Toxicology Research

REVIEW

In vitro models for neurotoxicology research

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The nervous system has a highly complex organization, including many cell types with multiple functions, with an intricate anatomy and unique structural and functional characteristics. The study of its (dys)functionality following exposure to xenobiotics, neurotoxicology, constitutes an important issue in neurosciences. Despite the extensive use of *in vivo* models to reveal the neurotoxicological phenomena, the existence of difficulties related to the increasing cost and time required for neurotoxicity studies with experimental animals, as well as the animal ethical concerns, have limited their use. Consequently, *in vitro* alternatives, providing an understanding of the mechanistic basis, at the molecular and cellular level, have earned a notable consideration in the field of neurotoxicological research. In this field, the selection of the most appropriate *in vitro* neuronal system relies on specific endpoints that are of particular relevance for the neurotoxicological phenomena that will be studied. Furthermore, application of specific endpoints to various neuronal cellular models should be done in a careful way to build reliable and feasible testing strategies. This review addresses the use of *in vitro* models for neurotoxicity research, aiming to contribute to a better understanding and guidance of *in vitro* neurotoxicological studies. As such, subcellular systems, namely isolated mitochondria and synaptosomes, and cellular models, including immortalized cell lines, primary cultures, co-cultures, organotypic cultures, neural stem cells and blood–brain barrier models, as well as their inherent advantages and limitations, are discussed.

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1. Introduction

The field of neurotoxicology has emerged from the integration of toxicology, pharmacology, psychopharmacology and experimental psychology, and involves the study of changes in the function and/or structure of the nervous system, as a result of chemical exposure or other environmental influences, and an interpretation of the consequences and adversity of those changes. Recent advances in our understanding of neuroscience have opened up new lines of research for detecting xenobiotic-induced neurotoxicity, dissecting underlying mechanisms, and developing potential protection/prevention strategies against neuronal injury. The use of *in vitro* neuronal models to understand issues relevant to the neurotoxicological field has the potential to advance our understanding of brain-related biological processes, including neuronal function and toxicity. This review addresses the use of *in vitro* models for neurotoxicity research, aiming to contribute to a better understanding and guidance of *in vitro* neurotoxicological studies. The first part of this review is focused on the general advantages and limitations involving the use of *in vitro* approaches for neurotoxicological studies. Then, *in vitro* neuronal models available for neurotoxicological studies, as well as their particular advantages and limitations are presented. In addition, important issues involving the use of *in vitro* neuronal systems as models that may contribute to a better understanding of the molecular and cellular mechanisms mediating a neurotoxic response, are described.

2. Neurotoxicity

Neurotoxicity can be defined as any adverse effect on the chemistry, structure or function of the nervous system, during development or at maturity, induced by chemical, biological or physical influences.[1] A large number of compounds have been shown to cause neurotoxicity, including metals (*e.g.* lead),[2] industrial chemicals (*e.g.* acrylamide),[3] solvents (*e.g.* toluene or *n*-hexane),[4–6] natural toxins (*e.g.* domoic acid),[7] pharmaceutical drugs (*e.g.* doxorubicin),[8] drugs of abuse [*e.g.* 3,4-methylenedioxymethamphetamine (MDMA; "ecstasy")][9] and

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pesticides (*e.g.* organophosphates).[10] The nervous system is particularly sensitive to toxic insults as a result of a number of intrinsic characteristics, such as the dependence on aerobic metabolism for a constant oxygen supply, the presence of axonal transport, or the processes of neurotransmission.[11] A neurotoxic effect can be the direct alteration of the neuronal structure or activity or can be the result of a cascade of effects due to glia activation and glia–neuron interactions. In addition, a neurotoxic effect can manifest immediately or only years later following the insult. The neurotoxicity can be permanent or reversible, and it can affect the whole nervous system, or only parts of it.[11–13]

From a general perspective, neurotoxicants can be divided into four groups: those which cause neuropathy, thus damaging the whole neuron, those which target the axon and cause axonopathy, those inducing myelopathy, and those affecting neurotransmission.[1] A number of chemicals may cause toxicity that results in the loss of neurons (neuronopathy), either by necrosis or by apoptosis. Such neuronal loss is irreversible and may result in a global encephalopathy, and when only subpopulations of neurons are affected, it results in the loss of particular functions. An example is 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which causes degeneration of dopaminergic neurons, resulting in Parkinson's disease-like symptoms,[14] or trimethyltin, which targets hippocampal, amygdala and pyriform cortical neurons, resulting in cognitive impairment.[15] Chemicals can cause neuronal cell death by a variety of mechanisms, including disruption of the cytoskeleton, induction of oxidative stress, Ca^{2+} overload, or by damaging mitochondria. A large number of neurotoxic chemicals, including *n*-hexane,[4] acrylamide[16] or docetaxel,[17] mainly target the axon, and cause axonopathy. The axon degenerates, and with it the myelin sheath surrounding the axon. In this case, the cell body remains intact. Nevertheless, axonal degeneration may lead to neuronal death.[18] In addition, the toxicant may cause a chemical section of the axon at some point along its length, and the axon distal to the section, which is separated from the cell body, degenerates. The result is most often the clinical condition of peripheral neuropathy, in which sensation and motor strength are first impaired in feet and hands. If the insult occurs in the peripheral nervous system, there is a good possibility for neuronal regeneration and recovery.[11] Other chemicals may target myelin, causing intramyelinic edema or demyelination. While neurons are structurally unaffected, their functions are altered. Triethyltin[19] and hexachlophene[20] are examples of chemicals that cause intramyelinic edema, leading to the formation of vacuoles and spongiosis in the brain. Finally, there are neurotoxicants that interfere with neurotransmission.[11,21] They can inhibit the release of neurotransmitters, such as the botulin toxin, which inhibits acetylcholine (ACh) release, act as agonists or antagonists of specific receptors, such as the marine neurotoxin domoic acid, which activates a subtype of glutamate (GLU) receptor, or atropine, which blocks muscarinic receptors, thus interfering with signal transduction processes. The effects resulting from an interference with the synaptic functioning are usually reversible, but, nevertheless, of toxicological relevance, as they may lead to severe acute toxicity or even death.[22,23]

2.1. Neurotoxicity assessment

The estimation of the compounds' neurotoxicity profile is controlled by regulatory guidelines (for a better understanding of the current testing guidelines for standard neurotoxicity testing see ref. 24–26). Nowadays, neurotoxicity assessment required by guidelines relies primarily on behavioral and histopathology evaluation of the nervous system, which is expensive, time consuming and unsuitable for screening a large number of chemicals. In addition, such *in vivo* tests are not always sensitive enough to predict human neurotoxicity and

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often do not provide information that facilitates regulatory decision-making processes.[26,27]

