce the noise in the acquired signals, the confocal images are deconvolved prior to analysis. Deconvolution uses an iterative algorithm that considers the acquired signal, the parameters of the microscope, and the refractive indices of the materials that light moves through, as each is capable of bending light to refocus the light that has scattered during imaging (19). The end result is a sharper image allowing for a more accurate assessment of signal in these images (see Fig. 2), and ultimately improving quantitative results.

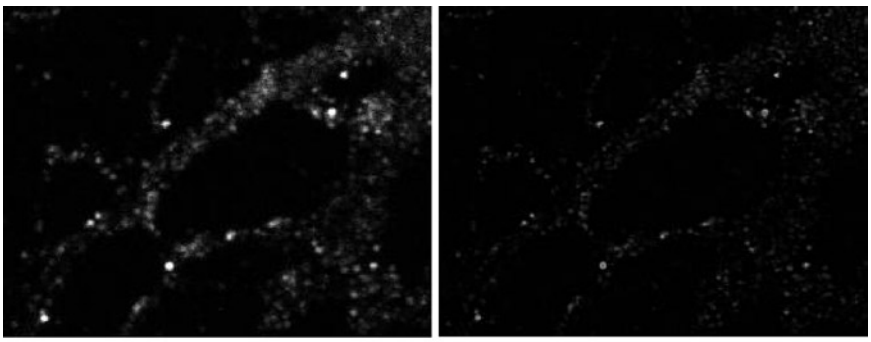

Fig. 2. Raw confocal image versus deconvolved confocal image. Shown are neurite extensions of a hippocampal neuron grown for 14 days in culture and immunolabeled for PSD-95. The wave nature of light results in diffraction and signal spreading in the acquired image. Without deconvolution (*left*), the fluorescence signal obtained from the microscope results in a somewhat cloudy and blurred image around the signal perimeter. After deconvolution (*right*), the signal is refocused and refined without a loss of relative intensity differences. Shown is a portion of one slice of a z-series taken from the red channel and enlarged to 200%.

There are numerous methods used to quantify the overlap of fluorescent signals, but when deciding which method to use for measurement, one should first consider the specific question being asked. Synapses are clearly objects with structure, consisting of not only clusters of distinct proteins localized into pre and postsynaptic terminals, but also the two structures in apposition forming a synapse. Because our interest is to quantify both the number of pre- and postsynaptic clusters and where they overlap, a three-dimensional method that defines structural objects is appropriate.

Three-dimensional object analysis uses all of the confocal information obtained and renders the surface of objects to show where they are located in three-dimensional space. This method of analysis improves on often-used methods that project the z-series of images into one two-dimensional plane. Projection of confocal stacks may introduce confounded results, since signals that are not near each other in vertical space may appear to be overlapping once projected into one plane. Additionally, colocalization, the analytical method that quantifies the overlap of labeled pixels from two different fluorescent channels, would provide information only about the overlap of individually labeled proteins without regard to the clustering or aggregation of those signals into structures. The determination and study of synapse formation can only benefit by methods that use all of the available data to assess the actual structure.

The three-dimensional object analysis program used here relies on an intensity threshold to automatically render the surface of objects. The outline of the surface is generated by connecting pixels that are at the intensity of the threshold value, or higher, with those adjacent pixels that meet the same parameters. Because the threshold has an effect on the dimensions of the objects, its determination is an important part of the analytical process (20). The method of threshold determination described in this chapter incorporates important concepts and tasks from a number of sources with modifications for this particular experimental question (20, 21). To determine the threshold, the mean intensities of 30 puncta from each channel in 15 different images are selected and automatically measured. Although the selection of puncta is manually determined, the overall sample size is quite high and results in a consistent three-dimensional reconstruction of objects that reflect what is seen in the confocal image (20) (see Fig. 3), an important consideration for object analysis thresholding.

Once the threshold is determined, the three-dimensional object analysis program is used to automatically calculate the number of pre- and postsynaptic puncta, and differences in how they overlap, offering intriguing information about how astroctyes promote the development of pre- and postsynaptic structures

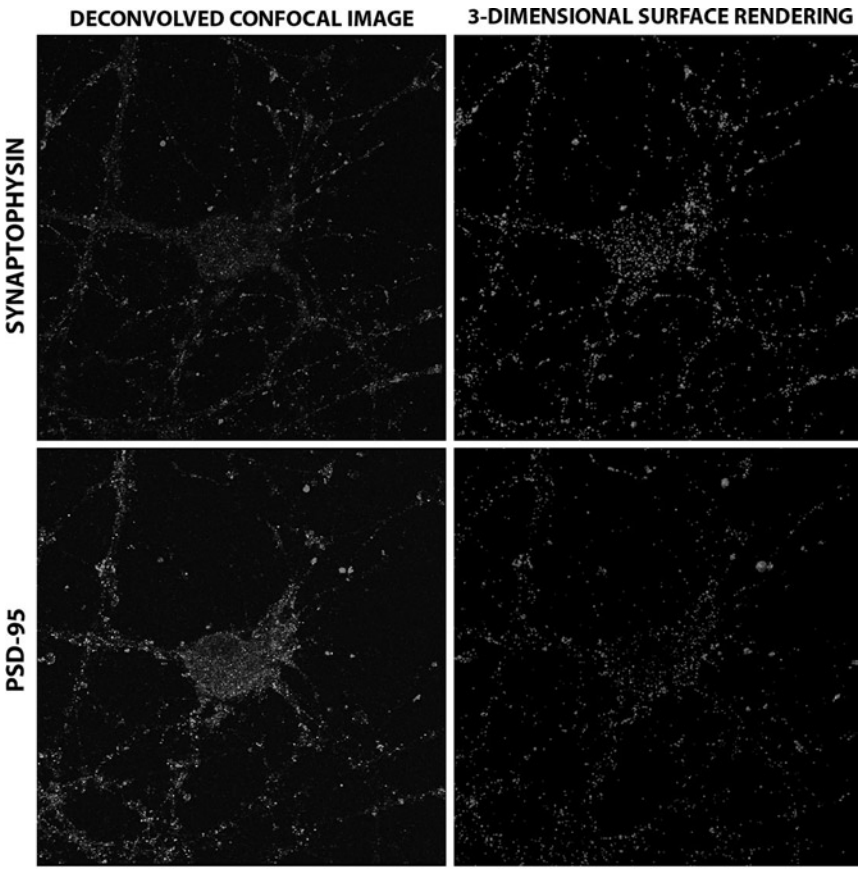

Fig. 3. Three-dimensional surface rendering of synaptic puncta after thresholding. The manual method used to generate a threshold for both the synaptophysin channel and the PSD-95 results in a three-dimensional surface rendering (*right*) that is reflective of the actual deconvolved confocal image (*left*). Shown is one slice of a deconvolved, confocal z-series, split by channel (*left column*) of a hippocampal neuron grown in culture for 14 days and cocultured with carbachol-treated astrocytes (1 mM) for 24 h. The neuron was labeled for the presynaptic protein, synaptophysin (*top left*), and the postsynaptic protein, PSD-95 (*bottom, left*). A total of 450 puncta per channel were manually selected from 15 images, and the mean intensities were averaged to determine the threshold value.

and their progression into synapses, as well as how the astroctye treatment may mediate that effect. The ability to quantify synaptic structure formation is important, and a coculture system of highly pure neurons cocultured with astrocytes allows for the ability to investigate the effect of astrocytes on synaptogenesis. It should be noted, however, that while this method quantifies the synaptic structure, additional information regarding the functionality of this structure could and should be gained by utilizing electrophysiological approaches.

2. Materials

2.1. Preparation of Neuron Coverslips

1. Glass coverslips for neuronal cultures: 12 mm, No. 1, round microscope glass coverslips (see Note 1).
2. Wax spacers: Paraffin wax is melted and wax spacers are attached to the glass coverslips using 1-mL syringes and 20 gauge, 1 in. needles.
3. 1 M hydrochloric acid (HCl) to clean (12) the glass coverslips; use 1 mL HCl per coverslip.
4. Coverslip substrate: 10 mg/mL poly-L-ornithine hydrobromide (PLO) dissolved in sterile water and stored at −20°C in 100 mL aliquots (see Note 2).

2.2. Hippocampal Neuron Preparation and Culture

1. Embryonic day twenty-one fetuses (E21) from Sprague-Dawley rats.
2. Parital Medium: Neurobasal-A Medium (Gibco) supplemented with 30 mM D − (+) glucose solution (30 mM), 0.5% fungizone, 100 μg/mL gentamicin, and 1% B-27 Supplement.
3. Complete Medium: Add 3 mM GlutaMax to the partial medium no more than a day prior to its use during the neuronal dissection and plating (see Note 3).
4. Hank's Balanced Salt Solution (HBSS) supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES) buffer solution (HBSS + HEPES).
5. Calcium- and magnesium (CM)-free HBSS supplemented with 10 mM HEPES buffer solution (HBSS − CM-free + HEPES).
6. Dissolve 4 mg/mL deoxyribonuclease-1 from bovine pancreas (DNase) in HBSS − CM-free + HEPES, sterile filter, and store in 4 mL aliquots at −20°C. This stock solution is added to the neuronal digestion solution as required.
7. Enzymatic Digestion Solution: Prepare 2 mg/mL papain from papaya latex at the start of the hippocampal dissection by dissolving the powder in 4 mL of HBSS − CM-free + HEPES. DNase (1:500) and 5 mM sterile magnesium chloride are added to the solution. The enzyme mixture is gently inverted two or three times to mix and placed in a 36°C water bath until use to assist in solubilization of the papain. This enzymatic solution will be sterile-filtered immediately prior to use. Papain powder is stored at −20°C (see Note 4).
8. From a 1 mM stock of cytosine β-d-arabinofuranoside (ARAC) in HBSS + HEPES, make a working stock of 100 μM ARAC in HBSS + HEPES and sterile filter. Store at −20°C.

2.3. Cortical Astrocyte Preparation and Culture

1. HBSS without calcium chloride or magnesium sulfate and supplemented with 10 mM HEPES buffer solution (HBSS – CM-free + HEPES).
2. Dulbecco's Modified Eagle's Medium (DMEM) with low glucose supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin and 100 µg/mL streptomycin (P/S).
3. 0.25% trypsin in dH_2O; sterile filter and store at –20°C in 20 mL aliquots (see Note 5).
4. 100-µm nylon mesh filter.
5. Sterile 75-cm^2 flasks precoated with 40 µg/mL poly-D-lysine (PDL); prepare PDL in dH_2O, sterile filter, and store at 4°C (see Note 6).

2.4. Cortical Astrocyte Plating for Coculture

1. Established culture of primary astrocytes (*see* Subheadings 2.3 and 3.3) between 1- and 2-weeks old (see Note 7), passed at a density of 0.250×10^6 per well of a 24-well plate (see Note 8) using DMEM-FBS (see the Subheading 2.3).
2. 0.25% trypsin in dH_2O, sterile-filtered, and stored at –20°C.
3. Wells of a 24-well plate coated with 40 µg/mL sterile-filtered PDL in dH_2O. Store PDL at 4°C.

2.5. Astrocyte–Neuron Coculture

1. Serum-deprivation media: DMEM supplemented with 0.1% albumin from bovine serum (BSA) and P/S (DMEM-BSA) (see Note 9).

2.6. Neuronal Immunocytochemistry

1. Cell Fixation: Add 4% (w/v) paraformaldehyde (PFA) to HBSS + HEPES with a stir bar. Use care when preparing and working with the solution, as PFA is toxic. Prepare in a fume hood and dispose of used PFA and pipettes as hazardous waste. Cover the solution with aluminum foil and heat, stirring, in a fume hood to 70°C. Add one drop of 1 M NaOH; continue heating until 80°C is reached. At this temperature, the solution should clear; lower the heat and continue stirring for 5 min. Place in a covered glass bottle and let cool to room temperature. Keeping covered with aluminum foil, adjust the solution to pH 7.4. Filter to remove nondissolved particles. Store for a maximum of 2 weeks at 4°C in a covered glass container clearly labeled as toxic. Prior to each use, adjust the pH to 7.4 and warm to 36°C.
2. Blocking and Permeabilization Solution: Add 5% BSA and 0.1% iso-octylphenoxy-polyethoxyethanol (Triton-X) to phosphate buffered saline (PBS) solution. Prepare 1 mL per coverslip, plus 5 extra mL to ensure an adequate volume.
3. Day 1 Wash Solution: Prepare 1 mL PBS per coverslip supplemented with 0.05% Triton-X.

4. Primary Antibodies: Rabbit monoclonal antibody to synaptophysin (Abcam). Mouse monoclonal antibody to PSD-95 (Affinity Bioreagents). Aliquot stock to smaller volumes and store both antibodies at −20°C.
5. Primary Antibody Dilution Buffer: Prepare the primary antibody dilution buffer using PBS supplemented with 1% BSA. Add the primary antibodies (Synaptophysin, 1:200; PSD-95, 1:250). To conserve the primary antibody, prepare 100 µL per coverslip plus an additional 100 µL.
6. Day 2 Wash Buffer: PBS supplemented with 0.1% Triton-X. Prepare enough Day 2 wash buffer to complete six washes. Each wash will require 1 mL/coverslip.
7. Secondary Antibody Dilution Buffer: Secondary antibodies contain fluorophores which are light-sensitive, so prepare dilution buffer in the dark. Add both donkey antirabbit Alexa 488 and donkey antimouse Alexa 555 (1:500 each) to PBS in a volume of 100 µL of PBS per coverslip plus 100 additional microliters (see Note 10). Store the antibody stock at 4°C in the dark.
8. Nuclear Stain: Prepare the nuclear stain fresh before each use by adding 1 µg/mL trihydrochloride, trihydrate (Hoechts 33342) dH_2O. Hoechts 33342 is light-sensitive, so work in the dark. Prepare enough working solution so that 200 µL per coverslip can be used (see Note 11). Store the stock solution at 4°C in a light-resistant container for 4–12 months.
9. Glass microscope slides: 75 × 25 × 1 mm.
10. Glass microscope cover glass: 22 × 40 mm, No. 1½.
11. Mounting medium: Vectashield mounting medium for fluorescence (Vector Laboratories). Store the stock solution at 4°C in darkness.
12. Coverslip sealant: Nail polish.

2.7. Neuronal Confocal Imaging

Confocal laser-scanning microscope: This protocol uses the Olympus Fluoview-1000, but successful imaging is not limited to this particular microscope. To obtain images to proceed with this analysis, the following parameters must be met: lasers that excite at 405 nm (blue emission), 488 nm (green emission), and 561 nm (red emission), a 60× oil immersion objective for high-resolution imaging, the ability to acquire signal from each channel sequentially, and an averaging option to reduce noise (see Note 12).

2.8. Deconvolution of Confocal Images

1. Deconvolution Software: There are many programs that perform deconvolution of images. This protocol uses Huygens Professional (Scientific Volume Imaging, Hilversum, the Netherlands, http://www.svi.nl). Regardless of the program used, a point-spread function (PSF) is generated for the iterative

algorithm used to deconvolve the image. For this determination, programs require information about the parameters of the imaging milieu, including the numerical aperture (NA) of the objective, the type of microscope (confocal), the sample size and sampling interval, plus the refractive indexes of the immersion and mounting medium (see Note 13).

2.9. Thresholding of Pre- and Postsynaptic Puncta: ImageJ Software

1. Java image analysis program, ImageJ, (available at http://rsbweb.nih.gov/ij/index.html). Be sure to include the appropriate plug-ins necessary for the importation of microscope-specific file formats (see Note 14).

2.10. Three-Dimensional Image Analysis

1. 3D Image Analysis Software: Object Analyzer from Huygens Professional, Scientific Volume Imaging.

3. Methods

The process of assessing the effect of astrocytes on hippocampal neuron synaptogenesis using this coculture system requires the management of two distinct culture systems, each of which requires specific tasks that occur at different times throughout the overall experimental time-span (see Table 1). Briefly, primary hippocampal neurons are grown for 14 days in culture, and established astrocyte primary cultures are passed 1 week prior to coculturing with the neurons. Both the culture systems require additional steps in the days prior to either the neuronal preparation or astrocyte plating. While the methods described here allow for the determination of the effect of astrocyte-secreted factors on synaptic structure formation, astrocytes may also be treated with an experimental compound to determine how that treatment may mediate the astroctye synaptogenic effect (see Note 15). Each method of the process is presented in detail below. The experimental plan shown in Table 1 combines the major methods and treatments necessary for each culture in a simplified day-to-day task list.

3.1. Preparation of the Coverslips

1. Two days prior to the neuronal preparation, melt the paraffin wax in a small beaker at high heat, stirring constantly. Work with caution while using the molten wax; wear goggles. Use a warmed needle and syringe to place four small, round wax balls, spaced evenly and close to the edge of the coverslip (see Note 16). Make three coverslips per treatment group. Proceed through all of the coverslips and ensure that each ball is adhered by touching each with tweezers. Add additional wax balls as needed.
2. Using tweezers, place the coverslips, with spacers facing up, in the wells of a 24-well plate. Add 1 mL of 1 M HCl to each

Table 1
Experimental timeline: Coculture of primary hippocampal neurons and primary cortical astrocytes

			0–1 *Week prior*			
			A: Culture astrocytes			
			Day 0	*Day 1*	*Day 2*	*Day 3*
	N: Prepare coverslips	N: Wash and coat coverslips	N: Culture primary neurons	N: Change neuronal media	N: Treat with ARAC	
Day 4	*Day 5*	*Day 6*	*Day 7*	*Day 8*	*Day 9*	*Day 10*
	N: Change 1/3 neuronal media		A: Pass astrocytes	A: Change media	A: Change media N: Change 1/3 media	
Day 11	*Day 12*	*Day 13*	*Day 14*	*After Day 14*		
A: Serum-deprive	A: (Optional: treat astrocytes)	A: (Optional: stop treatment)	A and N: Stop coculture	Complete immunocytochemistry		
	N: Change 1/3 media	A and N: Coculture	Fix with PFA	Mount		
			Store in PBS or incubate in 1° Ab	Image, deconvolve, and analyze		

Day 0 day of neuronal preparation, *N* neuron culture task, *A* astrocyte culture task, *ARAC* cytosine β-D-arabinofuranoside, *Ab* antibody

coverslip to clean the glass (10, 12). To ensure complete coverage and no floating (13), use a sterile needle to push the coverslip to the bottom of the well. Place the plates in a sterile hood overnight.

3. On the day prior to the hippocampal preparation, wash the coverslips with sterile water five times for 30 min each time, ensuring at each step that the coverslips are submerged using the sterile needle. Coat coverslips with 1 mL of filtered 0.015 mg/mL poly-L-ornithine. Place the plates in a 36°C incubator with 5% CO_2 overnight.

3.2. Hippocampal Neuron Preparation

1. Remove the coverslips from the incubator and wash them twice with sterile water. Replace with HBSS + HEPES. Leave in a laminar flow hood at room temperature until neuronal seeding (see Note 17).