In the field of neurobiology and neurotoxicology, *in vitro* neuronal models have been successfully developed and employed to address specific questions of cell biology and nervous system functioning. In addition, *in vitro* studies may provide the most appropriate approach when, in many cases, these studies cannot or are difficult to be conducted in live animals. For example, the study of mechanistic pathways and target molecules for neurotoxicants is difficult to perform in a whole animal. In such cases, *in vitro* systems allow the examination of mechanistic processes under isolated conditions, and facilitate the characterization of the modes of action in target tissues by elucidating information on cellular and molecular alterations caused by neurotoxicant exposure. The mechanistic understanding is also valuable in designing directed, hypothesis-driven, *in vivo* experiments.[28] A range of *in vitro* systems of increasing biological complexity is available for neurotoxicity testing, from subcellular systems (*e.g.* isolated mitochondria and synaptosomes), single cell types (*e.g.* immortalized cell lines) to systems that preserve some aspects of the tissue structure and function [*e.g.* primary mixed neuronal and glial cultures, three-dimensional (3D) cultures or organotypic brain slices]. The selection of any particular system depends on the question addressed, the intended use of the data and the available information on the suspected mechanism of neurotoxicity.[1,27]

However, as pointed below, it is generally recognized that *in vitro* systems often provide partial answers to complex conditions. Therefore, though *in vitro* studies cannot fully replace *in vivo* conditions, they can complement investigations with laboratory animals. In addition, the information obtained might also be used in the refinement of future *in vivo* studies of neurotoxicity.[27,29–31]

2.1.1. Advantages and limitations in using *in vitro* systems for neurotoxicity assessment. The use of *in vitro* systems for neurotoxicity testing has been discussed in numerous reviews.[1,26,31–37] Experimental systems for the understanding of toxicant-induced damage to the nervous system are often reductionist in nature, in order to increase the specificity (ability to identify the true negatives) and sensitivity (ability to identify the true positives) of the measured endpoints.

The main advantages and limitations in using *in vitro* neuronal models for neurotoxicological studies are summarized in Table 1. Of note, *in vitro* systems lack the ability to assess behavioral neurotoxicity endpoints, which is a major neurotoxic outcome of concern. Lacking this ability, the value of *in vitro* systems lies in their potential to respond mechanistically to a toxicant in a similar manner to that occurring *in vivo*. In addition, the appropriate age and developmental state of the nervous system at the time of exposure are extremely difficult to approximate in culture.

Although *in vitro* methods are associated with limitations and drawbacks, which must be considered when designing studies and extrapolating data to the dose–response paradigm, they play an important role in experimental research and provide valuable opportunities for mechanistic based risk assessments.[38] Thus, when combined with *in vivo* approaches, they allow a better understanding of the mechanistic basis for a neurotoxic effect.

2.1.1.1. Access to the cellular environment. The physicochemical environment of cells may be easily manipulated *in vitro*. Chemicals can be added or removed from the culture medium, allowing precise temporal analysis of the sequence of events. The concentration of the test compound can be controlled in terms of the amount being delivered to the entire cell population or to an individual target cell. However, this concentration must be consistent with the *in vivo* level of exposure to be meaningful.

Physicochemical properties of compounds, such as solubility, volatility, pK_a, binding to components of the culture medium, including protein binding, and osmolality, are criti-

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Table 1 Advantages and limitations of *in vitro* neuronal systems for neurotoxicological studies

Advantages
Reduced cost[97]
Tight control of the neurotoxicant concentration[34]
Control of the extracellular environment[34]
Fewer ethical issues, except for human embryonic cells[97]
Direct observation and measurement of cellular responses to neurotoxicants[34]
Possibility to compare concentration–response curves for different compounds[34]
Study of toxicants' effects at different maturation and differentiation stages[34]
Easy to maintain and manipulate[28,34]
Control of the exposure time[34]
Study of single cell types[34]
Higher reproducibility between independent experiments[364]
Observation of direct interactions between the neurotoxicant and the test system[34]
Ability to address questions of interspecies selective toxicity[72]
Precise temporal analysis of the sequence of events[34]
Limitations
Lack of integrated functions[34]
Lack of the BBB function[34]
Absence of systemic endocrine control[34]
Difficult to determine and reproduce the compensatory mechanisms observed *in vivo*[28,34]
Fail to account for the route of administration, distribution and biotransformation of the neurotoxicant in the body[34]
Impossibility to assess behavioral endpoints[34]
Unknown target concentration[34]
Limited ability to mimick heterogeneous cell–cell interactions[34]
Lack of the nutritional support provided by the blood circulation[34]
The *in vitro* conditions in which neuronal systems grow are a poor substitute for the intricate neuronal environment of the whole animal brain[34]

cal for predicting their toxic effect in an *in vitro* system. Therefore, tight control of their concentration is required over time. On the other hand, it is difficult to evaluate *in vitro* compounds that are either insoluble in aqueous systems or at neutral pH, thus forming insoluble particles or precipitating over time. To expose neuronal systems to such insoluble compounds, additives (*e.g.* dimethylsulfoxide or ethanol) are often added to the culture medium. However, the presence of these additives may modify the neurotoxicological effect of the tested compound, since these additives may also exhibit toxic effects.[39–42] To overcome these problems, compounds may be solubilized using physiological carriers, such as albumin or lipoproteins. The addition of the chemical under study to the medium can result in a direct reaction between the test compound and a component of the culture medium (*e.g.* protein denaturation or precipitation), thus affecting the availability of the toxicant or essential nutrients, and modifying signals to the cultured cells. Any protein-binding properties of drugs tested can directly alter the microenvironment of cultured cells in a fashion that may not occur *in vivo*.[34]

2.1.1.2. Exploring toxicokinetics in vitro. Toxicokinetics is defined as the generation of pharmacokinetic data, either as an integral component in the conduction of non-clinical toxicity studies or in specially designed supportive studies, in order to assess systemic exposure. These data may be used in the interpretation of toxicology findings and their relevance to clinical safety issues.[43] The dose needed to induce toxic effects depends on these pharmacokinetic parameters. For example, the route by which a compound enters into the body can substantially alter the quantity absorbed and, consequently, modify the dose required to cause neurotoxicity. Metabolism of xenobiotics usually causes detoxification in the organism, but for some compounds metabolism generates neurotoxic metabolites.[9,44] This process can take place in non-neural tissue (*e.g.* liver),[9,45] but also in the brain.[9,44] Nevertheless, whereas non-neuronal cells possess xenobiotic inactivation systems,[46] neuronal cells present a lower cytochrome P450-mediated metabolic ability.[47] Within the organism, the compound may be bioactivated and/or detoxified before its release back into the blood circulation, being posteriorly distributed throughout the body.

Differences in drug metabolism and excretion are considered to be an important determinant for the large species' differences in both the toxic dose and the type of neurotoxic response following drug or chemical exposure.[48,49] However, in culture, a test compound either remains unaltered or is relatively slowly modified. These conditions allow examination of the intrinsic toxicity of a substance to a cell-based system in the absence of any metabolites. Thus, *in vitro* test systems lacking the metabolizing activity can overestimate or underestimate the toxicity that a compound would have *in vivo*. For example, in the field of MDMA-induced neurotoxicity, hepatic formation of neurotoxic metabolites, followed by their uptake in the brain, is thought to contribute to the neurotoxic actions of this drug.[9] On the other hand, there are also compounds that following peripheral administration do not cause neurotoxicity *in vivo*, but are neurotoxic *in vitro*. One such example is the metabolite of MPTP, *N*-methyl-4-phenylpyridinium ion (MPP^+). Whereas MPTP easily crosses the BBB *in vivo*, MPP^+ cannot reach the brain, since it does not cross the BBB, and, therefore, peripheral administration of MPP^+ is not associated with neurotoxicity.[44,50] However, in an *in vitro* system containing dopaminergic neurons expressing the dopamine transporter (DAT), it will be highly toxic.[50,51] In addition, in the brain, the metabolism of MPTP to MPP^+ is catalyzed by the astrocytic enzyme monoamine oxidase B, thus indicating that in systems lacking the above mentioned metabolic ability, MPTP will not produce neurotoxic effects.[44,50] Therefore, it is critical to understand the *in vivo* metabolism, distribution, and effects of potential metabolites on the nervous system, in order to interpret the data obtained from both *in vivo* studies and *in vitro* systems.

To compensate for the lack of metabolic competence of *in vitro* neuronal systems, different approaches have been proposed. These include the addition of metabolically competent sources, such as S9 liver fractions (short-term experiments), hepatocyte-conditioned medium, the direct co-culture with hepatocytes from different species, or the transfection of cells with phase-I biotransformation enzymes.[33,52] However, any artificial system used to simulate normal metabolism of compounds should preferably be comparable to the *in vivo* con-

ditions. Moreover, attention should be paid to the differences between species in metabolic and kinetic parameters, which are important determinants for the extrapolation of results obtained in animal models to the human situation.[33,53] S9 liver fractions should be used with caution, since the amounts of S9 fractions generally needed for the occurrence of metabolism might exhibit toxicity. However, this problem may be prevented by using tissue culture inserts, which allow clear separation between cultured cells and the S9 fraction using a microporous membrane.[54] Alternatively, when glial cell-mediated metabolism is critical for neuronal toxicity,[44,50] co-cultures of neuronal and glial cells may be a good approach. However, the biotransformation processes in these cell culture systems may be incomplete or proceed by pathways different from those operating in whole organisms. Furthermore, the metabolic pattern is determined by the type of metabolic system, as well as by the species and gender from which the metabolic competent cells are isolated.

Traditionally, the effective concentration obtained from a cell culture neurotoxicological study takes into account the nominal concentration of a test chemical added to the medium at the start of the experiments. Nevertheless, for volatile toxicants, some studies have found their evaporation from assay plates, resulting in higher effective concentrations, as compared to the effects observed in sealed assay materials.[55,56] Other studies have also demonstrated that additional factors, namely the presence of serum proteins in culture medium, microtiter plate plastic and cell lipids, significantly bind and, consequently, reduce the free concentration of hydrophobic compounds under test in *in vitro* experiments.[57–61] At this level, solvent-free dosing systems have been established, thereby compensating for the loss of test compounds in *in vitro* experiments over time.[62–64] However, these partitioning approaches assume that the equilibrium is rapid in comparison with the duration of the experiments, which for some compounds might not always be observed for the partitioning into the cells.[65] Another important aspect related to the partitioning of compounds in *in vitro* cell-based experiments is the dynamics of the cells in response to the compound during the assay. The main factor is the growth of the cell population, thereby resulting in alterations in the partitioning during the experiments and, consequently, in the toxic effect experienced by the cells.[66] Thus, although the use of such systems might be useful in controlling the real concentration of the test compound over the *in vitro* experiment, other factors related to the system, by themselves, might modify the partitioning of test compounds over the experimental procedure.

2.1.1.3. Exposure time. The chemical-induced neurotoxic effects *in vivo* are largely dependent on the dose and time of exposure. Neurotoxic effects may be observed shortly after exposure (acute effects) or only after days, months or years of exposure (long-lasting effects). In some cases, indicators of neurotoxicity may be observed only after repeated or prolonged exposure. The appearance of such effects may not only depend on the type of chemical and dose used, but may also depend upon the biological processes underlying the neurotoxic response. Certain compounds interact directly with accessible cellular components, such as the cellular membrane or vital enzymes. As a result, these structures will be functionally affected shortly after exposure.[67] For example, by inhibiting acetylcholinesterase (AChE), organophosphorus compounds and carbamates lead to the accumulation of ACh at cholinergic synapses, causing a cholinergic syndrome characteristic of the intoxication by these compounds.[68] For these effects, the peak concentration and exposure schedule are of critical importance. On the other hand, a delay in the occurrence of neurotoxic effects may be due to the initiation of an irreversible cascade of reactions, which may culminate in neuronal death. For example, some organophosphorus compounds (*e.g.* diisopropylphosphorofluoridate, cyclic tolyl saligenin phosphate, phenyl saligenin phosphate, mipafox, dibutyl dichlorovinyl phosphate or di-octyl-dichlorovinyl phosphate) can cause delayed polyneuropathy, which is related to the irreversible inhibition of other esterases, such as neuropathy target esterase.[68–70]

Depending on the type of *in vitro* system, neuronal cells may survive for only a few hours or several months. Furthermore, as the degree of cellular maturation and differentiation of the cultured cells is influenced by time, this will influence the exposure window among the different culture systems, as a result of differences in the survival and differentiation state of the cells. Therefore, though the majority of *in vitro* neuronal systems employed are valuable for predicting acute responses, they may not be appropriate to examine some effects that are progressive or delayed in nature.

2.1.1.4. Lack of cellular homeostatic mechanisms. Considering the high functionality of the nervous system, perhaps one of the most important limitations of *in vitro* neuronal models is the lack of homeostatic mechanisms that are observed *in vivo*, such as the nutritional support provided by the blood circulation, regulatory control of the neuroendocrine system, interactions with adjacent cells and the intercellular components that create a unique microenvironment.[34] For example, in the whole animal, the presence of an intact BBB constitutes an important factor influencing the drug's access to the brain,[71] as observed for MPTP,[44,50] thus limiting the extrapolation of *in vitro* results to the *in vivo* scenario. Therefore, the use of *in vitro* systems provides the ability to study a discrete nervous system area or cells isolated from the homeostatic mechanisms observed *in vivo*. Nevertheless, *in vitro* systems offer much in the way of assessing mechanistic questions in a defined controlled system.[38]

2.1.1.5. Multiple interspecies comparisons. One of the main advantages in using *in vitro* systems for neurotoxicological studies relies on the opportunity for the investigator to examine similar cell types from multiple animal species.[72] Therefore, this approach allows addressing questions of selective toxicity among species. In addition, since the manipulation of *in vitro* models is easier than that of *in vivo* conditions, the use of such models to compare toxic effects between species makes this approach more representative of the *in vivo* scenario. However, comparisons may be limited by

the inherent differences in the origin and types of cell lines that are totally unrelated to the species of origin.