2. Prepare the Complete Medium. Add 3 mM GlutaMax® to the previously prepared Partial Media and warm in a 36°C water bath until use.
3. Prepare the enzymatic tissue digestion solution. Store in water bath until use.
4. Under sterile conditions, dissect the hippocampi from E21 rat pups. Place the tissue in a 35-mm dish over ice, containing 1.5 mL of HBSS – CM-free + HBSS.
5. Remove the papain enzymatic solution from the water bath and gently mix by inversion. Sterile filter.
6. With a pipette, carefully remove as much extra HBSS as possible from the 35-mm plate without losing the tissue. Using sterile surgical scissors, begin the dissociation process by cutting the tissue into small pieces. Transfer the tissue to a 15-mL conical tube using the papain solution to wash the plate. Add the remaining papain solution to the tissue; invert gently to mix. Place in a 36°C water bath for 30 min to digest the tissue. Check the cells after 10 min and add DNase (1:500), if necessary (see Note 18). Return the tube to the water bath for the remaining 20 min.
7. Centrifuge the cells at approximately $150\times g$ at 4°C for 5 min.
8. Trituration of Tissue: Aspirate off the papain solution and replace with 2 mL of warmed Complete Medium. Add DNase (1:500), if necessary (see Note 19). Triturate with a long, glass Pasteur pipette (see Note 20) and rubber bulb gently for 15 times, taking up the cells from the bottom of the conical tube and returning them along the side of the tube to minimize harm to the fragile cells. Let the cells settle for 2 min.
9. Filter the supernatant, which now contains dissociated, suspended cells, through a 40-μm nylon filter into a 50-mL sterile tube taking care not to disturb the settled tissue.
10. Add 2 mL of Complete Medium to the remaining tissue; add DNase, if necessary (see Note 19). Cut off a small portion of the tip of a 200-μL pipette to widen the mouth and attach it to a Pasteur pipette so that it is secure, but not too harshly that the glass tip breaks (see Note 21). Triturate the tissue as before (15 times, gently). Let the remaining tissues settle for 2 min. Filter the supernatant, adding it to the previously filtered cells.
11. If the tissue remains, add 2 mL of complete medium and DNAse, if necessary (see Note 19). Using a 1,000-μL pipette and tip, resuspend 15 times, as before, or until the tissue is no longer visible. Combine the cells with others by passing through the filter.

12. Transfer the filtered cells to a 15-mL tube and add more medium until a final volume of 10 mL is reached.
13. Centrifuge at approximately 150 × *g* at 4°C for 5 min.
14. Resuspend gently in 10 mL of Complete Medium and count both live and dead cells using trypan blue (see Note 22).
15. Seed each coverslip at a density of 80,000 cells/mL (see Note 23). When plating the neurons, do so gently; release the volume slowly against the side of the well, not directly into the well. Ensure that the coverslip is not floating.
16. Culture in the incubator (37°C, 5% CO_2) overnight (see Note 24). In the morning, wash the cells one time with warmed HBSS + HEPES (1 mL) and replace with 1 mL of Complete Medium.
17. To increase the neuronal purity of the culture, add ARAC, at a final concentration of 2.5 μM, to neuronal cultures after 2–3 days (see Note 25).
18. Every 4–5 days, remove one-third of the neuronal media and replace with warmed Complete Media (see Note 26).

3.3. Cortical Astrocyte Preparation

1. Remove the fetuses (E21) under aseptic conditions. Working in a sterile hood, decapitate one animal at a time. Remove the cerebral hemisphere and place on sterile filter paper and dissect away the mid-brain and olfactory bulbs. Isolate the cortex and carefully remove the meninges (see Note 27). Place tissue in a 100-mm plate containing 5 mL of HBSS-CM-free + HEPES.
2. After collecting the cortices from the entire litter, cut the tissue into small pieces using sterile surgical scissors. Pipette the solution of tissue into a 50-mL conical tube containing 20 mL of 0.25% trypsin. Place the conical tube in a 37°C incubator for 10 min.
3. To stop the enzymatic reaction, add 20 mL of the astrocyte culture medium (DMEM-FBS), mixing well by pipette. Centrifuge the solution at 250 × *g* at 25°C for 10 min.
4. Aspirate off the supernatant and resuspend the pellet of cells in 20 mL of DMEM-FBS. Vortex the conical tube at a maximum speed for 1 min (see Note 28). Centrifuge for 10 min using the same settings.
5. Discard the supernatant and resuspend in 20 mL of DMEM-FBS and centrifuge for 10 min at the same settings. Repeat this step 3 additional times.
6. Remove the supernatant and resuspend in 20 mL of the same medium. Filter the solution of cells through a 100-μm pore nylon mesh filter into a sterile 50-mL conical tube to remove aggregated cells.

7. Count the cells and plate 4×10^6 cells in 40 μg/mL PDL-coated 75-cm^2 flasks in a total of 10 mL DMEM-FBS. Incubate the culture at 37°C in a humidified atmosphere of 5% CO_2.
8. Replace the medium the next day, after washing twice with PBS. Thereafter, change the medium every 2–3 days until the cells are confluent, which is usually 9 days (see Note 29).

3.4. Cortical Astrocyte Passage for Coculturing

1. Coat enough wells of a 24-well plate with sterile-filtered 40 μg/mL PDL for 10 min to obtain one well per neuronal coverslip.
2. Wash once with sterile water. Replace with 1 mL PBS until plating.
3. Aspirate the medium from each flask to be passed and wash twice with 10 mL PBS, rocking PBS over the bottom of the flask.
4. Add 2 mL of warmed 0.25% trypsin; incubate for 10 min at 36°C.
5. Holding the flask in one hand, hit the sides of it five to ten times with the other hand to aid in the release of the cells. Add 8 mL of medium (DMEM + FBS) to each flask to stop the enzymatic trypsin reaction.
6. Rinse the flask by pipetting eight to ten times; transfer to a 15-mL tube.
7. Spin for 10 min at $250 \times g$ at 25°C.
8. Resuspend the pellet in 10 mL of medium and count the cells with a hemocytometer (see Note 30).
9. Plate 1 mL of cells in each coated well of the 24-well plate at a density of 250,000 cells/mL and incubate.
10. The following day, wash the cells one time with PBS and replace with warmed DMEM + FBS.
11. Replace the medium the following day.
12. Forty-eight hours before coculturing or 24 h prior to the treatment of the astrocytes, serum-deprived astrocytes (see Experimental Plan, Table 1). Wash them two times with PBS and replace the medium with warmed DMEM + BSA. Culture in an incubator for 24 h (see Note 31).
13. Using warmed DMEM-BSA, replace the medium if astrocyte treatment is not being performed or prepare the treatment compounds in DMEM-BSA and treat the astrocytes. Incubate the astrocytes for 24 h.

3.5. Coculture of Astrocytes and Neurons

1. Prior to coculture or after the optional 24 h treatment of astrocytes, wash the astrocytes twice with PBS and replace with DMEM + BSA, the serum-deprived medium. Allow the

astrocytes to condition the medium for 3 h prior to coculture with neurons (see Note 32).

2. At the end of 3 h, wash the neurons two times with warmed HBSS + HEPES and carefully remove each coverslip using a needle to lift the edge of the coverslip and tweezers to grasp the coverslip at the edge. Use care when tweezing the coverslip as you do not want to disturb the growing neurons. Invert the coverslip over the astrocytes so that the neurons are facing the astrocytes but are separated by the paraffin spacers. Do this carefully, placing one edge down and letting the coverslip fall gently over the monolayer to minimize the damage to the confluent layer of astrocytes.
3. Incubate together at 36°C for 24 h.

3.6. Neuronal Immunocytochemistry

1. After the 24 h coculture of astrocytes and neurons with tweezers, carefully transfer the coverslips, face up, to a new 24-well plate containing 1 mL of warmed HBSS + HEPES per well.
2. Wash two times gently with HBSS and fix the cultures using 1 mL of 4% PFA per coverslip. PFA is toxic, so work in a fume hood and treat as hazardous waste. Incubate at 36°C for 20 min. Remove the PFA from the wells under the hood and dispose of hazardous waste. Replace with PBS and wash for 5 min with agitation at room temperature. Repeat the PBS wash. Carefully remove the paraffin wax spacers (see Note 33). Wash one more time for 5 min to remove any wax residue released from the spacers during removal (see Note 34).
3. Block and permeabilize cells with PBS supplemented with 5% BSA and 0.1% Triton-X for 1 h at room temperature on a rocker.
4. Wash one time for 5 min in PBS with 0.1% Triton-X on a rocker at room temperature.
5. Prepare the primary antibody solution, and in a new 24-well plate, dispense 95 μL of primary antibody solution for each coverslip. Carefully lift the coverslip from the wash solution using a needle and tweezers as during the coculturing (see Subheading 3.5, step 2). While holding carefully, blot the edge of the coverslip on a Kimwipe to remove the excess liquid, as additional liquid can dilute the antibody concentration. Invert the coverslips carefully so that the neurons are floating face down on top of the primary antibody buffer previously placed in the wells. To avoid damage to the neurons, do not force the coverslip down (see Note 35). Wrap the plate in parafilm and place on a slow rocker at 4°C overnight, ideally for 18 h or more.
6. Secondary Antibody Staining: Remove coverslips from primary antibody solution and place in Day 2 Wash Buffer with

the neuronal side face up. Wash three times for 5 min on a rocker at room temperature.

7. As the secondary antibodies are light-sensitive, the remaining steps should be performed in the dark. Prepare the Secondary Antibody Solution and microcentrifuge at the highest speed for 3 min. Dispense 95 µL per coverslip, avoiding the pellet.
8. Remove the glass coverslips as before and blot the edge on a Kimwipe. Place the coverslip neuron-side-down, inverting it over the Secondary Antibody Solution previously dispensed. Wrap the plate in aluminum foil and incubate for 1 h at room temperature with light rocking (see Note 36).
9. Remove the coverslips and place them neuron-side-up in 24-well plate containing 1 mL of Day 2 Wash Buffer. Wash three times for 5 min each on a rocker at room temperature.
10. Counter stain with Hoechts solution by applying 200 µL per coverslip on top of the face-up coverslips. Place the foil-covered plate on a rocker for 10 min.
11. Wash twice for 5 min each time with dH_2O.
12. Mount the coverslips on slides in the dark. Remove the coverslips and blot on a Kimwipe to remove excess water. Stagger the three coverslips of one treatment group, neuron-side-up, on a glass slide so that a cover glass will fit over them completely. Add one drop of Vectashield mounting medium to each coverslip and place the rectangular coverglass over the fixed neurons (see Note 37). Apply nail polish to the perimeter of the coverglass to both stabilize it and seal the aqueous mounting medium.
13. Store the mounted slides at 4°C in light-protected slide case. Image as soon as possible to minimize the loss of signal with time.

3.7. Neuronal Confocal Imaging

1. Using a 60× oil immersion lens, set the field size as 1,024 × 1,024 pixels with a two times zoom. These parameters yield a 0.103 µm per pixel resolution and provide a field close enough for the imaging of one cell without the inclusion of its neighbors. This resolution is effective for thresholding and postimaging analysis. With the lasers set to the lowest possible intensity, use the slide with the brightest signal to set the detector gain parameters as per the microscope instructions so that all the signal intensity values will be captured (21) (see Note 38). Hold these settings constant throughout the experiment so that all signal intensity values are captured.
2. Using epifluorescence to excite the Hoechts stain, identify the healthy cells that are in similar environments and are at least two cell bodies apart. For each treatment group, image at least five healthy cells per coverslip for a total of 15 fields,

each containing one cell per field. To minimize the bleed-through of overlapping spectra, acquire the image sequentially, using a 488-nm laser to excite the labeled synaptophysin protein (green) and a 561-nm laser to excite the labeled PSD-95 protein (red).

3. Use a 0.3-μm step size and set the z-series distance. Start the series just below the point where the extensions are visible on the coverslip, and end the series one step above, where the last signal from the extensions disappears (see Note 39).
4. Acquire the z-series images from the green and red channels and save.
5. Without the 488- and 562-nm lasers, turn on the 405-nm (blue) laser and acquire one plane of the labeled nuclei (see Note 40).

3.8. Image Deconvolution

1. Using deconvolution software, enter the appropriate parameters used to acquire the fields, including the numerical aperture of the objective, the type of microscopy performed, the refractive indexes of the immersion oil, and the mounting medium. Enter the laser information as requested, as well as the microscope parameters. Using a theoretical algorithm, set the signal-to-noise ratio (see Note 41) and the number of iterations to 40. Load the images to be deconvolved and set the batch processor to begin.

3.9. Image Thresholding

1. Determine the threshold for each channel separately using the ImageJ program. From the group of coverslips that were used to set the gain for confocal imaging, import one deconvolved stack of images. When prompted, split the channels so that a threshold can be obtained for each. Import them in grayscale; do not select the "RBG Colorize Channels" option (see Note 42). Each channel will open in a separate window and include the stack of images acquired for that channel. Scroll to the slice located in the middle of each stack.
2. Enlarge each channel to 200% for a more accurate selection of puncta.
3. Using the round selection tool, choose the brush option and set it to the number of pixels that would fit the average of the smallest-sized puncta of the two channels (18). This value is usually two to three pixels, with the red channel or the PSD-95 puncta being the smallest (see Note 43).
4. On the middle image in the stack of one channel, starting at about 5 μm from the cell body, manually select ten puncta on three different extensions for a total of 30 puncta per imaged field. Select those puncta that are well-clustered and distinct, being sure to select a representative sample of intensities, not simply those that are the brightest (see Note 44).

5. After 30 puncta have been selected, add them to the Region of Interest (ROI) Manger. Save the ROI, renaming it to conserve the image and channel information (see Note 45).
6. Using the ROI Manger, split the renamed ROI into its 30 distinct selections. Delete the original ROI and measure the mean intensity of each selection separately. Save the results in a file that retains the image and channel information of the field.
7. Repeat the process on the second channel. Continue until all of the fields of the treatment group used to set the confocal parameters have been completed and the results saved.
8. Combine all of the mean intensities obtained for each channel into one spreadsheet and determine the threshold of each channel separately by averaging the puncta intensity values. This value will be used during the three-dimensional analysis (see Note 46).

3.10. Three-Dimensional Object Analysis

1. Open a deconvolved file in Huygens Professional and select the Object Analyzer.
2. Set the threshold of each channel using the values obtained in the Subheading 3.9 (see Note 47).
3. Record the number of starting puncta in each channel after setting the threshold.
4. Filter out the nonoverlapping puncta and record the number of puncta remaining in each channel.
5. Of the overlapping puncta that remain, use a filter to determine how many pair with puncta from the second channel. Record this value, as it reflects the number of synaptophysin and PSD-95 structures that are in apposition to one another, suggesting the number of synapses.
6. Holding the threshold value constant for all treatment groups, repeat the process until each field has been analyzed.
7. Determine the average number of starting puncta for each channel in each treatment group, as well as the average number of overlapping pairs.
8. Graph and compare the ratios of each treatment group compared to control. Perform statistical analysis (see Note 48).

4. Notes

1. It has been reported that the quality of the glass coverslips is important for neuronal attachment, growth, and ultimate survival. These particular coverslips have been reported to be of consistent quality and work well for primary neuron

cultures (12). If coverslips from other manufacturers are used, these factors must be taken into consideration, especially if the cultures do not adhere, cells are lost after washing, or the cultures do not survive. While there are many factors that may contribute to unsuccessful outcomes in this process, this may be one factor that is controllable.

2. It is important to avoid excessive freeze and rethaw of the stock concentration to ensure a consistent concentration of the substrate. With each thaw, the hydrophobic poly-L-ornithine will adhere to the storage Eppendorf and affect the consistency of the substrate concentration between experiments, and ultimately the attachment of cells and extent of neuronal extension development.
3. GlutaMax allows for a controlled release of L-glutamine, a necessary component of neuronal growth and development, into the culture. When L-glutamine is added to medium in its pure form, it rapidly degrades to ammonia, which is toxic to cells and so ultimately affects the cell viability. The GlutaMax supplement, however, allows for the gradual release of L-glutamine and L-alanine from a dipeptide and so reduces the production of ammonia (22). However, since GlutaMax does eventually break down to L-glutamine, it is reasonable to assume that in stored media, without the metabolic activity of cells to control the slow release of ammonia, it may accumulate. Due to the sensitivity of primary neurons, GlutaMax is added to the partial media just prior to its use in the neuronal preparation as a precaution. The volume of Complete Media necessary for the neuronal preparation depends on the overall experimental plan. One milliliter of medium per coverslip is used for plating cells, and each preparation of one litter of pups requires approximately 25 mL of medium to complete the cellular dissociation. Additionally, the medium is replaced the day following the initial plating, and it is helpful to include that volume when preparing the Complete Medium. Overall, ~25 mL plus two times the volume needed for the coverslip plating should be a sufficient volume to prepare. Adding a few more milliliters is helpful to account for pipetting error. Keep the aliquots of the medium covered while working to ensure that the pH is maintained (10), and store any medium remaining after the preparation at 4°C.
4. Papain is used for this process instead of other commonly used digestive enzymes, such as trypsin, because it is less damaging to the sensitive primary neurons and more effective. Initially, the papain in the HBSS will appear insoluble, but will solubilize during the time spent in the water bath while the hippocampal dissection is completed. This volume of

digestion solution (4 mL) is sufficient for the hippocampi of a litter of pups, up to 14 or 15, and has been effective without causing unacceptable cell death with tissue from as few as five pups. This neuronal preparation yields on average about 1×10^6 cells per pup. If the experimental plan requires more cells than are obtainable from one litter, the volume of the enzymatic solution should be increased for effective dissociation.

5. Avoid more than three freeze and rethaws of trypsin aliquots. Thawing will cause the enzyme to weaken and effectively reduce the yield of astrocytes obtained per flask.
6. Coating for each flask will require 10 mL of PDL. Cortical astrocyte passage (see Subheadings 2.4 and 3.4) will require 1 mL of PDL stock per well. The volume of the stock solution stored at 4°C will depend on the individual experimental design and the need for astrocytes. The stock should be stored in a glass container for no longer than 1 week.
7. The number of flasks necessary will depend on the experimental design and the confluence of the available astrocyte culture flasks. Once plated for coculturing, the astrocytes will remain in the experimental culture for one additional week. Using cells older than two weeks in culture may affect the response, the viability of the astrocytes, and the number of nonastrocytic cells. As these cultures age, the number of both microglia and fibroblasts increases. It is important to visualize each flask before use to verify that the culture is astrocyte-rich and suitable for use.
8. The number of flasks necessary for passage depends on the age and confluence of the astrocyte primary cultures, as well as the freshness of the trypsin used to release the cells.
9. The described method includes an optional 24 h astrocyte treatment. If the astrocytes are to be treated with an experimental compound, the treatment solutions should be prepared in DMEM-BSA at the time of use and sterile-filtered prior to use. After 24 h, astrocytes should be washed twice with PBS and DMEM-BSA medium replaced. The astrocytes should be allowed to condition this media for 3 h prior to coculture.
10. While the method has been optimized for these specific secondary antibodies, using donkey antirabbit Alexa 555 and donkey antimouse 488 would not be expected to cause a problem. Microcentrifuge either the prepared secondary antibody buffer solution or the stock solution of the secondary antibody vials to minimize nonspecific staining. Over time, the antibody fluors form particles that are difficult to break up and can be spun out of the buffer in this way. Although it is not visible, avoid the pellet when dispensing the secondary antibody solution to wells.