This approach may be particularly useful to evaluate the neurotoxic potential of new compounds in the human scenario. For example, if a compound exhibits neurotoxicity in *in vitro* systems derived from a mouse or rat and also *in vivo*, but not in an *in vitro* system derived from humans, it can be hypothesized that this compound may have a low impact on human health. As a good example, in a study developed by M. E. Culbreth and co-workers,[72] the effects of chemicals on cell proliferation and apoptosis were compared in human and mouse stem cells. It was found that some tested chemicals, including methylmercury, cadmium, dieldrin, chlorpyrifos oxon, *trans*-retinoic acid (RA) and trimethyltin decreased cellular proliferation either in ReN CX (human stem cell line) or mCNS (mouse stem cell line) cells, as assessed by the BrdU incorporation assay.[72] On the other hand, none of the tested chemicals activated caspase 3 or p53 in ReN CX cells, while methylmercury, cadmium, dieldrin, chlorpyrifos oxon, trimethyltin and glyphosate induced at least a doubling effect in these apoptotic markers in mCNS cells.[72] Compared to the control, cadmium, RA, and trimethyltin decreased cell viability by at least 50% in ReN CX cells, while cadmium, dieldrin, and methylmercury decreased the viability by at least 50% in mCNS cells.[72] Based on these results, the authors concluded that human cells are more sensitive than mouse cells to chemical effects on proliferation. By contrast, caspase 3 and p53 were altered by environmental chemicals tested in mouse, but not in human cells.[72] Therefore, the use of *in vitro* neuronal models from different species may help in understanding the molecular and cellular basis of chemical-induced neurotoxicity among different species and their impact on human health.

2.1.1.6. Heterogeneity of the nervous system. *In vivo*, the development and differentiation of the nervous system depend on the interaction between neuronal and non-neuronal cells.[73,74] Not all compounds directly interact with individual cells, but may cause injury by indirect pathways, such as modifications of cell–cell interactions.[75] Although much work has been focused on obtaining enriched cultures representative of neuronal populations, it has been demonstrated that rarely a primary neuronal culture exists in the absence of glial cells and that dynamic interactions exist between the cell types in culture. In fact, in many cases, neuronal survival *in vitro* is poor or improbable in the absence of astroglial cells.[73,76] When early postnatal cerebellar astroglial cells are cultured in the absence of neurons, they show a flat, undifferentiated morphology and proliferate rapidly. However, the presence of neurons in such cultures arrests glial cell growth and induces glial morphological differentiation into profiles resembling cerebellar glia observed *in vivo*.[77,78] In addition, it has been reported that the inhibition of glial cell proliferation in culture may be modulated by the availability and type of serum in the culture media.[79]

Studies performed in the past few years have also established the existence of bidirectional signaling between neurons and astrocytes,[80] thus influencing the cellular differentiation and synaptic transmission,[81,82], and potentially influencing the functionality of neurons.[83,84] Therefore, although mixed cultures of neurons and glial cells make difficult the evaluation of a particular response of neurons to a neurotoxicant, the presence of glial cells is determinant to maintain a healthy neuronal population, which being more representative of the *in vivo* scenario, constitutes a basic requisite to obtain valid results.

2.1.2 Use of *in vitro* neuronal systems for mechanistic studies. *In vitro* systems present high usefulness to understand the mechanistic basis of neurotoxic effects that occur at the molecular or cellular level. Since the nervous system presents a high complexity, generally no single *in vitro* preparation can be used to detect all possible endpoints. However, depending on the knowledge about the neurotoxicity of a certain compound, and of specific questions that need to be addressed, complementary studies with different models may be carried out.[38]

In the context of mechanistic *in vitro* neurotoxicology, one can point out studies investigating mechanisms of neurotoxicant-induced neuronal cell death,[85,86] inhibition of cell proliferation,[87] alterations of signal transduction pathways,[88] modulation of neurotoxicity by cell–cell interactions,[44,89] alterations of inhibitory or excitatory circuits[90] and many others. Therefore, there is no doubt that *in vitro* systems play a most relevant role in addressing the mechanisms of neurotoxicity. For example, neuroblastoma cell lines,[91] PC12 cells[92] or primary cells,[85,86] among other single cell-based systems, may be useful in addressing the interaction of neurotoxicants with ion channels or receptors. Slice cultures, due to their more complex organization, may be useful to study whether neurotoxicants affect certain excitatory or inhibitory circuits.[93] In addition, cultures of Schwann cells represent a good model to study the effects of neurotoxicants on myelination.[94] However, in all cases, the extrapolation of *in vitro* findings to the *in vivo* scenario still requires important considerations, as several factors might influence it, such as the dose selection,[95] the importance of metabolism and toxicokinetics in the development of neurotoxic effects,[9,44] and the BBB permeability,[33] among other factors.

In some cases, characteristic mechanistic knowledge may lead to the selection of a specific *in vitro* neuronal system to assess particular endpoints of neurotoxicity. For example, by inhibiting AChE, some organophosphorus compounds (*e.g.* paraoxon, chlorpyrifos-oxon, dichlorvos or trichlorfon) and carbamates lead to the accumulation of ACh at cholinergic synapses, causing a cholinergic syndrome. On the other hand, some organophosphorus compounds (*e.g.* diisopropylphosphorofluoridate, cyclic tolyl saligenin phosphate, phenyl saligenin phosphate, mipafox, dibutyl dichlorovinyl phosphate or di-octyl-dichlorovinyl phosphate) can cause delayed polyneuropathy, which is related to the irreversible inhibition of other esterases, such as neuropathy target esterase.[68–70] Therefore, the selection of specific *in vitro* neuronal models with differential expressions of these enzymes may be useful in assessing the mechanistic basis of their acute and delayed neurotoxicity.

2.1.3. Use of *in vitro* systems for neurotoxicity screening. As already mentioned, a second primary objective of *in vitro* systems is to provide a rapid, relatively inexpensive and reliable way for screening chemicals for potential neurotoxicity. However, though screening tests can provide much information about potential neurotoxicants, a more comprehensive study of these compounds is followed by more specific and complex tests, both *in vitro* and *in vivo*. The criteria used for *in vitro* neurotoxicity screening are the same general criteria used for other *in vitro* screening approaches, namely low incidence of false positives and false negatives, high correlation with the data provided by *in vivo* studies, sensibility, simplicity, quickness, economy, and versatility.[1]

The selection of a specific *in vitro* model for neurotoxicity screening is not always easy. However, this selection is primarily influenced by the endpoint of neurotoxicity that will be measured. A common belief is that for screening purposes one should examine general cellular processes, such as cell viability, proliferation or axonal/dendritic growth. However, each test requires careful considerations. For example, basic tests for cytotoxicity and viability assessment, including measurements of cell death and proliferation, membrane permeability or mitochondrial function are common to most cell types. However, though a chemical can affect these endpoints, we cannot conclude that the chemical is neurotoxic, but only that it displays cytotoxicity in this model.[38]

The use of different *in vitro* models may provide additional information whether the chemicals display differential effects, or present different potencies, in neuronal *versus* non-neuronal cells.[96] In addition, the relative sensitivity of each *in vitro* model to the toxic effects of compounds can also provide additional information about the possible targets and mechanisms involved in compound-induced neurotoxicity.[38]

3. *In vitro* models for neurotoxicity assessment

For evaluation of altered nervous system functioning, isolated models have been selected based on key biochemical, functional and morphological features, which are specifically targeted by neurotoxicants *in vivo*. The use of *in vitro* models for predicting neurotoxic events offers several advantages, including reduced cost, the ease of use, fewer ethical issues and better control over experimental variables.[97] Primary cultures allow for visualization of individual living cells and for monitoring both morphological and electrophysiological features. It is believed that neuronal and glial cells migrate to re-arrange themselves on the substratum, and differentiate according to their function and abilities. However, the tissue organization is lost as a result of the dissociation procedure needed to obtain the isolated brain cells. In addition, *in vivo*-like structures cannot be obtained by this technique. Despite these limitations, primary cultures are more accessible to experimental manipulation than slice cultures, and they are easier to manipulate and stable for long periods. It is possible to obtain and correlate biochemical, morphological, electrophysiological, and molecular data from a single cell. Additional purification methods can be used to enrich a cell preparation obtained by dissociation in a particular cell type.[28,98]

On the other hand, cell lines of tumoral origin provide homogeneous cell populations in large quantities in a very reproducible manner. Usually, the common cell lines of choice are those that continue to express in culture the properties of their normal adult cell counterpart. It must be noted again that these cell culture systems represent cells that are no longer part of an integrated neural network and may develop an altered appearance, metabolism and response to chemicals. Some believe that these concerns can be addressed by experimental design and choice of endpoints. No such efforts, however, will compensate for the isolation of a system from the natural neural environment and for the lack of systemic influences . In addition, the ability of *in vitro* neuronal systems to predict the neurotoxicity of a compound *in situ*, or the potency of a series of structurally related analogs, can depend on whether they express the molecular/cellular target for neurotoxicants.[28,98]

Below, for each *in vitro* neuronal model, the respective particular advantages and limitations, as well as its main characteristics, are described.

4. Subcellular systems

Despite the extended use of cell-based systems as *in vitro* models in neurotoxicological research, in the last few decades, sub-cellular systems have been extensively used in evaluating signaling pathways and receptor-mediated signal transduction, as well as in studying specific points that may be addressed out of the cellular environment.

4.1. Isolated mitochondria

The isolated brain mitochondrion model has emerged as an important *in vitro* approach in the field of neurotoxicology for understanding the role of this organelle in many toxicological mechanisms. Mitochondria preparations from whole brain are heterogeneous. Thus, distinct populations of brain mitochondria can be isolated from a single homogenate preparation: two from the synaptosomal fraction, heavy and light mitochondria, and one from the nonsynaptic origin, the so-called free mitochondria. It is possible, however, to separate the different types of mitochondria from different brain areas.[99]

The major advantage of this model is its simple preparation. However, during mitochondrial isolation, the homogenization procedure may cause rupture of mitochondrial membranes leading to potential direct effects on mitochondrial function: escape of soluble mitochondrial molecules, disruption of the inner mitochondrial membrane, mitochondrial swelling or dilution of the matrix components. Despite this, in their isolated state, mitochondria maintain many functional characteristics observed in their *in situ* and *in vivo* environ-

ments.[100] Another important limitation of this model is their contamination with cellular components.[99] Mitochondria free of contaminant membranes should have negligible activities of marker enzymes for other subcellular fractions, such as glucose-6-phosphatase for the endoplasmic reticulum, acid hydrolases for lysosomes and catalase and D-amino acid oxidase for peroxisomes. On the other hand, mitochondria should be highly enriched with cytochrome *c* oxidase (seems to be a rather stable mitochondrial enzyme, whose activity is not subject to fluctuations and pathological changes) and succinate dehydrogenase.[100,101]

The isolated mitochondrion model has been routinely used in the field of neurotoxicology to study the effect of several neurotoxicants on mitochondrial bioenergetics.[102–104] Working with this model, other studies have assessed the influence of many neurotoxicants on Ca^{2+} homeostasis.[105] In addition, in isolated brain mitochondria, increased ROS formation has been demonstrated to be implicated in the neurotoxicity elicited by several xenobiotics.[106] Therefore, isolated mitochondria constitute a useful model in addressing neurotoxicological mechanisms *in vitro* and provide a means for screening many drug candidates or chemical molecules that may be mitochondrial toxicants in the brain.[107]

4.2. Synaptosomes

One of the most used subcellular systems in neuropharmacological and neurotoxicological studies is the synaptosome model, whose preparation was introduced more than 50 years ago by Gray and Whittaker[108] and De Robertis and coworkers.[109]

Synaptosomes are subcellular fractions, derived from neurons, prepared from brain tissue by homogenization and function as small anucleated cells that retain neuronal vesicles and enzymes,[110] usually with a size ranging from 0.5 to 1 μm, one or more mitochondria, as well as extremely active ion transport systems across their membrane.[110,111] Synaptosomes can use glucose, either aerobically or anaerobically, and pyruvate, as an oxidative metabolite. Despite the high content of GLU and aspartate in the synaptosomal cytoplasm, these cannot be readily utilized as energy sources.[110] When maintained at 37 °C, the adenosine 5′-triphosphate produced is rapidly degraded, leading to a fast decrease in synaptosomal viability. However, when kept on ice and with the availability of nutrients, their functionality is maintained for several hours.[112,113] Carefully prepared synaptosomes show high respiratory control and maintain the plasma membrane potential ranging from −60 to −80 mV, in low K^+ medium,[114] with an average cytoplasmic free Ca^{2+} concentration of about 0.1–0.2 μM.[115] It may, thus, be concluded that the synaptosome model is energetically viable.[110]

The major advantage of this model is its simple preparation. One of the major limitations is the inherently heterogeneous neurotransmitter content, since even the most closely defined anatomical region contains a wide variety of neurotransmitters.[110,116]

Synaptosomes have been routinely used in the field of neurobiology to study metabolic pathways,[117] energy production and ion movements,[118] neurotransmitters' storage and synthesis,[119,120] and mechanisms involved in neurotransmitters' release,[120–124] oxidative injury to macromolecules[125–129] or neuronal mitochondria,[118,130,131] as well as to mimic the mitochondrial deficits found in neurodegenerative diseases.[132,133] Therefore, given their simplicity and functionality, they constitute an important *in vitro* model for neurotoxicological studies that does not require the functionality of intact cells.