11. For accuracy, it may be necessary to make a larger volume of the nuclear stain solution.
12. Sequential scanning of each channel using separate lasers is necessary to reduce bleed-through of fluorophores that overlap in their excitation or emissions signal. By imaging each channel separately, you are reducing the artifacts that will be collected if they are imaged at the same time (23). Alternately, to reduce the amount of overlap between fluorophores, choose two fluors that have greater separation between their signals. Invitrogen offers a helpful, interactive program called, SpectralViewer, which can be found at its Web site (http://www.invitrogen.com/site/us/en/home/support/Research-Tools/Fluorescence-SpectraViewer.html). It provides information about the extent of excitation and emission wavelength overlap. Additionally, it is important to ensure that bleed-through, nonspecific staining or autofluorescence do not produce artifacts during signal detection. To do this, prepare staining controls by including additional neuronal coverslips for use during the immunocytochemistry steps. During the staining process, incubate one coverglass containing neurons separately with primary and secondary antibodies. To detect autofluorescence, include an additional coverglass with no antibody added. Visualize these slides and if signal is detected, take measures to reduce this nonspecific staining (23). For a thorough explanation of staining techniques, confocal imaging, fluorescence, and deconvolution, see *The Handbook of Confocal Microscopy*, edited by James W. Pawley (24).
13. The values of these parameters depend on the specifics of the objective or immersion oil used. It is important for successful deconvolution that you obtain this information, which is normally readily available from the manufacturer.
14. Many commonly used plug-ins, developed by users to perform specific functions, are included in the initial download, including those used to open file formats specific to the Olympus Fluoview-1000 confocal microscope. If files obtained from your microscope are unable to be imported, browse through the Web sites plug-in list and download one that will open your particular file format. There are plug-ins for files generated by both Zeiss and Leica microscopes, as well as others. While ImageJ will be used for the determination of the threshold for each channel, the program has numerous applications. Be sure to explore the Web site and plug-in list for additional information and applications that may be of use to you.
15. The experimental plan (Table 1) and methods section (Subheading 3) include instructions for a 24 h astrocyte

treatment, where neurons are never exposed to the treatment compound. These details could easily be modified to fit your experimental plan, and these cultures may be used to assess the effect of toxicants on neurons alone, without the coculture. This 24 h coculture is successful, but longer cocultures have not been attempted.

16. These act as spacers to separate the neurons and astrocytes and have been commonly used in the "sandwich" coculture system (12–14). Attempt to be consistent with the size of the wax spacers, both between coverslips and between experiments, for consistent analysis. Additionally, these balls are removed just prior to the mounting on slides for imaging; it is helpful if they are as round as possible, i.e., they are secure enough to last through the time in culture, but are also able to be removed cleanly. When round, there is less surface area touching the glass coverslip which will help minimize the damage to the neurons during removal and ensure that the final cover glass will rest as flat as possible. If wax remains and the glass does not lie flat, imaging will be more difficult. When dispensing the wax, it helps to have a very hot needle and syringe. Use the solution of molten wax to warm the needle for a few seconds and when warmed, pull approximately 0.200 mL of wax into the syringe and dispense it back into the beaker about three to four times quickly to warm the syringe. A similar volume should be used for dispensing; if more is used, it will solidify rapidly and make dispensing not only difficult, but also unsuccessful as the wax may be too cool to adhere to the glass. Usually, three to four wax balls for each volume of wax will retain an appropriate temperature that when dispensed will result in a strong enough connection to retain their hold throughout the experiment. At the end of each dispensing session, quickly return any wax remaining in the syringe to the beaker. With the needle heating in the wax, reheat the syringe by filling and emptying it as before. To achieve the roundness of the wax ball, dispense a small drop at the tip of the needle and tap it onto the glass coverslip. Obtaining relatively standard-sized spacers and understanding the behavior of molten wax as a material will come with practice. Coverslips can be made in advance and stored in a covered container until needed.

17. Water may be cytotoxic to neurons, so it is important to remove as much as possible and use an isotonic solution after the washes. Some methods use PBS or complete media after the final rinse. Both medium and HBSS + CM + HEPES have been used in this protocol, with no discernable difference between the two solutions. If medium is used instead of HBSS, store the plates in the incubator until use.

18. The disruption of cells by cutting releases negatively charged DNA from the cells that have been damaged. This may cause the tissue in the solution to interact and result in coalescing into a clump that floats and is not easily separated. Check the cell solution after 10 min in the water bath. If the tissue is en masse and not clearly separated into pieces, add DNase (1:500) to the solution and gently invert three to four times. Return to water bath for the remaining 20 min. This will degrade the DNA and allow for a cleaner separation of individual cells during the digestion and a higher yield of cells.
19. It may not be necessary to add more DNase. As you repeatedly perform this preparation, you will become more familiar with the rate of dissociation of the tissue and the need for DNase. The freshness of both the DNase and papain in the initial water bath dissociation step, as well as the amount of tissue in the solution, may modify the ease or difficulty in manual trituration, and hence the need for DNase. DNase can be very helpful in this process, yet using high amounts of DNase on a small amount of tissue has been observed to be harmful to the cells and may lower the overall viability of the primary neurons. With practice, it should be clear whether more DNase will help with the digestion of the tissue. During resuspension, the tissue will noticeably break down and cells will enter the solution, as evidenced by the slight clouding of the media.
20. The trituration will proceed through the steps that include using tools with progressively smaller openings at each step. This gradual decrease in size will help minimize the harm to these fragile cells (14). If no tissue remains between steps, the next successive trituration process may not be necessary. Usually, no visible tissue remains after the three trituration steps. If the tissue does remain, however, repeat the addition of Complete Medium and the trituration using the 1,000-mL pipette until the tissue is no longer visible.
21. Usually, a gentle twist will help retain the tip without breaking the glass pipette.
22. Count both live and dead cells. On average, the number of cells per the hippocampi of one pup using this method is about a million. Higher or lower yields may indicate problems with any of a number of steps in the process. It is helpful to track the yield of each preparation and make adjustments to the technique where needed. Some common reasons that a yield may be lower than expected include the use of too much DNAse, not enough DNase, incomplete trituration, which leaves the cells aggregated and then lost during the filtering process, or trituration that is too harsh. Additionally, it may be an indication that a reagent is contaminated, mixed

inaccurately, or the temperature is either too hot or too cold for these sensitive primary neurons. Experience has shown that cell death greater than 15–20% of the total number of cells may indicate that something has gone wrong in the process and will usually not produce a successful culture.

23. This density provides enough neuron-to-neuron support to produce growth and survival in these culture conditions, yet still allows for enough distance between neurons to image single cells for analysis.
24. Most cultures of primary neurons call for a wash and change of medium after 1 h. This is appropriate when the substrate for the neurons is PDL. It has been observed in this system that neurons adhere more slowly to poly-L-ornithine than to PDL. Washing too early may cause the loss of cells and ultimately reduce the ability of the remaining neurons to survive in the culture. However, the timing of the first wash may be adjusted from overnight to an earlier time point if it is evident that the cells have adhered.
25. ARAC is an antimetabolic agent that is capable of damaging DNA during its synthesis. For those cells that are rapidly dividing, this damage is harmful to their survival. Since neurons growing in the culture are postmitotic, they are less affected than astrocytes, which are rapidly dividing. Treating with ARAC will aid in producing a highly pure neuronal culture. Experiments have shown that the combination of using the neuronal-specific B-27 supplement in the media, along with the one-time treatment with ARAC (2.5 μM final concentration), produces neuronal cultures that are 94% nonastrocytes. Using a higher concentration (5 μM ARAC) resulted in increased neuronal death and complicated long-term survival. B-27 alone, without ARAC treatment, also produced a relatively pure culture, but that purity was increased with the addition of ARAC without compromising the long-term survival and development (Data unpublished).
26. Neurons release factors that aid in the development and survival of the entire culture. By removing only one-third of the media, new nutrients are gained from the replacement media without removing all of the important neuron-released factors necessary for development (12–14).
27. By eye, the meninges appear as a slightly reddish membrane. By carefully rolling the cortex on the filter paper with a sterile surgical tool, you will notice that the meninges adhere to the filter paper, as evidenced by the reddish hue. Roll until the tissue is free of the reddish color. Meninges-free tissue will aid in reducing the number of fibroblasts in the astrocyte culture and so will increase the culture purity.

28. This step will aid in destroying the more sensitive neurons in the solution, with little harm to the more resilient astroctyes.
29. Inspect these cultures at each change of medium. In addition to astroctyes, some neurons will be present, but they usually die after trypsinization during the passage of the cells for coculturing. Additionally, microglia will be seen and their numbers can be decreased by dislodging the loosely attached cells at each media change by using your hand to firmly hit the side of the flask. This is an important step, as microglia release cytokines that are toxic to neurons (14). These astrocytes can also be purified by rocking the astroctye flasks overnight prior to their passage. If fibroblasts are present, use the cultures cautiously as they multiply rapidly.
30. The yield of astroctyes will depend on the age and freshness of the trypsin as well as the amount of manual dissociation.
31. Serum deprivation stops the proliferation of the astrocytes and forces them all into the same stage of the cell cycle. If a 24 h astrocyte treatment is not being performed, change the media after 24 h.
32. DMEM is an astrocyte-specific medium. By allowing astrocytes to condition the medium, astrocytes will release factors that will be supportive of neuronal growth and will enhance their survival, as opposed to placing them in the DMEM medium without necessary survival factors.
33. The spacers should detach easily. With a needle, gently stabilize the coverslip by pushing down at its edge while grasping one wax ball with a pair of tweezers. With a gentle twisting motion, lift the ball off the coverslip. Take care not to touch the coverslip with the tweezers. Discard the detached spacer. If more force is necessary to remove the wax, grasp the wax ball and use force toward the outer edges of the coverslip so that the growing cultures on the interior of the coverslip are not disturbed. Alternately, this step is made easier when the coverslips have been stored before immunostaining at 4°C after fixing and washing with PBS (see Note 34). Be sure to wash the fixed cultures an additional time after removing the wax balls to remove any small particles of wax.
34. At this point, coverslips can be stored preferably overnight, but up to a week, at 4°C in PBS with the plate wrapped in parafilm. While you may be able to retain them longer prior to immunostaining, you risk the growth of bacteria on your fixed cells. If they are stored, wash the cells one time for 5 min on the rocker at room temperature prior to immunostaining to remove any settled debris.

35. If performed gently, the neurons should be fine for imaging when the mounting process is complete. Alternatively, coverslips can remain neuron-side up if 200 μL of primary antibody solution is placed on top of them to insure the coverage. This, however, doubles the amount of costly antibodies used for each staining.
36. Use care, as it is quite difficult to manipulate the very fragile coverslips with tweezers during these important steps, especially in the dark. After dispensing the secondary antibody and wrapping the plate in aluminum foil, the overhead lights can be turned on, but must be turned off with subsequent washes and mounting.
37. Avoid air bubbles in the mounting medium, which can complicate imaging. Take care not to move the coverglass, once placed, to avoid harm to the fixed neurons.
38. There are many experimental questions, where it is valid to optimize each field to obtain the best possible image, making it appropriate to adjust the imaging parameters with each new field. However, when assessing the effects of treatment on cells, the question of interest becomes one of comparing the relative changes across the treatment groups. These differences can only be observed if the laser intensity and gain settings of the microscope remain constant for each treatment group imaged. Selecting the brightest treatment group to set the imaging parameters insures that every other treatment will have signal within the appropriate detectable range and enable the comparison of the intensity and structure between the groups. If a less-intense slide is chosen to determine the settings, oversaturation of the signal could occur when you reach a treatment group with a more intense signal, resulting in the loss of data and difficulty in quantifying any differences (25).
39. A portion of the cell body will still be visible in the image. This step size yields a z-series stack that ranges from 12 to 18 slices per neuron and captures all the necessary data throughout the neuronal extensions. When deconvolving the image (see Subheading 3.8), a 0.3-μm step size yields three-dimensional puncta that are slightly stretched in the z-plane. This stretch can be diminished by decreasing the step size (0.15 μm). This, however, would require the acquisition of twice the number of images per field. Because each laser channel is acquired sequentially, one 18-slice stack requires the acquisition of 36 images, and it takes approximately 3–4 h to complete the required fields of one treatment group. The trade off for obtaining a less-stretched z-plane is an increase in the amount of time required to image and deconvolve

a larger stack. The 0.3-μm step size produces consistent, statistically significant results.

40. This one plane, which identifies the nucleus of the cell, can be merged with the other two channels for use in figures to identify the cell body of the neuron. It will not be used in analysis, so one plane of the blue channel is sufficient. Because a z-series of this channel would require the scanning of the image for an additional 12–18 times, obtaining one plane saves imaging time.
41. Depending on the amount of background noise generated in the image by either the detector, nonspecific staining, or autofluorescence, the signal-to-noise ratio of particular experiments or images may need to be adjusted and the images redeconvolved. A ratio of 12–15 is usually adequate for this staining method. For more information, see "Signal-to-Noise Ratio in Confocal Microscopes" by Sheppard et al. (25).
42. Working in grayscale will minimize operator bias that may occur when working with colored images, as certain colors are easier to detect by eye (20).
43. It is important that the selection circle matches the puncta size. If too large of an area is selected, pixels from the dark background will be included which will decrease the mean intensity of the puncta inappropriately. This will ultimately cause a reduction in the overall threshold used to render the puncta in three dimensions and result in an increase in the number of puncta that is not reflective of what is actually present. If the smallest puncta in either channel is less than two pixels in diameter, it is possible that either the immunocytochemistry was unsuccessful or that the neurons have not matured to an appropriate stage development.
44. This is a manual determination of the threshold, and so may be subject to user bias (20, 21). To decrease this bias, it is important to select a range of puncta that reflect the particular extension used, being sure not to choose only the most intense signal. However, do not select those areas that are diffusely labeled or have indistinct structures, as is often seen within the cell body. While diffuse signal may be indicative of pre- or postsynaptic proteins, the fact that it lacks intensity and structure raises doubt as to whether it is indicative of a synaptic structure. Diffuse signal may be protein that is either being produced, concentrated in the cell body awaiting transport, or in the process of being trafficked and so not yet part of a structure. With experience, the ability to discriminate the actual puncta from indistinct signal will become easier. It should be noted that while the puncta are selected

manually, the sample size obtained after all images are measured is high, increasing the reliability. Measurements of the mean intensity of each puncta will be obtained and averaged, totaling 450 measurements for the determination of the threshold for each channel. Additionally, the overall accuracy of the threshold will be confirmed by comparing the deconvolved image with the three-dimensional rendering after thresholding (18).

45. This saves the entire 30 puncta as one overlay, which can be reloaded onto the stack at any time. Saving the ROI is important for both validation and so that an experimental record of the process exists.
46. In the three-dimensional analysis process, the threshold is used to render the surface of each puncta. Pixels that are determined to be part of a cluster of synaptic proteins must be at the intensity of the threshold or higher. A border is drawn around those pixels that meet the threshold value and any of their neighbors of the same or higher intensity. Setting a threshold too low will yield more structures or structures that are larger than what is actually represented; setting a threshold too high will exclude the signal that may in fact be a structure.
47. In the beginning, it is helpful to compare the deconvolved image with the automatic surface rendering obtained using the three-dimensional analysis software. This ensure that the result of the manual selection of puncta reflects what is actually present. If the puncta selected include a representative sample of the range of intensities, the surface rendering after thresholding should be reflective of what is actually present (see Fig. 3). If there are too many objects rendered or abnormally large objects compared to the deconvolved image, the threshold was set to low, which could be an indication of a selection tool that was too large or signal that was weakly stained and not representative of an actual puncta that was included. Additionally, if the threshold was set too high, there will be a reduction in the number or size of puncta in the surface-rendered neuron compared to what is seen in the deconvolved image. Confirming that the rendered surface area represents the confocal image is a good test to verify that the manual selection of puncta is in fact performed in an unbiased manner.
48. There will be some variability in the number of pairs within a treatment group. However, 15 images per treatment group are capable of capturing statistically significant differences between treatment groups when an ANOVA analysis is used to assess these differences.

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Chapter 26

Volume Measurements in Cultured Primary Astrocytes

Michael Aschner

Abstract

Damage to the central nervous system (CNS) is selective, likely reflecting the intrinsic properties of individual cell types. Targets of chemical injury are diverse hence assessing neurotoxicity is extremely difficult. Overcoming this obstacle requires a general screen or "marker" for injury that reflects cellular damage. The "marker" must be reliable and represent a biochemical event which broadly reflects cellular stress and damage. One such "marker" is cell swelling; it occurs in response to a diversity of insults, such as physical damage, disease (ischemia, trauma, and hypoxia), and chemicals (methylmercury, lead, 1,3-dinitrobenzene, and triethyltin). In astrocytes, a type of glia, astrocytic swelling can be measured with several methods. Commonly, freshly isolated astrocytes are grown to confluence on coverslips, a period requiring 3 weeks in culture. At this time, astrocytic volume can be measured using either an impedance technique or 3-*O*-methyl-D-glucose to assess cell volume. This review will briefly detail these methods and provide insight into molecular mechanisms associated with cell swelling and the ensuing regulatory decrease (RVD).

Key words: Astrocytes, Swelling, Electrical resistance, 3-*O*-methyl-D-glucose, Neurotoxicity

1. Introduction

The pioneering work of Klatzo published in 1967 (1) led to the distinction between two types of brain edema, referred to as vasogenic edema, where injury to the vessel wall and compromised function of the blood–brain barrier (BBB) leads to escape of water and plasma exudates into the brain parenchyma, and (2) cytotoxic edema where toxic compounds directly affect the cells and lead to intracellular swelling in the absence of changes in the restrictive properties of the BBB. Cytotoxic edema is the most common cause of brain swelling. Given that astrocytes are commonly swollen in brain trauma of various etiologies, the review will be restricted to swelling mechanisms in these cells. This will

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be followed by detailed methodologies for the measurements of cell volume using an electrical resistance method and 3-*O*-methyl-D-glucose.

Astrocytes occupy approximately 20% of the gray matter's volume, extending their processes around synapses and in close apposition to the nodes of Ranvier, axon tracts, as well as the capillaries. Mature astrocytes elaborate and secrete neurotrophic factors, control the interstitial pH, concentrate uptake and metabolize neurotransmitters, modulate neuronal signals and immune responses, and repair damaged tissue, just to name a few. During development, they also provide cues for migrating neurons and provide the scaffolding for neuronal migration. Review of the plethora of astrocytic functions is beyond the scope of this paper. Additional reading on this topic can be found number of comprehensive reviews (2–6).