5. Cellular systems in monolayers

With the development of new approaches to study the nervous system, cell cultures began to gain a more prominent and important position in neurobiology and neurotoxicology fields. Since 1907, studies of the nervous system in tissue culture have provided invaluable insight into the embryological origin of nerve cells, the development of their cytological and biochemical identities, their functional connectivity and their interactions with glia and other cell types within the nervous system.[98]

5.1. Immortalized cell lines

Cell lines of limited lifespan often undergo genetic changes, after which their growth potential is modified and, consequently, their proliferation becomes unlimited. These cell lines are termed immortalized cell lines.[98]

The main advantages and limitations in using immortalized cell lines as *in vitro* models for neurotoxicological studies are summarized in Table 2. One of the major limitations of immortalized cell lines is the difficulty in inhibiting cell division experimentally to obtain a stable population of differentiated cells. This contrasts with the *in vivo* situation, where the end cell in a differentiation stage usually does not divide. In many cases, differentiation is induced by chemicals or deprivation of substrates. However, it is not known whether this differentiation process is comparable to the differentiation that occurs *in vivo*.[134] Still, the genetic stability of any given phenotype is critical for the reproducibility of experimental findings. However, *in vitro* differentiation of neuronal cell lines constitutes another contributing factor for phenotypic variability, as a result of differences in the effectiveness of the differentiation process.[98,134,135]

Most of the cell lines presently available for neurobiological and neurotoxicological research do not express some key aspects of neuronal differentiation. Though there are cell lines that express many of the individual characteristics of differentiated neurons, including neurotransmitters, ion channels, receptors and other neuron-specific proteins, they are not good models for all specific neuronal phenotypes.[136] For example, PC12 cells, which can come as close as any cell line to mimic a specific population of differentiated nerve cells, do not develop distinct axons and dendrites or form

Table 2 Advantages and limitations in using immortalized cell lines as *in vitro* models for neurotoxicological studies

Advantages
A large amount of cells can be easily grown[98]
Retain their main properties along time[98,134]
Proliferation in culture for long periods without contamination[98]
Enable studies with homogenous populations of neuronal cells uncontaminated with glia[98]
High homogeneity of the cell populations[98]
Can be stored indefinitely in liquid nitrogen and used when needed[98]
Possibility to obtain and correlate biochemical, morphological, electrophysiological, and molecular data from a single cell[98]
Limitations
Lack the growth regulation seen *in vivo*[98,134]
Limited cellular interactions resulting from the high homogeneity[98]
In general, cell lines are more resistant than primary cell cultures to neurotoxic insults[136]
The genetic amenability and response to a neurotoxicant may be changed, as compared to primary cells[98,134]
Possible development of phenotypic alterations[98,134]
Do not possess all features of adult differentiated cells[98,134,135]
Difficulty in inhibiting experimentally cell division to obtain a stable population of differentiated cells[134]
The majority of immortalized cell lines have not been studied in depth to identify all the idiosyncratic changes imposed by genetic manipulation and multiple passages[134]

synapses like the sympathetic neurons they otherwise resemble.[137]

Cell lines exist that are representative of neurons, including neuroblastoma cell lines (*e.g.* SH-SY5Y, Neuro-2A or IMR-32 cell lines),[138] and glia, such as oligodendrocyte cell lines (*e.g.* CG4, OLN-93 or HOG cell lines), Schwann cell lines (*e.g.* 33B, IMS32 or NF1 T cell lines), astrocyte cell lines (*e.g.* C6, SFME or U87MG cell lines) or microglia cell lines (*e.g.* HAPI, BV-2 or CHME-5 cell lines).[139] With some rapidly dividing cell types, such as glia, a large cell population can be derived from a single cell. Although cell lines lack the growth regulation observed *in vivo*, the critical point for experimental use is whether the cells express the differentiated characteristic of interest identical to those observed *in vivo*. If they do, information on the binding characteristics of the tested compound to the target receptor or binding site molecule may be studied as an important first step in the neurotoxicity evaluation. However, the use of cell lines for neurotoxicological studies, even if they express the differentiated characteristic of interest, should be exercised with caution. As the genetic amenability and response to a test compound may be dramatically changed in cell lines, as compared to the original primary cells, unforeseen cell line specific effects may occur. Thus, the data obtained from these studies might not be directly applicable for extrapolation to the *in vivo* conditions.[98,134]

Actually, cell lines may be acquired from several commercial companies, including European Collection of Cell Cultures, American Type Culture Collection, Invitrogen or Sigma-Aldrich, among others.

5.1.1. Cell lines of neuronal origin

5.1.1.1. Neuroblastoma cell lines. The first neuroblastoma cell line emerged over 50 years ago. Human neuroblastoma cell lines correspond to adrenal neuroblasts, arrested at different levels during the morphogenesis of the adrenal medulla.[138] The differentiation of sympathoadrenal precursor cells toward either sympathetic neurons or chromaffin cells may be promoted by several factors and hormones. Particularly, glucocorticoids are involved in the differentiation of the precursor cells toward chromaffin cells.[140] This finding is indicative of the sensitivity and dependence of neural crest-derived cells on steroid hormones. Cell lines derived from human neuroblastomas represent a good model of human immature neurons and have been widely used for the study of mechanisms involved in neuronal function and differentiation.[138,141] These cell lines also synthesize *in vitro* proteins unique to the neuronal phenotype [*e.g.* neuron-specific enolase (NSE) and neurofilaments (NFs)], receptors for neuroactive factors, as well as enzymes required for the biosynthesis of several neurotransmitters [choline acetyltransferase, tyrosine hydroxylase (TH) and dopamine (DA)-β-hydroxylase]. In addition, they form homogenous populations and proliferate rapidly in chemically defined media and extend neuritic-like processes.[142–145] Neuroblastoma cells may be differentiated *in vitro*, from immature into mature neuronal cells, by different natural and chemical agents, allowing, thus, the study of the evolution of several biologic processes at different stages of cellular development and differentiation. However, when neuroblastoma cell lines are being used, it is important, and at times difficult, to distinguish between morphologic differentiation and cytotoxicity.[138,141] A large part of the data present in the literature using neuroblastoma cells has been obtained with the human SH-SY5Y cell line.

5.1.1.1.1. SY-SY5Y cells: a human neuroblastoma cell line

The SH-SY5Y neuroblastoma cell line is a thrice cloned subline of SK-N-SH cells which were originally established from a bone marrow biopsy of a neuroblastoma patient with the sympathetic adrenergic ganglial origin, in the early 1970s.[146] This cell line has been widely used as a neuronal model since the early 1980s, as these cells possess many biochemical and functional properties of neurons.[147–150]

The main characteristics of the SH-SY5Y cell line are summarized in Table 3.

SH-SY5Y cells may differentiate upon treatment with a variety of agents, including RA,[147–151] 12-*O*-tetradecanoylphorbol-13-acetate (TPA),[147–150,152] brain-derived neurotrophic factor (BDNF),[153] dibutyryl cyclic adenosine 5′-monophosphate (dBcAMP)[154] or staurosporine.[155] Nevertheless, differentiation of SH-SY5Y cells may also be induced by lowering the serum content in cell culture medium.[151,156,157] Although SH-SY5Y cells have been used either in their undifferentiated or differentiated state, the use of undifferentiated cells involves some limitations, as the proliferation during the course of the experiment, which makes it difficult to find out whether neurotoxic agents influence the proliferation rate or the rate of cell death.[158] Moreover, undifferentiated cells do not express high levels of DAT, apart from that they exhibit less sensitivity

Table 3 Characteristics of SH-SY5Y, PC12 and C6 cell lines

SH-SY5Y cell line
Thrice cloned subline of SK-N-SH cells[146]
Expresses DAT[148,150,151]
Expresses DA-β-hydroxylase and TH[504]
After differentiation, presents a functionally mature neuronal phenotype[147,148]
Limited synthesis of catecholamines (DA and NA) as a result of the deficiency in dihydroxyphenylalanine decarboxylase[504]
Exhibits specific uptake of NA[503]
Expresses proteins of NFs[503]
Expresses opioid, muscarinic, and nerve growth factor (NGF) receptors[503]
May differentiate upon treatment with a variety of agents[147–155] or substrates' deprivation[151,156,157]

PC12 cell line
Established from a rat pheochromocytoma[187]
Expresses receptors coupled to G-proteins[191]
Expresses AChE, ChAT and ACh synthase[505]
May differentiate upon treatment with a variety of agents[186,189,191–194,197–199]
Low DA-β-hydroxylase activity[187]
Expresses Na^+, K^+, and Ca^{2+} channels[191]
Ability to synthesize and store ADR and DA, and sometimes NA, which may be released upon depolarization in a Ca^{2+}-dependent manner[190]
Difficulty in adherence to tissue culture plates[200]

C6 cell line
Express Ras, Ras GTP activator protein[506] and wild-type p53[507]
Increased expression of PDGFb,[511] IGF1[512] and epidermal growth factor receptor,[513] compared to astrocytes
Do not express GFAP[510]
Express S100B protein[508] and variable levels of vimentin,[509,510] a protein of the NFs
Reduced expression of IGF2 and fibroblast growth factors 9 and 10, compared to astrocytes[510]
After differentiation, C6 glioma cells acquire a more characteristic glial phenotype[217–221]

to neurotoxins and neuroprotective agents than primary neurons.[148]

In contrast, SH-SY5Y differentiated cells possess more biochemical, ultrastructural, morphological and electrophysiological similarity to neurons and express a variety of neuronal-specific markers, including receptors for neurotrophic factors, growth-associated protein 43, NSE, neuronal nuclei, synaptophysin, and neuronal-specific cytoskeletal proteins, including microtubule-associated protein Tau. In fact, Tau, growth-associated protein 43, neuronal nuclei, and synaptophysin are classical markers of mature neurons.[151] However, according to the differentiation agent used, cells may acquire different phenotypes. SH-SY5Y cells differentiated with RA present a mature cholinergic phenotype, with no significant differences in DAT and TH expression.[148,151] However, they present higher expression of choline acetyl transferase (ChAT)[159] and vesicular monoamine transporter,[148] thus confirming the enhancement of a cholinergic phenotype. On the other hand, SH-SY5Y cells differentiated with TPA acquire a more characteristically adrenergic neuronal phenotype, as observed by increased expression of TH, neuropeptide Y (NPY), as well as NSE, and increased NA biosynthesis. Indeed, both NA and NPY are two important neurotransmitters produced in adrenergic neurons of the sympathetic nervous system.[148,160] Despite this, SH-SY5Y cells differentiated with RA followed by TPA specially develop a dopaminergic phenotype, expressing higher levels of DAT and TH,[148,150] but lower levels of vesicular monoamine transporter,[148] compared to their undifferentiated state. Furthermore, RA/TPA-differentiation increases the density of D2 and D3 receptors on the cell surface.[148] In addition, a higher ability of RA/TPA-differentiated SH-SY5Y cells for DA uptake and its retainment was observed, compared to their undifferentiated state[147,148] as well as their lower resistance to the neurotoxic actions of dopaminergic toxins, such as MPP^+ (ref. 148), thus confirming the enhancement of a dopaminergic phenotype. Representative phase-contrast images of differentiated SH-SY5Y cells under control conditions or after exposure to the toxin rotenone are shown in Fig. 1.

Among the different agents that can differentiate SH-SY5Y cells are also included compounds of the family of neurotrophins, including nerve growth factor (NGF) and BDNF.[153] In addition, several other growth factors, including basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF) 1, glial cell line-derived neurotrophic factor and ciliary neurotrophic factor, also induce differentiation of SH-SY5Y cells.[161,162] However, SH-SY5Y cells generally lack functional neurotrophin receptors. Nevertheless, pre-treatment with RA up-regulates the neurotrophin receptors, making, thus, SH-SY5Y cells responsive to differentiation by neurotrophins.[153] Despite the well-known effectiveness of neurotrophins as differentiation agents, the neuronal phenotype that is promoted remains unclear. Reports indicate that SH-SY5Y cells differentiated with RA and BDNF increase the expression of general neuronal markers and cholinergic markers [NPY, vesicular acetylcholine transporter (VAChT) and ChAT], thus suggesting that after differentiation cells acquire a typically cholinergic neuronal phenotype.[163] However, other authors indicate that SH-SY5Y cells differentiated with RA and BDNF exhibit a characteristically dopaminergic phenotype, with high TH and DAT activity and functional uptake and release of DA.[164] Therefore, further studies on SH-SY5Y cells differentiated with RA and BDNF are needed for better characterization of their phenotype.

Staurosporine is another agent used to differentiate SH-SY5Y cells, inducing a mature adrenergic phenotype characterized by up-regulation of TH, DAT and NPY activities and increased NA content.[152,155] SH-SY5Y cells differentiated with guanosine and guanosine 5′-triphosphate presented a dual dopaminergic/adrenergic phenotype characterized by cell-cycle arrest and increased TH and DAT expression.[165] On the other hand, dBcAMP differentiates SH-SY5Y into an adrenergic neuronal phenotype, characterized by increased NA synthesis and TH expression.[154]

As previously stated, after differentiation, SH-SY5Y cells present a modified susceptibility to neurotoxins. These changes appear to be most closely related to the differentiation agent used. Reports indicate that SH-SY5Y cells differentiated with RA are more resistant to certain neurotoxins like MPP^+, 6-hydroxydopamine or β-amyloid protein than undifferentiated cells.[151,166,167] Other reports indicate that differentiation with