The availability of pure primary astrocyte cultures has been instrumental to the understanding of the mechanisms of astrocytic swelling. Though still generally ill-defined, at least in its exaggerated form, it seems that astrocytic swelling has deleterious effects resulting in frank pathology. Persistent astrocytic swelling can be viewed as a pathological extension of more limited and controlled volume changes which appear otherwise to be part of the normal homeostatic function of astrocytes in their control of the extracellular milieu. Swelling of astrocytes is a complex phenomenon, and four different contributing mechanisms have been ascribed to this phenomenon, likely operating in tandem (Fig. 1). The first mechanism is referred to as acid–base or pH-driven, and it involves the simultaneous operation of Cl^-/HCO_3^- and Na^+/H^+ exchange transport systems, with H^+ and HCO_3^- cycling from the intra- to extracellular spaces via membrane-permeant CO_2 (7, 8) (Fig. 1). The second mechanism is referred to as glutamate-driven swelling and it operates by increasing the production of the metabolic products CO_2 and H^+, thus leading to swelling by similar processes to those described above. In addition, astrocytes transport glutamate intracellularly by a Na^+-dependent mechanism with a likely stoichiometry of 1 glutamate and 3 Na^+ transported inwardly, and 1 K^+ transported outwardly to offset the negative charge of glutamate (Fig. 1). The resulting net uptake of Na^+ and glutamate associated with these processes leads to increased intracellular osmolarity and swelling. Increased extracellular potassium concentration $[K^+]_o$ (>20 mM), which is inherent to both stroke and head injury, causes astrocytic swelling by increased uptake of KCl due to Donnan forces (9), referred to as Donnan-induced swelling (third mechanism of cellular swelling). K^+ uptake occurs via K^+ channels, of which there are several types in astrocytes (10) and Cl^- uptake occurs largely via Cl^- channels. Finally, cell swelling in astrocytes can occur as a result of nonspecific breakdown of the selective permeability of the plasma

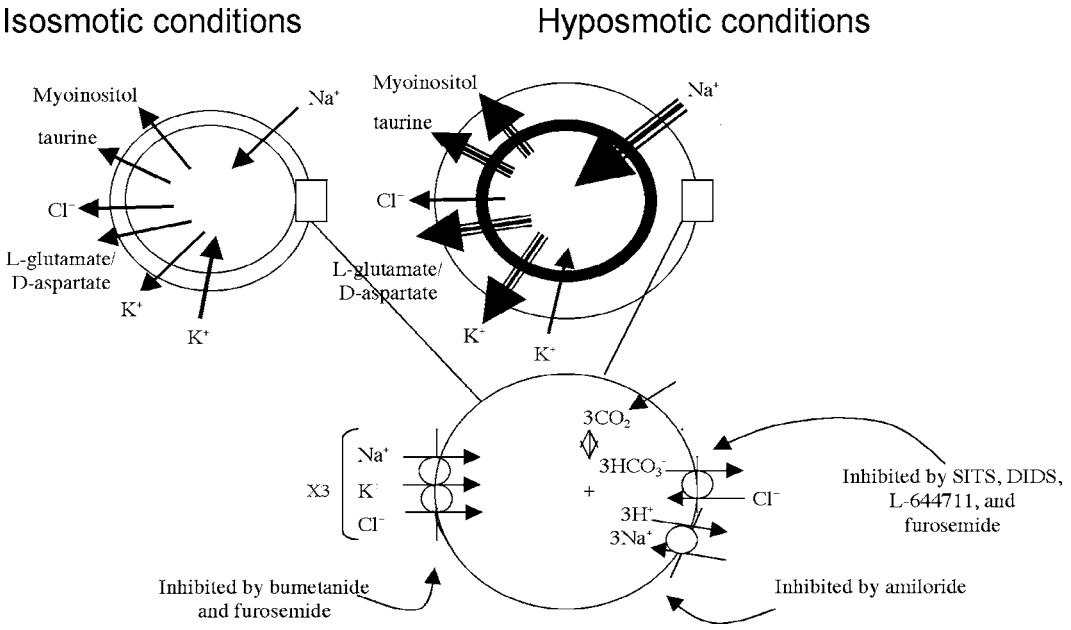

Fig. 1. Schematic representation of the effects of hypotonicity on amino acid and ion transport. *Insets* represent various transport systems associated with swelling and their specific inhibitors. For additional information, refer to Subheading 1.

membrane, referred to as nonspecific swelling. This most likely reflects increased generation of intracellular reactive oxygen species (ROS). Changes in cell volume are amenable to experimentation. In the next section, I will describe two such methods, the electrical resistance (impedance) method and the 3-*O*-methyl-D-glucose method. These are well suited for measurements of volumetric changes in astrocytes cultured under in vitro conditions.

2. Materials

2.1. Electrical Impedance Method

1. Plexiglas.
2. Lexan polycarbonate plastic (GE Plastics, Pittsfield, MA).
3. Apiezon M grease (SPI Supplies/Structure Probe, Inc., West Chester, PA).
4. Lock-in amplifier Model 5301 (Princeton Applied Research, Princeton, NJ).
5. No. 1 glass coverslips (30×13.8 mm; BD Biosciences, Franklin Lakes, NJ).
6. A freezing point osmometer (Advanced Instruments, Inc., Needham Heights, MA).

The following solutions are needed for coverslip preparation:

7. 70% Ethanol.
8. 0.2% (w/v) Gelatin solution: Dissolve gelatin powder in sterile MilliQ water by gently swirling mixture for 15 min in a 60°C water bath. Cool the 0.2% gelatin solution at room temperature, and, while still warm (~37–40°C), filter it through a 0.45 μm cellular acetate membrane (CA). We usually prepare 100 ml at a time and store 50 ml aliquots of the filtered gelatin solution at 4°C.
9. Borate Buffer: add 1.9 Borax (sodium tetraborate) to 400 ml double-distilled water, add 1.24 boric acid to borax solution, adjust pH at 8.4 with NaOH. Dilute to desired molarity with double-distilled H_2O.
10. 10× Poly-L-Lysine (Sigma) stock solution: 25 mg poly-L-lysine in 25 ml borate buffer. Store at 4°C for up to 2 months.
11. 1× Poly-L-lysine solution: 5 ml 10× poly-L-lysine solution in 45 ml borate buffer. Filter sterilize (0.2 μm) and store at 4°C for no more than 1 day (see Note 1).

2.2. 3-O-Methyl-D-Glucose Method

1. 3-*O*-Methyl-D-[1-^{3}H]glucose (Aqueous solution, sterilized; 74–555 GBq/mmol, 2–15 Ci/mmol; 37 MBq/ml, 1 mCi/ml) (GE Healthcare Bio-Sciences Corporation, Piscataway, NJ).

3. Methods

3.1. Fabrication of the Impedance Chamber

1. Mill out a channel [7.0 cm (length) × 1.8 cm (width) × 1.0 cm (height)] in inert translucent Plexiglas.
2. Mill Out a second channel (7.0 cm × 1.4 cm × 250 pm) at the bottom of the chamber, leaving a 2-mm lip on each side.
3. Place a piece of Plexiglas (2.5 cm × 1.8 cm × 1.0 cm) on the lip of the chamber, forming the roof of the enclosed channel into which the cells will be placed.
4. Fit the edge of this piece of Plexiglas closely to the side of the chamber, allowing virtually no current flow through the sides. The final dimensions of the channel should be 2.5 cm × 1.4 cm × 250 μm.
5. Secure gold-plated, low-impedance, inert electrodes to the bottom of each chamber on either side of the channel with a dab of silicone grease.
6. To make the gold electrodes, evaporate gold through a mask, at 10m^4 Torr, onto clear Lexan polycarbonate plastic strips.
7. Leads from the gold electrodes are made of insulated copper wire, soldered to the silver with pure indium and the connections

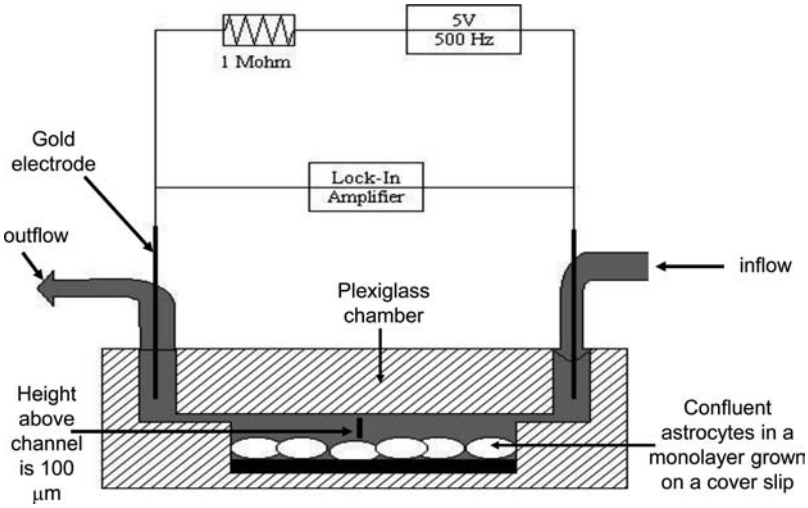

Fig. 2. Schematic representation of the electrical resistance (impedance) apparatus for measuring astrocyte cell volume.

are covered with wax (a molten solution of 50% Apiezon M grease and 50% paraffin wax).

8. Connect the gold electrodes through a large external resistor (1 MΩ) to a lock-in amplifier (5301; Princeton Applied Research, Princeton, NJ) that supplied a 500 Hz, 5-V signal to the system (Fig. 2).

3.2. Isolation and Culturing of Astrocytes

The culture technique is based on the method first published by Frangakis and Kimelberg (11), with several modifications instituted in our laboratory. The protocol was previously published in detail Allen and Aschner (12). Briefly, astrocytes are isolated by a method involving enzymatic dissociation of tissue with a bacterial neutral protease (dispase). Next, they are recovered by repeated removals of dissociated cells from non-dissociated sedimenting tissue. This method is less traumatic than other techniques which are based on mechanical isolation and, therefore, leads to a greater cellular yield. Though tissue dissociation by trypsin is more rapid and produces a cellular yield similar to dispase, we opt to utilize the latter as in our hands the digestion with dispase leads to higher viability of astrocytes when compared with dissociation with trypsin. (For extensive details on the culturing methods, notes, and troubleshooting, refer to ref. 12).

We commonly isolate astrocytes from neonatal day 1 rat pups (8–12 pups/litter). We purchase time-pregnant rats that are delivered to the animal facility on gestational days 15–17, thus minimizing the per-diem expenses (rat gestation is 21 days).

Waiting for the dams to naturally deliver has also multiple advantages, alleviating the need for surgeries during pregnancy, thus saving time and reducing trauma. The above described protocol (12) routinely yields from a single litter sufficient number of cells (100,000,000) for approximately 15 culture dishes (100 mm) and likely hundreds of coverslips. Cells from these preparations can be directly seeded onto coverslips (see below) that are placed in larger culture dishes for the measurement of cell volume using the electrical resistance (impedance) method. For the 3-*O*-methyl-D-glucose method, it is best to seed the cells in 6- or 12-well plates. The number of required plates should be determined a priori, keeping in mind the need to replicate each experimental point and treatment concentration in a minimum of triplicates from three (or more) independently isolated astrocyte cultures. Cells are cultured in standard conditions and reach confluency in ~3 weeks. They are >95% positive for glial fibrillary acid protein (GFAP), a cytoskeletal intermediate protein marker, which is solely expressed in astrocytes (see ref. 12 for extensive experimental details).

3.3. Measurements of Astrocytic Volume with the Electrical Resistance (Impedance) Model

Astrocytic swelling is measured by a modified dynamic method based on the measurement of electrical resistance (13). The premise of the electrical resistance (impedance) measurement is simple (Fig. 2). The volume of the solution within the channel available for current flow decreases proportionally to cell swelling. As cells swell their volume increases such that flow decreases, translating to increased resistance in the channel above the cells. Given Ohm's law that $V = IR$ (where V is voltage, I is current, and R is resistance) and I is constant (500 Hz), changes in V are directly proportional to changes in R. Since the resistance of the chamber itself is <0.02 MΩ, this arrangement essentially provides the system with a constant current source (13).

The chamber's height is 100 μm above the cells (Fig. 2). Since we measure the percentage change in voltage (and resistance), a 1% change in the measurement translates to approximately 1 μm change in the average cell height of the monolayer. A recorded increase in the voltage (and thus resistance) means that the volume through the channel above the cells available for current flow has decreased by the same amount as the volume of the monolayer cell height has increased.

3.3.1. Preparation of Coverslips for Cultured Astrocytes

As a general rule, cultured cells do not adhere well to glass and thus cell growth on glass coverslips can be difficult. However, many microscopic, histological, or other procedures are necessarily performed on glass and therefore a procedure for promoting attachment of astrocytes by coating coverslips with gelatin followed by poly-L-lysine is given below (see Note 2).

1. Place coverslips in an autoclavable container in layers between Kim-Wipe tissues. *Do not crowd coverslips, you do not want them to overlap during autoclaving. You may make multiple layers of coverslips.*
2. Autoclave coverslips.
3. In a laminar flow hood, remove coverslips and place into individual wells of a 6-well culture plate with sterile forceps.
4. Add 1.5 ml sterile room temperature gelatin solution to the surface of the coverslip. Allow gelatin to coat the coverslips for 30 min at room temperature.
5. Aspirate gelatin solution and add 1.5 ml of 1× poly-L-lysine solution to the surface of each coverslip.
6. Place plates containing coverslips at 4°C overnight to allow adherence of poly-L-lysine and the gelatin coating.
7. After isolation of cells the following day and ~1 h before seeding cells.
8. Aspirate poly-L-lysine and wash with 1.5 ml sterile water three times.
9. Place plates with coverslips under ultraviolet light of the laminar-flow hood for 30 min to cross link poly-L-lysine and gelatin (Gelatin, Type A from Porcine Skin, Bloom 300).
10. Once isolated, grow the astrocytes on precut no. 1 glass coverslips (30 × 13.8 mm).
11. When confluent, at approximately 3 weeks in culture (postisolation) insert the coverslip into the channel of the Plexiglas chamber (Fig. 2).
12. In a typical experiment, the control isosmotic bathing medium for the experiments consists of the following: 22 mM NaCl, 3.3 mM KCl, 0.4 mM $MgSO_4$, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 10 mM D-(+)-glucose, and 25 mM HEPES (*N*-2-hydroxyethylpiperazine *N*′-2-etahesulfonic acid) and 200 mM mannitol.
13. Maintain HEPES-buffered solutions at pH 7.4 by the addition of 1N NaOH. The osmolality of the solutions should be ~300 mosmol. It should be measured by a freezing point osmometer (Advanced Instruments, Inc., Needham Heights, MA).
14. Determine the baseline cell volume after placing the cells in the channel and continuously perfuse with isotonic solution at 25°C, the flow of which is driven by the height difference of the solutions in the chambers that flank the channel (see Notes 3 and 4).
15. The height of the astrocytic cell monolayer can be calculated from the resistance measurement of the channel. Measure the

magnitude and phase of the voltage across the two electrodes, and calculate the resistance of the channel.

16. Normalize this resistance to 1.0, and subsequent changes in resistance, such as those occurring after perfusion with hyposmotic buffer are represented as a percent change from the initial value.
17. For experiments in which the effects of a Na^+-free hypotonic buffer is tested, apply to the cell a solution where Na^+ is replaced with *N*-methyl-D-glucamine chloride (NMDG). Maintain the solution at pH 7.4 by the addition of 10N HCl (see below for additional details). This can be also achieved with mannitol substitution for the Na^+ (see Note 5).
18. If desired, the chamber may be placed in an incubator and experiments can be conducted at 37°C. The flow through the chamber is driven by hydrostatic pressure (due to height difference between the solutions and chamber). A pump may also be utilized.
19. At the end of each experiment, remove the coverslip with the cells and insert a blank coverslip into the channel.
20. Calculate the difference between the channel resistance under the two experimental conditions. The difference equates to the difference in height due to the cell monolayer. In our hands, the resting height of the astrocytic monolayer is normally about 5 μm, as determined by this method.
21. For additional detail on calculations of cell volume, please refer to O'Connor et al., 2003 (14) (see Note 6).

3.4. Measurement of Cell Volume in Cultured Astrocytes with the 3-O-Methyl-D-Glucose Method

A second method for the estimation of cell is based on the procedure of Kletzien et al. (15). The method is based on the nonmetabolizable hexose 3-*O*-methyl-D-glucose as a marker for the intracellular space. Phloretin, an inhibitor of sugar transport is used to prevent the accumulated 3-*O*-methyl-D-glucose from leaving the astrocytes during the washing period. Analogous to the impedance method, the major advantage of this technique is that it permits determination of the intracellular water space in cells attached to a culture dish. However, the technique may also be of general use for the accurate determination of intracellular water space in cell suspensions.

1. For measurements of astrocyte volume, astrocytes cultured in 12- or 24-well dishes are used.
2. Replace the culture medium with HEPES-buffered Eagle's medium and incubate the cells in saline medium in the absence of glucose.
3. Quickly aspirate the medium and replace with medium containing 0.1 μCi of 3-*O*-Methyl-D-[1-^{3}H]glucose.

4. At the desired time point (commonly after 2, 5, and 10 min as you are trying to capture early changes in cell swelling) of incubation at 37°C, remove the medium by suction followed by a rapid wash (10 s) with nonradioactive medium containing 1 mM phloretin.
5. Dissolve the cells in 2 M KOH.
6. Remove 200 μl of the solution and use for protein determination by your referred method. The remainder of the solution should be used to count radioactivity.
7. Determine the cellular volume by determining change in radioactivity of 3-^{3}H over time, standardized to protein content (see Notes 7 and 8).

4. Notes

1. Poly-D-lysine is an acceptable substitution for poly-L-lysine and has been suggested to possibly be preferred due to decreased digestion of the D-isomer by cells. Regardless of the isomer, the large molecular weight (>300 kDa) preparations should always be used.
2. Issues related to astrocyte culturing have been extensively detailed. Please refer to Allen and Aschner (12). It is critical to use confluent astrocytes. This will assure that when swelling occurs the swelling will largely be propagated in the *y*-axis (height) rather than the *x*-axis (width).
3. The chamber is not available commercially; hence those interested will have to fabricate it. The procedure required a fine machine-shop as the dimensions, especially the height of the channel must be precise.
4. The lock-in amplifier should supply a 500 Hz, 5-V signal to the system. At this frequency, the cell membranes are insulating, and current will travel over and not through the cell monolayer. The electrode impedance is negligible. The lock-in amplifier is used because it can resolve small voltage changes with high noise rejection. Thus, apart from the solution in the cell channel, the two chambers were effectively insulated from each other (14).
5. The replacement of part of the NaCl with mannitol in isosmotic solutions has the important feature of maintaining the same electrolyte concentration in the respective iso- and hyposmotic solutions, assuring identical conductivity properties. However, a small correction has to be made for the small decrease in solution conductance due to mannitol, which is corrected for by adding a small amount of water (μl).

It is critical to balance all the experimental solutions to the same resistivity so that the resistance (and voltage) differences measured when the solutions are changed can be accounted for solely due to changes in cell volume.

6. For theory on the measurement of cell height changes due to anisotonic media exposure, the reader is further referred to O'Connor et al. (14).
7. It is possible that small amount of 3-*O*-methyl-D-glucose is not restricted only to the intracellular water space and thus it may be incorporated into or irreversibly sequestered by the cells. This would tend to overestimate cellular volume. Generally, this does not seem to be a problem (15).
8. In general, using either this or the impedance method, astrocytes exposed to a medium with decreased osmolarity respond rapidly, increasing cell volume by approximately 50% in 2 min. In the course of swelling, excitatory amino acids (EAAs) and K^+ are released from the astrocytes. A process referred to as regulatory volume decrease or RVD (16, 17) follows this rapid swelling phase where astrocytes shrink toward their normal size. RVD involves the activation of conductive K^+ and Cl^- channels, allowing for the escape of KCl and osmotically obligated water. Astrocytic exposure to reduced osmolarity also leads to the release of D-aspartate, L-glutamate, and taurine (17–19). Whereas taurine plays an important role as an osmolyte (17–20), both aspartate and glutamate are believed to have no contribution to RVD, and in fact may lead to the overstimulation of *N*-methyl-D-aspartate receptors and cellular damage in neurons (13, 21, 22). Reports on hypotonic-induced ion and amino acid release invoke phosphatidylinositol hydrolysis and elevated intracellular calcium concentrations $[Ca^{2+}]_i$ as regulatory mechanisms of RVD (23–25). cAMP, calmodulin, eicosanoids, protein kinases, leukotrienes, and the microfilament network have also been postulated to play a role in RVD (24). O'Connor et al. (13, 14) have reported that removal of external calcium can inhibit RVD, at least over the initial 8–10 min and Bender et al. (26) have reported that calmodulin inhibitors inhibit RVD in astrocytes. Nimodipine, a specific blocker of the L-type calcium channel, significantly inhibited volume regulation in our studies. When intracellular calcium was quenched, using BAPTA-AM (1, 2-bis-(2-aminophenoxy)-ethane-*N*, *N*, *N*′, *N*′-tetraacetic acid [tetra(acetoxymethyl) ester]), RVD was inhibited to an even greater extent. Likewise, trifluoperazine, a calmodulin inhibitor, also inhibited RVD. In all of these cases, [^{3}H]-D-aspartate release was significantly increased compared with hypotonic-induced release. In contrast, ^{86}Rb (a marker for K^+) efflux was significantly decreased by these agents.

The observation that astrocytes exposed to calcium-free media or nimodipine upon hypotonic-induced swelling exhibited some RVD, albeit at a slower rate than controls, points to a role for intracellular calcium mobilization in RVD (22). More recently, Mongin and Kimelberg (20) have established that ATP regulates volume-regulated ion channel (VRAC) activity via two separate Ca^{2+}-sensitive signaling cascades involving PKC and CaMK II and that cell swelling per se activates VRAC via a separate Ca^{2+}/calmodulin-independent signaling mechanism. Ca^{2+}-dependent organic osmolyte release via VRACs may also contribute to the physiological functions of these channels in the brain (20); see also Ye et al. (27).

Acknowledgments

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Chapter 27

Assessment of Cholesterol Homeostasis in Astrocytes and Neurons

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Abstract

Cholesterol homeostasis is highly regulated in the nervous system; dysregulation in cholesterol trafficking and content have been involved in the pathogenesis of neurodegenerative diseases (such as Parkinson's and Alzheimer's diseases). Furthermore, low cholesterol levels during brain development are associated with neurodevelopmental deficits and mental retardation. The methods described in this chapter can be used to investigate the effect of neurotoxicants on cholesterol homeostasis. Astrocytes and neurons are two major cell types in the brain in which cholesterol synthesis and efflux are highly regulated to keep a proper cellular cholesterol level. Disruption in cholesterol synthesis and/or cholesterol efflux may result in cholesterol deficiency or accumulation in these cells leading to brain dysfunctions.

Key words: Primary cortical astrocytes, Primary cortical neurons, Cholesterol synthesis, Cholesterol efflux, Thin layer chromatography

1. Introduction

Cholesterol plays an important role in the nervous system (1). With almost 25% of unesterified cholesterol in the human body and only 2% of the body mass, the brain has the highest cholesterol content compared with other organs (2). While high brain cholesterol is linked to Alzheimer's disease, low cholesterol levels during brain development are associated with neurodevelopmental deficits and mental retardation.

As the blood brain barrier (BBB) has a low permeability for cholesterol-containing lipoproteins, the brain practically *de novo* synthesizes all of its cholesterol (3, 4). Though neurons synthesize cholesterol, glial cells are the major cholesterol producer and supplier in the brain (5). As in other cell types, in astrocytes and

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neurons, cholesterol is synthesized from acetyl-CoA in a 37-step process, and the synthesis is greatly inhibited by compounds such as statins, which inhibit 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase). The free cholesterol thus synthesized is partially converted to cholesterol esters intracellularly, by the acyl-CoA: cholesterol acyltransferase (ACAT) (6). To control cellular cholesterol homeostasis, excess cholesterol is removed from the cells through passive diffusion as well as by active transport to cholesterol acceptors (7). High-density lipoproteins (HDL) acting as cholesterol acceptors, significantly increase cholesterol efflux in both astrocytes and neurons. In neurons, but not in astrocytes, cholesterol is metabolized into 24(*S*)-hydroxycholesterol which diffuses freely out of the cell membrane (8). Two developmental neurotoxicants, ethanol and 13-*cis*-retinoic acid, upregulate the levels of cholesterol transporters and cholesterol efflux in CNS cells leading to reduced cholesterol content in astrocytes (9), and this action may be involved in their terotogenic effects.

2. Materials

2.1. Cell Culture

1. Coating substrate: Poly-D-lysine hydrobromide with molecular weight 150,000–300,000 (Sigma-Aldrich, St. Louis, MO) is dissolved in deionized distilled (DD) water at 10 mg/mL and stored at −20°C.
2. Growth medium for astrocytes: Dulbecco's Modified Eagle's Medium (DMEM), low glucose (Invitrogen), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin.
3. Treatment medium for astrocytes: DMEM, low glucose, supplemented with 0.1% bovine serum albumin (BSA), 100 U/mL penicillin, and 100 µg/mL streptomycin.
4. Growth medium and treatment medium for neurons: Neurobasal™-A Medium (Invitrogen), supplemented with 2% B-27 serum-free supplement (Invitrogen), 10 mM glucose, 2 mM Glutamax™-I supplement (Invitrogen), 1.25 µg/mL amphotericin B (Fungizone® Antimycotic, liquid) (Invitrogen), and 100 µg/mL gentamicin.
5. Washing buffer for astrocytes: dilute 10× phosphate-buffered saline (PBS) to 1× with DD water, autoclave, and store at room temperature (RT).
6. 2.5% Trypsin, diluted in PBS to the working concentration 0.25% and stored in 5 mL aliquots at −20°C.
7. Hanks' Balanced Salt Solution (HBSS), 1×, calcium and magnesium free (Ca^{2+}, Mg^{2+} free), stored at RT.

8. Papain, lyophilized powder (Sigma-Aldrich), stored at −20°C.
9. 4 mg/mL Deoxyribonuclease I (DNase) from bovine pancreas, dissolved in HBSS (Ca^{2+}, Mg^{2+} free), stored at −20°C in aliquots.
10. 1 M Magnesium chloride, stored at RT.
11. Dissociation solution for neurons: 2 mg/mL Papain in HBSS (Ca^{2+}, Mg^{2+} free), supplemented with 40 μg/mL DNase and 20 mM magnesium chloride. Prepare the solution right before dissection, incubate at 37°C and filter before use.
12. Washing buffer for neurons: 1× Hanks' Balanced Salt Solution (HBSS) supplemented with 10 mM HEPES; store at RT.

2.2. Exogenous Cholesterol Efflux

1. [1,2-^{3}H(*N*)]Cholesterol, 1 mCi/mL, 54.2 Ci/mmol (PerkinElmer, Covina, CA), stored at −20°C.
2. 10 mM 22(*R*)-hydroxycholesterol [liver X receptor (LXR) agonist] in ethanol, stored at −20°C; 10 mM 9-*cis*-retinoic acid [retinoic X receptor (RXR) agonist] in DMSO, stored at −20°C.
3. Cholesterol acceptors: apolipoprotein A-I (apo A-I) and HDL, stored at 4°C.
4. Solvent for lipid extraction: hexane:isopropanol (3:2, v:v). Store in a glass bottle sealed with parafilm in inflammable cabinet.
5. EcoLume™ liquid scintillation fluid (MP Biomedicals, Solon, OH), stored at RT.

2.3. Endogenous Cholesterol Synthesis

1. [^{3}H]Acetic acid, 10 mCi/mL, 5 Ci/mmol (PerkinElmer), stored at −20°C.
2. 1 mM lovastatin (HMG-CoA reductase inhibitor) in DMSO, stored at −20°C.
3. Solvent for lipid extraction: hexane/isopropanol (3:2) as described in Subheading 2.2.
4. Cholesterol reference standard: 20 mg/mL cholesterol in chloroform, stored at −20°C.
5. 10 mg/mL Cholesterol ester and 10 mg/mL 24(*S*)-hydroxycholesterol reference standards, in chloroform, stored at −20°C.
6. Thin layer chromatography (TLC) plates: Polygram®, SIL G precoated on plastic, 20×20 cm (Macherey-Nagel, Bethlehem, PA).
7. The TLC running solvent: hexane:diethyl ether:methanol: acetic acid 50:12.5:4.2:0.625 (v:v:v:v).
8. TLC staining material: Iodine crystal (Sigma-Aldrich).
9. EcoLume™ Liquid Scintillation.

2.4. Endogenous Cholesterol Efflux

1. [^{3}H]Acetic acid, 10 mCi/mL, 5 Ci/mmol (PerkinElmer), stored at −20°C.
2. Cholesterol acceptor: HDL.
3. Solvent for lipid extraction, cholesterol reference standard, TLC supplies and scintillation fluid are as described above (see Subheading 2.3).

3. Methods

Radioactive labeling provides an effective and sensitive method for detecting cholesterol synthesis and efflux. Exogenous cholesterol efflux is measured by labeling cells with [^{3}H]cholesterol followed by treatment with agents that may affect cholesterol transporter levels. After 6–24 h, a incubation in the presence of cholesterol acceptors, the radioactivity in the medium corresponding to the effluxed cholesterol is quantified. To measure endogenous synthesis and efflux, cells are labeled with the cholesterol precursor [^{3}H]acetate and are treated with agents to be tested for their effect on cholesterol homeostasis. After lipid extraction and separation by TLC of both cell extracts and medium, [^{3}H]cholesterol, identified by comigration with cholesterol reference standard, is visualized by iodine vapor and quantified.

3.1. Cell Cultures

1. Primary rat neurons are prepared from the cerebral cortex of 21-day-old rat fetus (10).
2. Dissect the tissue, free of meninges, to 1–2 mm^3 sections in HBSS (Ca^{2+}, Mg^{2+} free), and incubate for 30 min in dissociation solution (see Subheading 2.1).
3. Sediment cells at 150 × *g* for 5 min at 4°C. Aspirate the supernatant and resuspend cells in growth medium supplemented with 40 μg/mL DNase.
4. Titrate cells with thin glass tips and filter through a nylon mesh of 40 μm pore size. Sediment again filtered cells at 150 × *g* for 5 min at 4°C, and plate in 75-cm^2 flasks precoated with 20 μg/mL Poly-D-lysine for 30 min in a humidified atmosphere of 5% CO_2–95% air in growth medium (cells from 3–4 pups per flask); during this time, glial cells begin to attach to the flask while neurons remain unattached. This is a purification step known as pre-plating, which enriches the cell suspension in neurons by removing astrocytes.
5. Discard flasks containing attached glial cells, and carefully collect the supernatant. Count cells, and plate them in 24-well plates (precoated with 100 μg/mL Poly-D-lysine overnight)

at $4 \times 10^5/cm^2$. Two hours later, wash cells once with HBSS, and add fresh growth medium to the culture. Medium is replaced with fresh growth medium again after 24 h. The neurons are cultured for 6 days before use for experiments.

6. Primary rat astrocytes are prepared from the cerebral cortex of 21-day-old rat fetus (11).
7. Dissect the tissue, free of meninges, to 1–2 mm^3 sections in HBSS (Ca^{2+}, Mg^{2+} free), and incubate for 10 min in 0.25% trypsin at 37°C. Terminate trypsinization by adding growth medium, and sediment cells at $250 \times g$ for 10 min; resuspend cells in growth medium, and vortex at maximum speed for 1 min.
8. After three washes at $250 \times g$ for 10 min each, resuspend cells in growth medium, filter through a nylon mesh of 100 μm pore size, plate them in Poly-D-lysine-coated (40 μg/mL) 75-cm^2 flasks at a concentration of 5×10^4 cells/cm^2, and incubate at 37°C in a humidified atmosphere of 5% CO_2–95% air in growth medium.
9. Replace the culture medium with fresh growth medium every 2 or 3 days.
10. After 8 days in culture, dissociate cells with 0.25% trypsin and plate them into 24-well plates (1.25×10^5 cells/cm^2).

3.2. Exogenous Cholesterol Efflux

3.2.1. Treatment

1. Incubate neurons (at 6 days in culture) and astrocytes (4 days after subculture) with 1 μCi/mL [^{3}H]cholesterol in growth medium for 24 h.
2. At the end of labeling, wash astrocytes twice with PBS; wash neurons once with HBSS. Treat cells with test compounds in treatment medium, then incubate with or without cholesterol acceptors for 6–24 h (see Note 1).

3.2.2. [^{3}H]Cholesterol Content in the Medium

1. At the end of incubation, collect medium from each sample and centrifuge at $800 \times g$ to remove detached cells; transfer the supernatant to a scintillation vial.
2. Add 5 mL scintillation fluid to each vial. Cap the vials and vortex briefly to mix the scintillation fluid with the medium.
3. Place the vials in a scintillation rack and use a liquid scintillation counter to measure [^{3}H] radioactivity (see Note 2).

3.2.3. [^{3}H]Cholesterol Content in the Cells

1. Wash neurons once with HBSS; wash astrocytes twice with PBS.
2. Add 500 μL extraction solvent (see Subheading 2.2) to the cell monolayer and incubate for 20 min on a shaker. Transfer the solvent from each well to a scintillation vial under a fume hood.

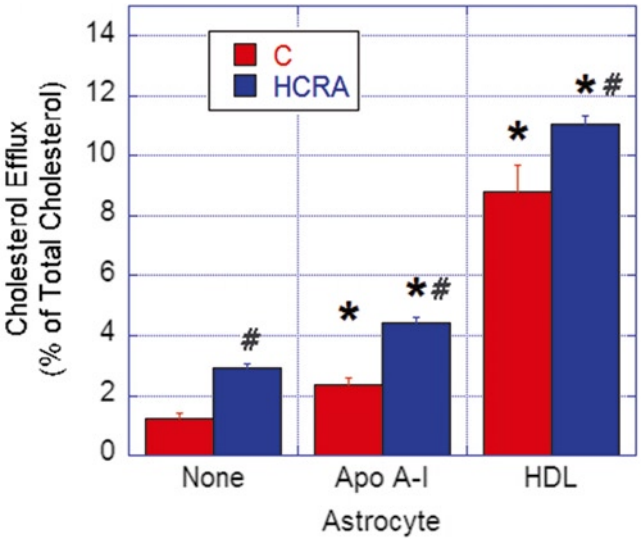

Fig. 1. 22(*R*)-Hydroxycholesterol and 9-*cis*-retinoic acid (HCRA) increase exogenous cholesterol efflux to primary astrocytes. Primary astrocytes labeled with 1 μCi/mL [^{3}H] cholesterol are incubated in the absence and presence of 10 μM HCRA for 24 h followed by 6 h incubation with or without the cholesterol acceptors apo A-I (10 μg/mL) and HDL (50 μg/mL). Values are expressed as percent of total cholesterol and represent the mean (±SD) of three determinations. HCRA significantly increases cholesterol efflux to both A-I and HDL in primary astrocytes.

3. Repeat step 2 and pool the extraction solvent from the same well together.
4. Let the solvent dry overnight in a fume hood. To each vial, add 5 mL scintillation fluid. Cap the vials, vortex vigorously to resuspend lipid extracts and store the samples overnight in the dark.
5. Use a liquid scintillation counter to measure [^{3}H] radioactivity. Count every 24 h until counts are stable (see Note 3). An example of results produced by this method is shown in Fig. 1.

3.2.4. Calculation

The cholesterol efflux is expressed as percentage of total cholesterol using the following formula:

$$\frac{[^3H]\,Cholesterol\ from\ medium\,(cpm)}{([^3H]\,Cholesterol\ from\ medium\,(cmp)+[^3H]\,Cholesterol\ from\ cell\,(cpm))}\times 100\%,$$

where [^{3}H]Cholesterol from medium = [^{3}H] Radioactivity measured from the medium in step 3 of Subheading 3.2.2 and [^{3}H] Cholesterol from cell = [^{3}H] Radioactivity measured from lipid extracts of cells in step 5 of Subheading 3.2.3.

3.3. Endogenous Cholesterol Synthesis

1. Wash neurons (at 6 days in culture) once with HBSS and astrocytes (4 days after subculture) twice with PBS. Incubate cells with 25 μCi/mL [^{3}H]acetic acid in the presence of test compounds in treatment medium for 4 h.
2. Aspirate the medium. Wash the neurons once with HBSS and astrocytes twice with PBS.
3. To each well, add 500 μL extraction solvent (see Subheading 2.3) to the cell monolayer and incubate for 20 min on a shaker. Transfer the solvent from each well to a glass tube.
4. Repeat step 2 and pool the solvent from the same well together.
5. To each Eppendorff tube, add 5 μL of cholesterol reference standard. Let the solvent dry in a vacuum centrifuge or under a fume hood (see Note 4).
6. TLC plate preparation: on each TLC plate, draw two lines parallel to the edge one 2 cm from the bottom and one 1 cm from the top. Activate TLC plates by heating in an oven at 100°C for 30 min.
7. In a glass tank, prepare TLC running solvent: hexane:diethyl ether:methanol:acetic acid (50:12.5:4.2:0.625). Cap the tank and let the solvent vaporize in the tank (see Note 5).
8. In another glass tank, put a few iodine crystals into the tank and cap the tank to form iodine vapor.
9. While heating the TLC plates, resuspend lipids in 50 μL of chloroform (see Note 6). Transfer 5 μL (1/10) of each sample to a scintillation vial to measure total lipid radioactivity. Apply the rest of each sample to a TLC plate on the line near the bottom and let spots dry. Samples are spotted 2 cm apart and no more than nine samples are spotted on a 20 cm wide TLC plate (see Note 7).
10. Insert the TLC plates in the glass tank containing the running solvent against the wall and run until the solvent front reaches the top line (about 1.5 h) (see Note 8).
11. Remove the TLC plates from the tank and allow the plates to dry in a fume hood (about 10 min). Enclose the dried TLC plates in the iodine tank until the spots are clearly stained (see Note 9). An example of TLC plate to which cholesterol, cholesterol ester, and 24(*S*)-hydroxycholesterol reference standards were applied, separated, and stained by iodine is shown in Fig. 2.
12. Circle and cut the stained cholesterol bands and transfer each band to a scintillation vial.

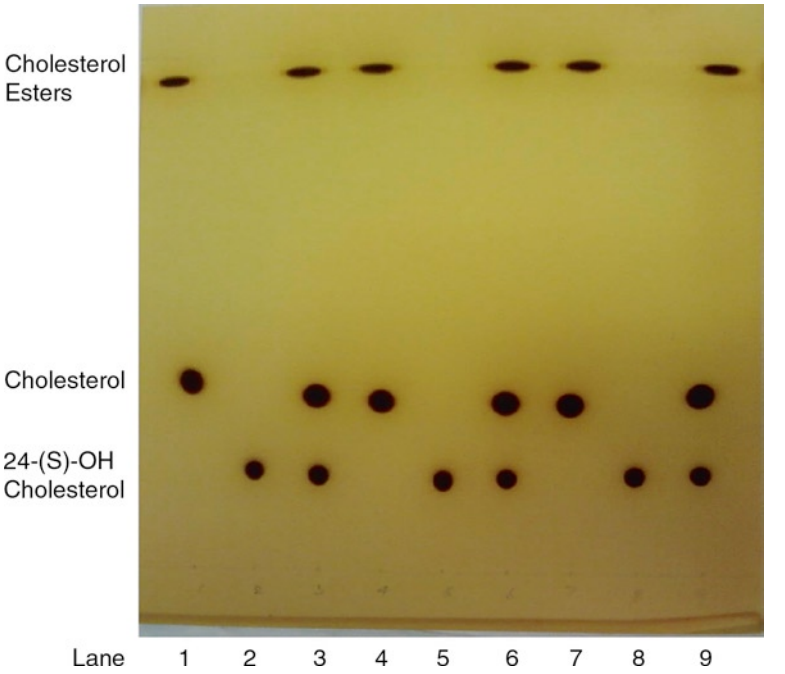

Fig. 2. Cholesterol, Cholestrol Esters, and 24(*S*)-hydroxycholesterol reference standards after separation by thin layer chromatography. Cholesterol and Cholestrol Esters reference standards were applied to lane 1, 4, and 7 along a line 2 cm from the bottom of the TLC plate; 24(*S*)-hydroxycholesterol was applied to lane 2, 5, and 8; all three reference standards were applied to lane 3, 6, and 9. The TLC plates were run in hexane:diethyl ether:methanol:acetic acid (50:12.5:4.3:0.625) and stained with iodine vapor overnight. Cholesterol metabolites and cholesterol were clearly separated by TLC by polarity.

13. To each scintillation vial, add 5 mL of scintillation fluid. Cap the vials and vortex vigorously for 3 min and store for 2–3 days in dark before counting (see Note 10).
14. Count samples in a liquid scintillation counter to measure [^{3}H] radioactivity. Count every 24 h until counts are stable (see Note 11). An example of the result produced by this method is shown in Fig. 3.
15. Endogenously synthesized cholesterol is expressed as percentage of total lipids using the following formula:

$$\frac{[^3H]Cholesterol\ from\ cell\ (cpm)}{9 \times ([^3H]Total\ Lipids)\ (cpm)} \times 100\%$$

where [^{3}H] Cholesterol from medium = [^{3}H] Radioactivity measured from the cholesterol band in step 14 of Subheading 3.3, and [^{3}H] Total lipids = [^{3}H] Radioactivity measured from lipid extracts in step 9 of Subheading 3.3.

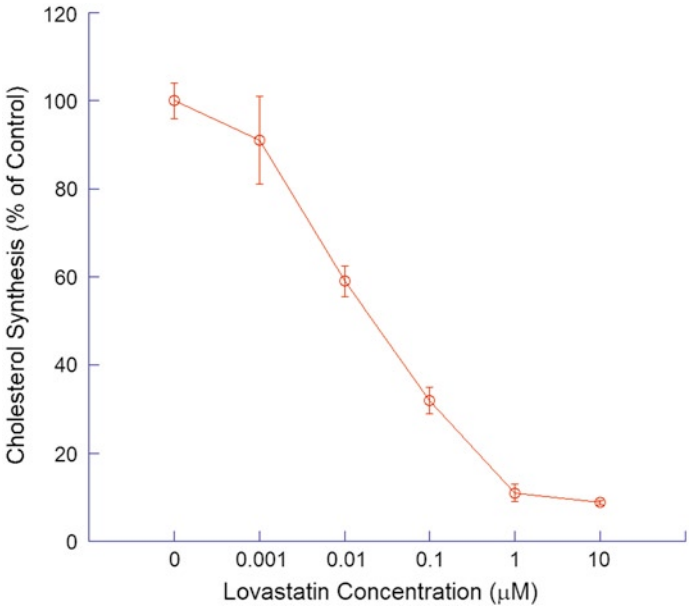

Fig. 3. Concentration–response of lovastatin on cholesterol synthesis in astrocytes. Primary astrocytes were labeled with 25 μCi/mL [^{3}H]acetic acid in the absence or presence of lovastatin (0.001–10 μM) for 4 h. Values are expressed as percent of control and represent the mean (±SD) of three independent determinations. Lovastatin clearly inhibits cholesterol synthesis in astrocytes.

3.4. Endogenous Cholesterol Efflux

1. Incubate neurons (at 6 days in culture) and astrocytes (4 days after subculture) with 25 μCi/mL [^{3}H]acetic acid in treatment medium for 4 h, followed by a 6–24 h incubation with or without cholesterol acceptors in the presence of the test compound.
2. At the end of incubation, collect medium from each sample in a 1.5-mL tube.
3. Add 500 μL extraction solvent (see Subheading 2.4) to the tube; shake vigorously for a few seconds.
4. Centrifuge at 10,000 × *g* for 5 min at RT. The mixture separates into a lower red, medium-isopropanol phase containing the hydrophilic components of the medium and a colorless upper hexane phase that contain lipids. Carefully transfer the upper hexane phase into a glass tube.
5. Repeat steps 2 and 3 and pool the extracted lipids in hexane from the same sample together.
6. To each Eppendorff tube, add 5 μL of cholesterol reference standard. Let the solvent dry in a vacuum centrifuge or under a fume hood (see Note 4).
7. Prepare TLC plates and TLC tanks as described above (see Subheading 3.3).

8. Resuspend lipids in 50 μL chloroform and apply the entire sample to a TLC plate (see Note 7). Run and stain TLC plates, then cut and transfer cholesterol bands as described above (see Subheading 3.3).
9. To each scintillation vial, add 5 mL of scintillation fluid. Cap the vials and vortex vigorously for 1 min and store overnight in dark before counting.
10. Count samples using a liquid scintillation counter to measure [^{3}H] radioactivity. Count every 24 h until counts are stable.
11. Lipid extraction, TLC separation, and scintillation counting are as described above (see Subheading 3.3).
12. Extract and separate lipids from the cells as described above (see Subheading 3.3).
13. The efflux of endogenously synthesized cholesterol is expressed as percentage of total cholesterol using the following formula:

$$\frac{[^3H]\,Cholesterol\ from\ medium\,(cpm)}{[^3H]\,Cholesterol\ from\ medium\,(cpm)+[^3H]\,Cholesterol\ from\ cell\,(cpm)}\times 100\%,$$

where [^{3}H]Cholesterol from medium: [^{3}H] Radioactivity measured from the cholesterol band in step 10 of Subheading 3.4, and [^{3}H]Cholesterol from cell = [^{3}H] Radioactivity measured from the cholesterol band as described in step 14 of Subheading 3.3.

4. Notes

1. Cholesterol efflux through active transport to cholesterol acceptors in the nervous system has been studied particularly in astrocytes. Apolipoproteins (e.g., apo A-I and apo E) and lipoproteins (e.g., HDL) increased cholesterol efflux through cholesterol transporters ATP binding cassette A1 (ABCA1) and G1 (ABCG1), which are upregulated by LXR/RXR agonists. Whether ABCA1 and ABCG1 are involved in active cholesterol efflux from neurons is still being investigated.
2. Set the scintillation counter to measure [^{3}H] radioactivity for 4 min and to record the average value expressed as counts per minute (cpm). If more than one scintillation counter is used, cpm should be converted to disintegrations per minute (dpm) using the following equation:

$$dpm=\frac{cpm\,sample-cpm\,background}{detector\ efficiency}.$$

3. Allow 4–12 h for the [^{3}H]cholesterol to be fully resuspended and equilibrated in the scintillation fluid. Count the samples at least twice 24 h apart. If [^{3}H]cholesterol is not fully equilibrated, the readings will continue to rise. Record the counts when the readings stabilize.
4. The solvent dries rapidly, and exposure to a nitrogen stream is not necessary. However, samples should not be stored in dried state; suspend them in chloroform as soon as the solvent is evaporated.
5. Prepare the solvents mixture in the tank 30 min to 1 h before running the TLC plates to allow for equilibration between vapor and liquid phase of the solvents in the chamber. Usually 70 mL of the running solvent (see Subheading 2.3) are sufficient to run four TLC plates.
6. Make sure to pre-wet pipette tips before transferring choloroform. Pipette chloroform up and down a few times to saturate the space above the liquid. Otherwise, chloroform will tend to drip from the tip of the pipette because of the low surface tension.
7. The samples could be applied individually using glass capillary tubes or 10 μL micropipette. For each sample, start spotting 5–10 μL a time and let the solvent evaporate before applying more sample. Continue this procedure until the entire sample is applied. This method of sample application allows to load a large volume of sample onto a small spot. The samples could also be applied using TLC autospotter (Analtech, Newark, DE) which applies up to nine samples concurrently.
8. Lipids and sterols will migrate along the TLC plates at different rates due to polarity differences; the solvent mixture moves up by capillary action. The polar lipids have higher retention in the stationary phase (silica), while the nonpolar lipids have less retention and migrate faster along with the mobile phase (running solvent).
9. The bands of cholesterol standard applied to the TLC plates will be lightly stained after 3–4 h and strongly stained overnight.
10. Cholesterol absorbed on the silica of the TLC plate does not easily solubilize in the scintillation fluid. Allow 2–3 days for the radioactivity to equilibrate. For counts to stabilize faster, increase vortex time.
11. The described TLC method separates cholesterol from cholesterol esters and 24(*S*)-hydroxycholesterol. Therefore, determination of cholesterol, cholesterol esters, and 24(*S*)-hydroxycholesterol levels can be carried out concurrently.

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Chapter 28

Dose–Response or Dose–Effect Curves in In Vitro Experiments and Their Use to Study Combined Effects of Neurotoxicants

Matteo Goldoni and Sara Tagliaferri

Abstract

Interactions among neurotoxicants in in vitro models, where the molecular mechanisms of toxicity are generally studied, represent today an emerging field in the experimental neurotoxicology. In this chapter, we define some general concepts about the optimization of in vitro experiments to assess the dose/concentration-effect/response relationships and to extrapolate the functions describing them. After describing the available models to study interactions (the Bliss independence criterion and the Loewe additivity model), we present a method to practically apply these models to experimental data. Finally, we provide some examples of the theory of interactions among neurotoxicants in in vitro models.

Key words: Dose–response curve, Dose–effect curve, Interactions, Synergism, Additivity, Antagonism, Combined exposure, Bliss independence criterion, Loewe additivity model, Hill equation

1. Introduction

Interactions among substances in vivo and in vitro are studied for the assessment of combined favorable/unfavorable effects of treatments in the pharmaceutical field (1). In recent years, however, there has been a growing interest in studying the interactions among environmental toxicants and food contaminants (2).

Several toxicants, at relatively low concentrations, can cause varying degrees of neurological effects particularly on the developing nervous system. Therefore, the study of interactions among neurotoxicants in in vitro models, where the molecular mechanisms of toxicity are usually studied, represents today an emerging field in the experimental neurotoxicology (3). In the literature, there is confusion on the concept of "additivity," "synergism,"

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and "antagonism." In fact, without the use of any mathematical model to describe interactions, "synergism" is erroneously defined as an increased effect as compared to the effect of single toxicants composing the mixture. However, in some cases, this observed effect could be additive or even antagonistic, depending on the curves that describe the response/effect of a toxicant on a cellular line, when its concentration increases. *Additivity* means that two or more toxicants act without any interaction among them. In other words, the overall effect is not different from what we expect from concentration-effect/response relationship of the single compounds (2). Additivity can change on the basis of the model used to study interactions (see below). On the other hand, *antagonism* and *synergism* will be present when the overall effect is, respectively, lower and higher than expected (2). Therefore, it is not possible to assess the interaction among toxicants without defining the expected additive effect as a comparison term for synergism and antagonism. From a molecular point of view, we can simplify the cell as a system having a number of binding sites for the toxicant. Every toxicant has a specific affinity for similar or different binding sites (*target site*). Each binding induces a quantifiable direct/nondirect effect. *Interaction* means that the affinity is positively/negatively modified by the presence of other toxicants. This may occur as a direct modification of binding constant, a modification of the cause/effect chemical pathway, or an amplification of the observed effect induced by the binding. The chemical pathways describing these phenomena can be very complex, with several in series and in parallel steps. However, they can be studied only when a specific molecular pathway is known. In most cases, when macroscopic effects are assessed in in vitro systems (viability, cell count, % of apoptotic or necrotic cells, oxidative stress, etc.), the molecular action of the toxicant cannot be entirely defined; however, the significance of interaction remains valid, as it quantifies the "deviation" from additivity, independent of its nature (4). Some relatively simple mathematical models to study interactions have been defined, though not all scientists agree on their biological plausibility (5). However, they are extremely useful to describe the interactions among toxicants in particular in in vitro models, where repeatability of the experiments is ensured and the system could be well described by mathematical functions. Aim of this chapter is: to define some general concepts of the optimization of in vitro experiments to assess the dose/concentration-effect/response relationships and to extrapolate the functions describing them; to briefly describe the available models to study interactions; to present a method to practically apply these models to the experimental data; and to review some samples on interactions derived from the recent in vitro neurotoxicology literature.

2. The Dose–Response and Dose–Effect Curves to Assess the In Vitro Toxicity: Optimization of the Experiments

2.1. Definitions

The term "dose–response relationship" defines the relationship between the amount of a toxicant administered and the subsequent effect. The *dose–response curve* (DR curve) is the mathematical function of best fit that changes as a function of the increase of dose of the toxicant. In in vitro models, one usually refers to concentration–response relationship and concentration–response curve. The effect measured in the in vitro system is usually a change in cell viability, an increase of cellular death (both necrosis and apoptosis), or a decrease in cell number.

In contrast, in in vitro experiments, a change in the magnitude of a continuously graded effect is usually measured, when one measures a change in continuous variables (e.g., enzymatic activity, biochemical endpoints, etc.). The *dose–effect curve* (DE curve) is the mathematical function of best fit which shows the trend of a specific effect as a function of the concentration–dose of the toxicant.

In this chapter, we use the nomenclature DR curve and DE curve also for in vitro models, bearing in mind that not all the scientists identify the term "Dose" with "Concentration."

2.2. The Experimental In Vitro Procedure: One Experiment with a Large Number of Replicates or a Large Number of Experiments with Few Replicates?

When we decide to perform a specific test to study the toxicity of a determined compound at any concentration, it is fundamental to find DR/DE curves describing the observed response/effect. Thus, we have to choose the conditions of the experiment to ensure the highest repeatability and experimental accuracy. In particular, the time of exposure and the nominal concentrations of the toxicant should be accurately checked. Finally, the mathematical curve describing the data should be extrapolated, as its definition is mandatory to study interactions.

The quality of the results is also ensured by the assessment of the number of replicates/experiments that should be performed at any fixed nominal concentration of the toxicant. There are essentially two possibilities: (a) performing the experiment once, but with a high number of replicates for each point; (b) performing the experiments more than once, with a relatively low number of replicates.

This choice is of fundamental importance, since the statistical approach to define central tendency and dispersion is essentially different. The number of degrees of freedom also differs.

Case (a) is the most simple; it is sufficient to calculate mean and SD, and use $n-1$ degrees of freedom (df) for statistical analysis. The statistical power is increasing with the number of replicates. The approach to consider all the experimental measures together, as if the experiment is one, is preferable, if the number of experiments is low (we suggest $N=3$). Case (b) could be

extremely useful, when repeatability is also checked. However, the statistical calculations are more complex.

If we perform k experiments, with $n_1,\ldots n_n$ replicates (this may vary from experiment to experiment), the overall mean of the experiments should be calculated, weighting the mean of each single experiment both for each own SD and the number of replicates. If every experiment $(1,\ldots,k)$ has mean (M_i) and standard error $\left(\mathrm{SE}_i = \frac{\mathrm{SD}_i}{\sqrt{n_i}}\right)$, the weighted mean is calculated as follows:

$$M_{\text{best}} = \frac{\sum_{i=1}^{k} M_i w_i}{\sum_{i=1}^{k} w_i}, \tag{1}$$

where $w_i = \frac{1}{\mathrm{SE}_i^2} = \frac{n_i}{\mathrm{SD}_i^2}$ is the weight of the mean of each experiment. The experiments with the lowest dispersion and/or the highest number of replicates have the highest weight. Obviously, the hypothesis underlying this calculation is that M_i are normally distributed.

The estimation of SD of M_{best} is more complicated and follows the equations presented by Bland and Kerry (6), with the complication that in a model where the effect is extrapolated by a continuous endpoint, we have to use the formulation of w_i given above. Briefly, one can proceed as follows:

$$\text{Weighted sum of observations squared } (s^2) = k\frac{\sum_{i=1}^{k} M_i^2 w_i}{\sum_{i=1}^{k} w_i}, \tag{2}$$

$$\text{Correction term } (C) = kM_{\text{best}}{}^2, \tag{3}$$

$$\text{Sum of squared about the mean } (S^2) = s^2 - C, \tag{4}$$

$$\text{Weighted estimation of SD } (\sigma_{\text{best}}) = \sqrt{\frac{s^2}{k-1}}. \tag{5}$$

In this case, however, the number of df for each statistical comparison is $k-1$.

A more approximated method would be to calculate the weighted mean of SD and to perform statistical tests of comparisons with $n-1$ df, only if all experiments have the same number of replicates.

2.3. The Steady-State Status and the Nonlinear Fitting

The use and interpretation of DR/DE curves in vitro requires particular attention, due to the nature of the system we are studying. With in vitro models, it is not possible to define an "equilibrium" state, where the system remains unmodified during a certain time. In fact, cellular growth of immortalized cell lines or the death of primary cells is governed by microkinetic parameters that can continuously alter the macroparameters of the system. Therefore, the shape and the slope of the curves depend on the kinetic processes both in growth/death and in toxicant action (toxicant uptake, toxicant binding, induction of an effect, etc.). In the absence of kinetic models describing the variations of the system, it is preferable to reach a "steady state" of the system, e.g., a state where all the parameters of the cellular population remain substantially unaltered for several hours, and to apply fitting functions, some of which require the equilibrium to be applied, in those conditions (4, 7). Kinetic processes should be studied with adequate instruments that take into account of time (toxicokinetics) (8).

Finally, in in vitro experiments, the functions that fit the data are often nonlinear, and a software dedicated to nonlinear curve fitting is necessary. Besides the algorithm used, the best-fit curve should have both biological and mathematical plausibility, and several functions have been reported. Nonlinear fitting allows to calculate several parameters, such as the effective median concentration (EC50) or the inhibitory median concentration (IC50), the same parameters at 10% effect/inhibition (EC10 – IC10), the slope of the phenomenon we are studying, and the data asymmetry. Without a best-fit curve, only the significance in differences between pairs of concentrations is calculable, but it is impossible to comprehend the overall phenomenon underlying the observed differences. It is impossible to mathematically define a threshold dose, as the benchmark dose (BMD) or to apply more sophisticated equations to study interactions (see below) (7).

2.4. The Hill Function

This function is the most used one to fit the DR/DE curves. Its biological plausibility and adaptability to several biochemical processes and its simplicity make it ideal to be used in combination studies. Its properties are summarized in the literature (4, 7, 9, 10).

The general formula $E = E(x)$ of the Hill Function can be generalized as follows:

$$E = A_1 + (A_2 - A_1)\frac{X^n}{(\mathrm{EC50})^n + X^n}. \tag{6a}$$

The model can be easily extended to the case where we have a decrease of a biological parameter (e.g., a decrease in cell viability) despite of an increasing effect:

$$V = A_1 + (A_2 - A_1)\left(1 - \frac{X^n}{(\mathrm{IC50})^n + X^n}\right) \tag{6b}$$

V is the viability, A_1 the background, and A_2 the amplitude of the effect or a biological decrease of a parameter, and n the so-called "cooperativity index" and it is bound to the steepness of the curve (7).

2.5. Conclusions

In conclusion, apart from the quality of experimental data, to define DR/DE curves, it is necessary to follow a number of steps, which are fundamental for defining the biological phenomenon we are studying: (1) statistical treatment of the data to define response/effect as a function of the concentration of the toxicant; (2) definition of a steady state of the system (on the contrary, kinetic aspects should be assessed using toxicokinetic models); and (3) the use of an appropriate function to fit the data (Hill function is particularly used). This allows the definition of specific threshold doses to compare the potency of the toxicants, to try an integrated approach, which includes different in vitro endpoints, and to compare in vitro with in vivo data. Finally, this approach allows the study of interactions among different toxicants.

3. The Study of Interactions in Combined Exposure

The models described below can be used, only when the system is in a steady state, as defined in Subheading 2.3. In fact, interactions can also alter the kinetic processes induced by toxicants without altering the final steady state, and therefore kinetics should be always studied with specific models of toxicokinetics.

3.1. The Bliss Independence Criterion and the Loewe Additivity Model

Given the hypothesis postulated above, two models are mostly used in several forms or equations to study interactions among toxicants.

3.1.1. The Bliss Independence Criterion

The main assumption of this model is that two or more toxic agents act independently from one another (5, 11). In other words, the target sites of the toxicants are never overlapping. Therefore, the mode and/or the site of action of the compounds in the mixture are completely independent. In some cases, this assumption is supported by data, in particular, when drugs with different target sites are used in in vitro models to precede further studies of polytherapy (12).

The noninteraction curve (additivity) is described in a n-dimension space by the following formula, when we observe a response/effect (E) increasing with the concentration of the n-considered toxicants (11, 13):

$$E(x_1,\ldots,x_n) = A_1 + (A_2 - A_1)\times\left[1 - \prod_{i=1}^{n}(1 - E(x_i))\right]$$
$$= A_1 + (A_2 - A_1)\times[1 - (1 - E(x_1))(1 - E(x_2))\ldots], \quad (7a)$$

where $E(x_1,\ldots, x_n)$ is the response/effect normalized between 0 and 1 in the combined effect at concentrations $x_1,\ldots, x_n$ of the toxicants; $E(x_i)$ is the response/effect observed for the single ith toxicant alone; A_1 is the background, and A_2 the amplitude of the effect (response is normally defined between 0 and 1 or 0 and 100%).

When we observe a decrease in a signal or in a frequency (e.g., cellular viability), the formula is further simplified. In fact, it is demonstrable that defining $V = 1 - E$ and therefore $V(x_1,\ldots, x_n) = 1 - E(x_1,\ldots, x_n)$ and $V(x_i) = 1 - E(x_i)$, the following equation is valid:

$$V(x_1,\ldots,x_n) = A_1 + (A_2 - A_1)\times \prod_{i=1}^{n} [V(x_i)] = A_1 + (A_2 - A_1)\times[V(x_1)V(x_2)\ldots]. \quad (7b)$$

With two toxicants and a normalized signal, the formulae become:

$$E(x_1,x_2) = E(x_1) + E(x_2) - E(x_1)\times E(x_2), V(x_1,x_2) = V(x_1)\times V(x_2). \quad (8a)$$

With three toxicants and a normalized signal, the formulas become:

$$E(x_1,x_2,x_3) = E(x_1) + E(x_2) + E(x_3) - E(x_1)\times E(x_2) - E(x_1) \times E(x_3) - E(x_2)\times E(x_3) + E(x_1)\times E(x_2)\times E(x_3),$$
$$V(x_1,x_2,x_3) = V(x_1)\times V(x_2)\times V(x_3). \quad (8b)$$

The hypotheses of this model are: (1) all the toxicants have an effect/response in the same range of values (A_1, A_2); (2) the combined effect/response is always reproducible with a single concentration of x_i in a single toxicant exposure.

This model is relatively simple to apply and could also be applied to single concentrations of toxicants in the absence of entire DR/DE curves (4).

As these equations define the noninteraction curve, every E significantly higher than expected in the combination experiments signifies synergism. In contrast, E significantly lower than that expected in the combination experiments is an indication of antagonism.

Unless the independence among toxicants is demonstrated, this model has important limitations (4): (1) In case of steep dose–response/effect curves, synergism could be overestimated. This is particularly true when toxicants are considered independent without any scientific evidence: the mutual dependence, not evaluated by the model, particularly when the effect/response is under 50% of the total, is considered as a synergistic effect. (2)

The biological plausibility of the model is poor: except for some specific cases, e.g., when some macroscopic parameters are measured (cell viability or death, oxidative stress, DNA damage, and so on), toxicants tend to have at least in part common target sites. The model gives therefore an excessive simplification of the noninteraction curve.

3.1.2. The Loewe Additivity Model

The main assumption of this model is that two or more toxic agents act on the same biological sites (or different indistinguishable binding sites) (5, 14). Even if the same mechanism of action is assumed (i.e., the difference between toxicants is only in their potency), this does not mean that the DR/DE curves of single toxicants are parallel (4).

The noninteraction curve (additivity) is described in a n-dimension space by the following formula, when we observe a response/effect (E) increasing with the concentration of the n-considered toxicants:

$$\sum_{i=1}^{n} \frac{x_i}{X_i} = 1, \tag{9}$$

where x_i is the concentration of ith toxicant at which the combined E is performed [$E = E(x_1,\ldots,x_n)$], and X_i is the concentration of the same toxicant at which the same E is observed, when it is used in a single combined exposure experiment (e.g., the pure DR/DE curves).

Sometime, the concentration of a toxicant is log-transformed and the DR/DE curves are calculated on $\log(x_i)$.

The hypothesis of the model is that $\exists X_1,\ldots, X_n$: $E(x_1,\ldots, x_n) = E(X_1) = E(X_n)$. In other words, it is not possible to entirely apply this model without defining the equation of the DR/DE curves of the single toxicants, and all these equations should be defined in the same finite range of values (e.g., 0–1, 0–100%, A_1–A_2, etc.). Mathematically:

$$\lim_{X_{i\to 0}} E(X_i) = A_1 \,(\text{finite}),$$

$$\lim_{X_{i\to \infty}} E(X_i) = A_2 \,(\text{finite}).$$

The model can be easily extended to the case where we have a decrease of a biological parameter (e.g., a decrease in cell viability): $V = V_0 - E$ despite of E. It is theoretically possible to apply the model also to the case where E (but more frequently V) is represented by linear or log-linear models. However, they are an approximation of nonlinear functions, because it is very rare that

$$\lim_{X_{i\to \infty}} E(X_i) = \infty \;\text{ or }\; \lim_{X_{i\to \infty}} V(X_i) = -\infty.$$

For this reason, only functions with finite values as extreme limits will be considered in this chapter.

The hypotheses are substantially the same as Bliss independence criterion. There will be synergism when $\sum_{i=1}^{n} \frac{x_i}{X_i} < 1$ and antagonism when $\sum_{i=1}^{n} \frac{x_i}{X_i} > 1$.

This model has also some limitations (4): (1) It should be used only with simple systems, where the main parameters are measurable. The most simple one is the effect of combined inhibitors on enzyme activity. Its use in toxicology is recommended with in vitro models, because the parameters of the experiments are relatively controllable, whereas its use in complex biological systems is only an approximation. (2) If dose–response/effect curves are steep, a partial independence of target sites is estimated by the model as antagonism, which can be therefore overestimated.

3.2. Which One Is the Best Model?

In the absence of specific knowledge on the mechanism of molecular interactions between a given toxicant and its target sites, a definitive agreement on whether the Bliss independence criterion or the Loewe additivity model is the best one to use, is still lacking. In some cases, the results they provide may differ, and lead to different or ever opposite conclusions. However, the Loewe additivity model is generally preferred, because of a higher biological plausibility (5). The Bliss independence criterion is preferred in specific fields of toxicology, such as combined irradiation (2), and when it is demonstrated that the two compounds have independent target sites. Several formulations of the Loewe additivity model exist and specific softwares capable of finding the noninteraction surface and/or establishing whether a combined effect is significantly synergistic or antagonistic have been created. When both models have been applied, often the general results and conclusions coincide.

Furthermore, as discussed in Goldoni and Johansson (4), the use of several in vitro tests of asynchronous exposure (15) and the summary of overall data could add important elements for the choice of a model over another (4).

3.3. The Applicative Basis of the Bliss Independence Criterion to the Loewe Additivity Model with Two Toxicants

With the in vitro combined exposure to two toxicants (X and Υ), we need to know the DR/DE curves of the single compounds and all the mathematical parameters which describe them [$E_1(X)$ and $E_2(\Upsilon)$]. The starting point is that $E(x,y)$ (combined exposure to known concentrations of both toxicants) $= E_1(X) = E_2(\Upsilon)$. In other words, it is mandatory that the amplitude of the effect for both toxicants and their combination has the same amplitude.

The use of the Bliss criterion is simplified by the fact that we can directly insert $E_1(X) = E_2(\Upsilon)$ in Eq. 8a.

The application of Loewe additivity model requires more algebraic passages. If $E_1(X)$ and $E_2(\Upsilon)$ are invertible functions, we can calculate:

$$X = F^{-1}(E_1) = G_1(E), \quad \Upsilon = F^{-1}(E_2) = G_2(E).$$

Substituting them in Eq. 9, we obtain the following equation for the noninteraction surface:

$$\frac{x}{G_1(E)} + \frac{y}{G_2(E)} = 1. \tag{10}$$

Greco et al. define the interaction index α, which is calculable with its confidence interval, as the result of the sum of Eq. 9 (5, 16):

$$\frac{x}{G_1(E)} + \frac{y}{G_2(E)} = \alpha \Rightarrow xG_2(E) + yG_1(E) = \alpha G_1(E)G_2(E). \tag{11}$$

The equation has been applied with a specific DR curve, the median-effect equation (see next sections). The iterative approach proposed by Whitehead et al. using the software SAS® is also very interesting and extensible to Bliss independence criterion (17). In this case, synergism/antagonism with Loewe additivity model is estimated by the following equation (two toxicants):

$$\left(\frac{x_1}{X_1}\right)^{\varepsilon} + \left(\frac{x_2}{X_2}\right)^{\varepsilon} = 1, \tag{12}$$

$\varepsilon = 1$ means additivity, $0 < \varepsilon < 1$ synergism, and $\varepsilon > 1$ antagonism. The SAS® script provides the calculation of the confidence interval of ε. Another approach, namely, isobolographic method has been already described and it is not reported here (4).

The approach we present is a direct application of Eqs. 8a and 10 and is only based on the best-fit parameters of single DR/DE curves (4, 18).

4. Studying Interactions: A Practical Example of the Application of Bliss Independence Criterion and Loewe Additivity Model

The method presented by Goldoni et al. and Vettori et al. (4, 18) is only one of the possible methods to study interaction, and it is described in detail with an example taken from Tagliaferri et al. (19), where the authors have investigated the viability decrease of neuronal SK-N-MC cells after exposure to single and combined exposures of 24 h, to two polybrominated diphenyl ethers (PBDEs), BDE-47 and BDE-99.

PBDEs are a class of flame retardants, largely used in different consumer products and they have been found in the environment, animals, food, and human biological samples. Many studies have provided indications about their toxic effects, in particular, in the early stages of central nervous system development, but the knowledge about the potential mechanism of action is still elusive.

Because of the absence of information about the mechanism of action of the two PBDEs congeners, both the Bliss independence criterion and the Loewe additivity model were chosen to study the interaction among them.

4.1. Defining DR Curves of Single Compounds

The authors have fitted experimental data of a single DR curve by describing the decrease in cell viability as a function of single BDE-47 and BDE-99 concentrations, with the Hill function (Eq. 6b) defining the following parameters:

$$A_2 - A_1 = V_{0,1} = 0.99 \pm 0.03; \quad n_1 = 2.0 \pm 0.2;$$
$$\mathrm{IC50}_1 = 10.8 \pm 0.7\ \mu\mathrm{M(BDE\ -\ 47)}, \tag{13a}$$

$$A_2 - A_1 = V_{0,2} = 0.97 \pm 0.02; \quad n_2 = 1.7 \pm 0.2;$$
$$\mathrm{IC50}_2 = 33.9 \pm 1.6\ \mu\mathrm{M(BDE\ -\ 99)}. \tag{13b}$$

However, being the $\lim_{\mathrm{PBDEs}\to 0} V = 1$ for both DR curves, we have normalized the functions in the following way:

$V_{0,1} = V_{0,2} = 1.00 \pm 0.03$, being 0.03 the maximum between the errors of $V_{0,1}$ and $V_{0,2}$, it means that the biggest variability is taken into account (Fig. 1).

Depending on the aim of the study, different ranges of concentrations can be chosen for the combination exposures. In this case, we suppose to consider the combined exposure to BDE-47 1 μM and BDE-99 5 μM, giving the experimental viability of $V_{\mathrm{exp}} = 0.76 \pm 0.07$. What about the theoretical noninteraction V (V_{theor}) applying the Bliss independence criterion and Loewe additivity model?

4.2. The Bliss Independence Criterion V Value of Noninteraction

In this case, the calculation is not complicated. We have only to consider the expected V(BDE-47 = 1 μM) and V(BDE-99 = 5 μM) obtained with the single DR curve described by parameters 13a and 13b:

$$V(\mathrm{BDE\ -\ 47} = 1\ \mu\mathrm{M}) = 0.992; \quad V(\mathrm{BDE\ -\ 99} = 5\ \mu\mathrm{M}) = 0.963.$$

From Eq. 8a, we have

$$V_{\mathrm{theor}} = V(\mathrm{BDE\ -\ 47} = 1\ \mu\mathrm{M}) \times V(\mathrm{BDE\ -\ 99} = 5\ \mu\mathrm{M}) = 0.955.$$

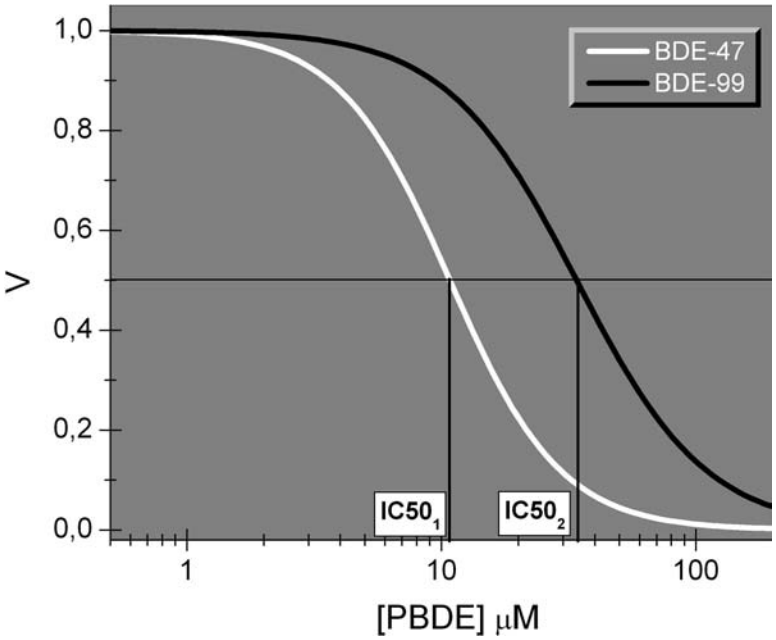

Fig. 1. The DR curves (Hill function) representing cell viability (V) as a function of BDE-47 and BDE-99 concentration on the basis of the parameters 13a and 13b, normalizing V_0 to 1. IC50s are also reported.

4.3. The Loewe Additivity Model *V* Value of Noninteraction

In this case, the calculation is more complicated. It is demonstrable that the application of Eq. 10 with two different Hill functions and with V as a varying parameter ($V_0 = 1$), we obtain (4, 18):

$$x \times \mathrm{IC50}_2 \times Z^{1/n_2} + y \times \mathrm{IC50}_1 \times Z^{1/n_1} = \mathrm{IC50}_1 \times \mathrm{IC50}_2 \times Z^{1/n_1 + 1/n_2}, \tag{14}$$

where x is the concentration of the first toxicant and y that of the second one in the combined exposure, $Z = (1 - V)/V$.

In the example with $x = 1$ μM (BDE-47) and $y = 5$ μM (BDE-99) and using the parameters reported in Eqs. 13a and 13b, we obtain:

$$1 \times 33.9 \times Z^{0.588} + 5 \times 10.8 \times Z^{0.5} = 33.9 \times 10.8 \times Z^{(0.588+0.5)}. \tag{15a}$$

In other words, we have to resolve the following equation:

$$33.9 \times Z^{0.588} + 54 \times Z^{0.5} - 366.12 \times Z^{1.088} = 0, \tag{15b}$$

whose solution is the root different from $Z = 0$. It can be graphically done (Fig. 2) or we can use specific softwares which calculate the roots of functions (e.g., functions available at the site http://www.numericalmathematics.com).

In this case, $Z = 0.077$ and therefore $V_{\mathrm{theor}} = 0.929$.

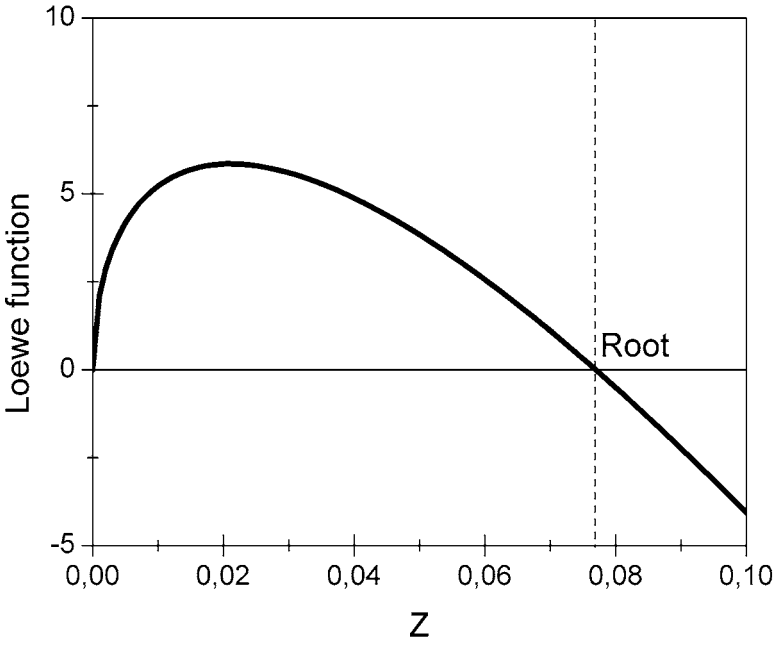

Fig. 2. The Loewe function represented by Eq. 15b, varying Z value. The root $\neq 0$ is also reported.

4.4. The Range (Min, Max) of V_{theor}

On the basis of the errors of the parameters kept variable in the fitting of the pure curves (13a and 13b), it is possible to find the minimum and maximum slope curves (Fig. 3), which define the interval of variability of single points of the noninteraction surface:

1. Maximum:

$$V_{\mathrm{MAX}} = (V_0 + \delta V_0)\left(1 - \frac{x^{n+\delta n}}{(\mathrm{IC50} + \delta \mathrm{IC50})^{n+\delta n} + x^{n+\delta n}}\right), \quad \forall x < x_{\mathrm{MAX}}, \tag{16a}$$

$$V_{\mathrm{MAX}} = (V_0 + \delta V_0)\left(1 - \frac{x^{n-\delta n}}{(\mathrm{IC50} + \delta \mathrm{IC50})^{n-\delta n} + x^{n-\delta n}}\right), \quad \forall x \geq x_{\mathrm{MAX}}, \tag{16b}$$

2. Minimum:

$$V_{\mathrm{MIN}} = (V_0 - \delta V_0)\left(1 - \frac{x^{n-\delta n}}{(\mathrm{IC50} - \delta \mathrm{IC50})^{n-\delta n} + x^{n-\delta n}}\right), \quad \forall x < x_{\mathrm{MIN}}, \tag{16c}$$

$$V_{\mathrm{MIN}} = (V_0 - \delta V_0)\left(1 - \frac{x^{n+\delta n}}{(\mathrm{IC50} - \delta \mathrm{IC50})^{n+\delta n} + x^{n+\delta n}}\right), \quad \forall x \geq x_{\mathrm{MIN}}. \tag{16d}$$

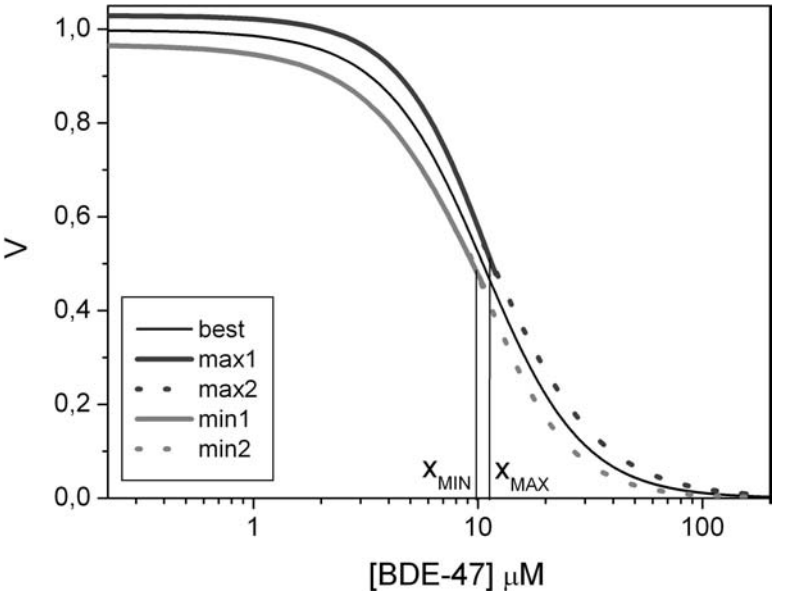

Fig. 3. The DR curve of BDE-47 as in Fig. 1 (best) with the maximum (Eqs. 18a = max1 and 18b = max2) and the minimum (Eqs. 18e = min1 and 18f = min2) curves. The intersections x_{MIN} and x_{MAX} are also reported.

where x_{MAX} and x_{MIN} are the cross points of the two curves of maximum and minimum, and d represents the fitting error of the parameters. These points vary both on the basis of the fitting curve and its goodness-of-fit. Based on the four equations given above, we can apply Bliss independence criterion or Eq. 14 by combining the two maximum curves for the first toxicant with the two maximum curves of the second one (four combinations) and the two minimum curves (other four combinations), obtaining so $V_{theor,MAX}$ and $V_{theor,MIN}$ for both Bliss independence criterion and Loewe additivity model, representing a confidence interval approximable to SD of V_{theor}:

$$\mathrm{SD}\,(V_{theor}) = \min(V_{theor,MAX} - V_{theor}; V_{theor} - V_{theor,MIN}). \qquad (17)$$

Look at the example:

Maximum (BDE-47):

$$V_{MAX} = 1.03\left(1 - \frac{x^{2.2}}{(11.5)^{2.2} + x^{2.2}}\right), \quad \forall x < x_{MAX}\,(\text{near IC50}), \qquad (18a)$$

$$V_{MAX} = 1.03\left(1 - \frac{x^{1.8}}{(11.5)^{1.8} + x^{1.8}}\right), \quad \forall x \geq x_{MAX}. \qquad (18b)$$

Maximum (BDE-99):

$$V_{\mathrm{MAX}} = 1.03\left(1 - \frac{x^{1.9}}{(35.5)^{1.9} + x^{1.9}}\right), \quad \forall x < x_{\mathrm{MAX}}(\text{near IC50}), \tag{18c}$$

$$V_{\mathrm{MAX}} = 1.03\left(1 - \frac{x^{1.5}}{(35.5)^{1.5} + x^{1.5}}\right), \quad \forall x \geq x_{\mathrm{MAX}}. \tag{18d}$$

Minimum (BDE-47):

$$V_{\mathrm{MIN}} = 0.97\left(1 - \frac{x^{1.8}}{(10.1)^{1.8} + x^{1.8}}\right), \quad \forall x < x_{\mathrm{MIN}}(\text{near IC50}), \tag{18e}$$

$$V_{\mathrm{MIN}} = 0.97\left(1 - \frac{x^{2.2}}{(10.1)^{2.2} + x^{2.2}}\right), \quad \forall x \geq x_{\mathrm{MIN}}. \tag{18f}$$

Minimum (BDE-99):

$$V_{\mathrm{MIN}} = 0.97\left(1 - \frac{x^{1.5}}{(32.3)^{1.5} + x^{1.5}}\right), \quad \forall x < x_{\mathrm{MIN}}(\text{near IC50}), \tag{18g}$$

$$V_{\mathrm{MIN}} = 0.97\left(1 - \frac{x^{1.9}}{(32.3)^{1.9} + x^{1.9}}\right), \quad \forall x \geq x_{\mathrm{MIN}}. \tag{18h}$$

As the considered concentrations (BDE-47 = 1 μM; BDE-99 = 5 μM) are lower than respective IC50s, we will combine only Eqs. 18a and 18c for the maximum, and Eqs. 18e and 18g for the minimum.

For the Bliss independence criterion:

$$V_{\mathrm{theor,MAX}} = 1.03V_{\mathrm{MAX}}(\mathrm{BDE}\text{ - }47 = 1\ \mu\mathrm{M}, V_0 = 1)$$
$$V_{\mathrm{MAX}}(\mathrm{BDE}\text{ - }99 = 5\ \mu\mathrm{M}, V_0 = 1) = 1.03 \times 0.995 \times 0.976 = 1.00,$$

$$V_{\mathrm{theor,MIN}} = 0.97V_{\mathrm{MIN}}(\mathrm{BDE}\text{ - }47 = 1\ \mu\mathrm{M}, V_0 = 1)$$
$$V_{\mathrm{MIN}}(\mathrm{BDE}\text{ - }99 = 5\ \mu\mathrm{M}, V_0 = 1) = 0.97 \times 0.985 \times 0.943 = 0.90.$$

Therefore, $V_{\mathrm{theor}} = 0.955\ (0.900 - 1.00) \sim 0.955 \pm 0.055$

For the Loewe additivity model:

$$V_{\mathrm{theor,MAX}} = 1 \times 35.5 \times Z^{0.526} + 5 \times 11.5 \times Z^{0.455}$$
$$= 35.5 \times 11.5 \times Z^{(0.526+0.455)}, Z = 0.057,$$
$$V_{\mathrm{theor,MAX}} = 1.03 / 1.057 = 0.974,$$

$$V_{\mathrm{theor,MIN}} = 1 \times 32.3 \times Z^{0.667} + 5 \times 10.1 \times Z^{0.555}$$
$$= 32.3 \times 10.1 \times Z^{(0.667+0.555)}, Z = 0.112,$$
$$V_{\mathrm{theor,MAX}} = 0.97 / 1.112 = 0.872.$$

Therefore, $V_{theor} = 0.929\ (0.872 - 0.974) \sim 0.929 \pm 0.057$.

The calculations, which could appear quite complicated, are in reality only algebraic and therefore easily incorporable into an automatic algorithm. The slight differences in the parameters respect to the data published by Tagliaferri et al. arise from the rounding.

4.5. The Statistical Comparison Between V_{exp} and V_{theor}

Now, we have to check if the experimental viability value in the combined treatment (0.76 ± 0.07) is significantly different from the theoretical ones (0.955 ± 0.055 calculated with the Bliss model, 0.929 ± 0.057 obtained with the Loewe one).

Degrees of freedom: since the combinations normally reflect experimental points used to calculate DR/DE curves, we will consider total degrees as the sum of replicates/experiments necessary to calculate the viability of single curve: eight replicates for BDE-47 and eight replicates for BDE-99 in our experiments $\Rightarrow N = n_1 + n_2 = 1$. The fitting curve implies the presence of five constraints: V_0 (common to both curves), n_1, n_2, $IC50_1$, $IC50_2$. Therefore, we have:

Degrees of freedom $(df) = n_1 + n_2 - 5 = 11$ in the example. V_{exp} will simply have $n_3 - 1df$ (7 in our example).

Now, we can do a simple Student's *t*-test and in our example the difference is highly significant with both Bliss Independence criterion and Loewe additivity model ($p < 0.001$).

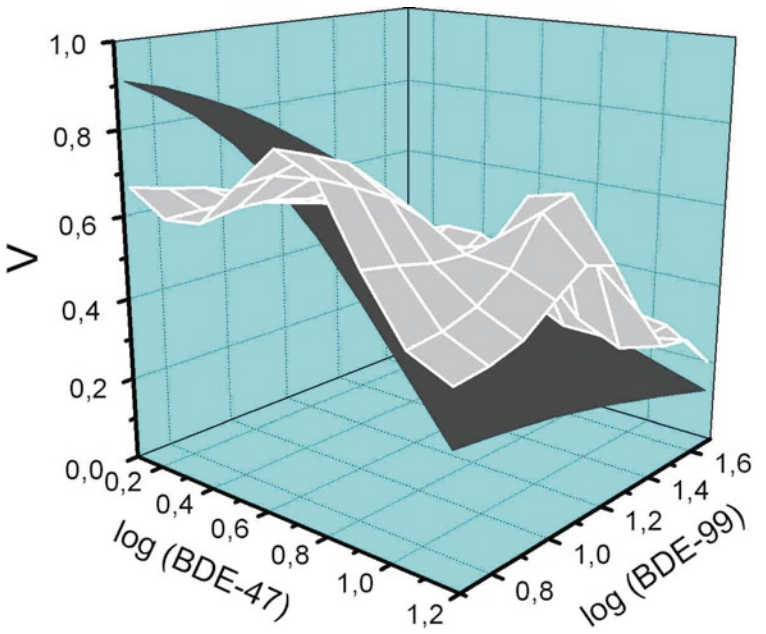

Fig. 4. The theoretical Loewe noninteraction surface built up starting from different combination points and the experimental surface obtained by plotting the 3D experimental points of single combinations (19). At low concentrations of BDE-47, a general synergism is evident, whereas at high concentration of both BDE-47 and BDE-99 antagonism is evident.

4.6. Conclusions

With the method proposed by Vettori et al. (18), it is possible to define the entire noninteraction surface with its minimum and maximum values with both models, and without any limitation (Fig. 4). It gives the advantage that all the range of concentrations of two toxicants can be studied.

5. Study of Interactions in In Vitro Neurotoxicology Studies

Relatively few in vitro studies have considered the potential impact of interactions in neurotoxicology, while several combined exposure experiments have been carried out in vivo in animals (20–22).

Stavenes Andersen et al. studied the interactions between binary mixtures of known neurotoxic environmental and food contaminants (23). This study investigated the interactions of methyl mercury (MeHg) in combination with polychlorinated biphenyls (PCBs) or brominated flame retardants (BFRs), examining their effects on the reuptake of glutamate by synaptosomes. To analyze the type of interaction, the Bliss independent criterion and the Loewe additivity model were used: mixtures were prepared at a fixed ratio of concentrations for each binary mixture (MeHg:PCB or MeHg:BFR), according to their respective IC25 values. Interestingly, even if the two models are based on opposite assumptions, with regard to the chemical's target sites, both approaches yielded similar results, suggesting similar additive or synergistic trends. More specifically, the Bliss model gave the best prediction for some pairs of compounds (e.g., MeHg:PCB153), whereas the Loewe approach was more appropriate for others. As already stated, the choice of one model over of another is due to differences between the mode of action of chemicals, such as their mechanisms of action, potency, or above all, and differences in the shape of the DR/DE curves of each compound.

The study by Vettori et al. highlights the importance of the shape of the dose–response curve of individual compounds when evaluating interactions (18). In this study, the interaction between two neurotoxicants, MeHg and PCB153, was evaluated. The dose–response curve of PCB153 was steeper than that of MeHg. To analyze PCB/Me–Hg interaction, only the Loewe additivity model was used with viability as an endpoint, but the validity of the model was confirmed by other endpoints (e.g., oxidative stress) and by the results obtained during an asynchronous exposure (4). In this study, only additive or antagonistic effects were observed. The absence of synergistic effects between MeHg and PCBs has also been confirmed in another study using a more empirical interaction model (24).

The Bliss independence criterion and the Loewe additivity model were used to assess the potential interaction between different PBDEs, BDE-47 and BDE-99 in vitro (19). The chemicals belong to the same family of contaminants, but their mechanisms of toxicity are still not completely understood. After defining the individual concentration–response curves, calculated by the Hill function, the parameters of the equation (IC50 and cooperativity index) were used to predict the additivity surface between the two toxicants. Various combinations of concentrations of the BDE-47 and BDE-99 were tested using cell viability of human neuronal cells as the end point. Both synergistic and antagonistic effects on decreased cell viability were observed, depending on the concentrations of BDE-47 and BDE-99. Specifically, low concentrations of BDE-47 combined with a wide range of concentrations of BDE-99 resulted in synergism. In contrast, antagonism was observed when high concentrations of BDE-47 were combined with a range of concentrations of BDE-99. Interestingly, the synergistic and the antagonistic interactions were confirmed by both the models, suggesting that the observed interactions accurately reflect the real behavior of the compounds. The interaction between BDE-47 and BDE-99 provided important information pertaining to the different behavior of the two contaminants at different ranges of concentration. The synergism observed at low doses is especially interesting, because it occurs at environmentally relevant concentrations.

Lau et al. assessed the potential interactions between pairs of four common food additives in the in vitro model (25). The Bliss independence criterion and the Loewe additivity model were used to calculate the theoretical noninteraction surface. Applying the Bliss model, the authors compared the single treatment with each food additive at a concentration that led the 20–25% inhibition of neurite outgrowth with a mixture supposed to produce the same effect of 20–25% inhibition (if their effects were simply additive). Significant synergistic interactions were observed between Brilliant blue and L-glutamic acid, and between Quinoline Yellow and the artificial sweetener aspartame.

In contrast, in the assessment of the interaction by the isobolographic method, they used concentrations of compounds which inhibited neurite outgrowth by 50% (IC50 value), in order to define the additivity straight line. By testing some combinations of concentrations of the chemicals, they obtained isobolograms, which allowed to describe synergism between L-glutamic acid and Brilliant Blu and between Quinoline Yellow and Aspartame. Also in this case, two different models, based on opposite assumptions, described the same kind of interaction.

6. Final Conclusions

In conclusion, in vitro models appear particularly useful to study combined neurotoxicity and to assess the molecular processes that could influence the interaction between neurotoxicants in vivo. Therefore, the choice of mathematically driven models to define DR/DE curves and to study interactions, despite their relatively high complexity, appears today as a necessary approach to assess interactions along wide range of concentrations of neurotoxicants. In the absence of specific knowledge about toxicity pathways, both the Bliss and the Loewe models should be used, even if the Loewe model appears to be more biologically plausible. Only one model may be used, if a multiendpoint analysis or other experimental evidence indicates its higher plausibility. We do not recommend the use of empirical models, based solely on significant differences between single and combined exposures.

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