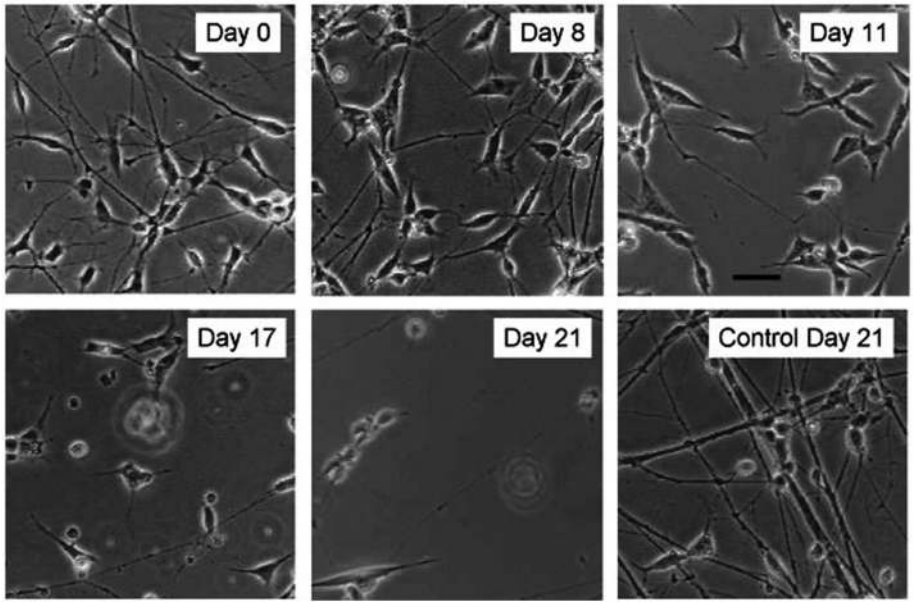

Fig. 1 Phase-contrast images of differentiated SH-SY5Y cells under control conditions (day 0) or after exposure to 50 nM rotenone over 21 days. Differentiated cells surviving in rotenone exhibited progressive loss of processes. Reproduced with permission from *Molecular Neurodegeneration* (M. K. Borland, P. Trimmer, J. Rubinstein, P. Keeney, K. Mohanakumar, L. Liu, *et al.* Chronic, low-dose rotenone reproduces Lewy neurites found in early stages of Parkinson's disease, reduces mitochondrial movement and slowly kills differentiated SH-SY5Y neural cells. *Mol. Neurodegener.*, 2008, **3**, 21. DOI: 10.1186/1750-1326-3-21).[522] Copyright © 2008, BioMed Central Ltd.

RA followed by TPA makes SH-SY5Y cells less prone to the neurotoxic actions of DA.[147] However, compared to cells differentiated with RA, RA/TPA-differentiated SH-SY5Y cells appear to be more sensitive to the neurotoxic effects of the MPP^+.[148] On the other hand, staurosporine-differentiated SH-SY5Y cells are more susceptible to the neurotoxic actions of several agents than in their undifferentiated state,[168] which emphasizes the similarity of RA/TPA- or staurosporine-differentiated SH-SY5Y cells to primary neurons. These changes of vulnerability might be attributed to various biochemical changes evoked by different differentiating agents, such as over-expression and down-regulation of anti- and pro-apoptotic proteins[168,169] or alterations in signaling pathways, including protein kinase B and tropomyosin receptor kinase B pathways.[170,171]

The SH-SY5Y cell line has been extensively used to establish the neurotoxic potential of several compounds, namely MDMA,[147,149,150] classical neurotoxicants like MPP^+ (ref. 148 and 172) and 6-hydroxydopamine,[173,174] or organic pollutants.[175,176] In addition, different mechanisms have been explored, such as disruption in intracellular Ca^{2+} levels,[150,177] mitochondrial dysfunction,[174] oxidative stress[147,149,150,173,174] or disruptions in neurite outgrowth,[175,176] thus making this cell line an important *in vitro* model in the field of neurotoxicology.

5.1.1.2. Pheochromocytoma cell lines. In recent years, there has been great interest in developing pheochromocytoma cell lines, which would be useful to further study cellular and molecular abnormalities involved in the pheochromocytoma appearance and development. In addition, the development of new pheochromocytoma cell lines provides useful research tools to understand the cellular and molecular mechanisms involved in drug-induced cellular adaptations and toxicity to the adrenergic system *in vivo*.[178]

The first human immortalized cell line derived from a sporadic benign human adrenal pheochromocytoma is the KNA cell line,[179] though earlier attempts by these same researchers resulted in four similar human cell lines with finite lifespans of up to one year in culture.[180,181] On the other hand, in addition to human cell lines, rat- and mouse-derived cell lines have been developed. The rat-derived cell line PC12, probably the best known pheochromocytoma cell line, has been extensively used in neuroxicology research.[182,183] Generally, pheochromocytoma cell lines show catecholamine storage and present a ability to release adrenaline (ADR), NA and DA into the tissue culture medium. The outgrowth of the neurotic processes may be stimulated by differentiation with NGF, in a dose-dependent manner,[184] thus inducing a neuronal phenotype.

The pheochromocytoma cell lines express a variety of chromaffin cell markers indicative of mature chromaffin cells, including secretory granules, chromogramins and related peptides and DA-β-hydroxylase.[179] Therefore, pheochromocytoma-derived cell lines present high exocytotic activity. Considering that the key dynamic event in neuronal communications is exocytosis,[184] these cell lines constitute suitable *in vitro* models for neurosecretory studies,[137] as well as for the assess-

ment of the potential neurotoxic effects of drugs that interfere with the process of neurotransmitters' release.[185,186]

5.1.1.2.1. PC12 cells: a rat pheochromocytoma cell line

The PC12 clonal cell line was established from a rat pheochromocytoma (adrenal medullary tumor) and has many properties in common with primary sympathetic neurons and chromaffin cell cultures.[187]

The merits and limitations of PC12 cells as a model for neurotoxicological studies have been reviewed.[188,189] The main characteristics of the PC12 cell line are summarized in Table 3. Though PC12 cells were originally reported to lack the ability to synthesize ADR,[187] their ability to synthesize and store ADR and DA, and sometimes NA was subsequently described.[190] Their low ability to synthesize NA appears to be related to the low activity of DA-β-hydroxylase in this cell line.[187] In addition, as they contain Na^+, K^+, and Ca^{2+} channels, and various membrane receptors including receptors coupled to G-proteins, they are useful to examine the interference of several compounds in the basic biological processes of neurotransmitter biosynthesis and secretion, neuronal differentiation, Ca^{2+} ionic flux, and signal transduction mechanisms.[191]

Upon differentiation with NGF, the PC12 cell line presents a phenotype that resembles the sympathetic ganglion neurons, thus acquiring a characteristically cholinergic phenotype. However, after removal of NGF, cells reacquire a dedifferentiated state. The differentiation with NGF was reported to increase the expression of the TH gene[189,192,193] and promote neurite extension,[189,193] as well as Ca^{2+} channel expression.[188,194] The mechanism by which NGF induces cell differentiation involves a number of protein kinases, including extracellular signal-regulated kinase.[195] In addition, intracellular iron, by increasing the extracellular signal-regulated kinase activity, promotes the NGF-stimulated differentiation of PC12 cells.[195] Therefore, the responsiveness to NGF by PC12 cells has allowed them to be used as a model of neuronal differentiation and pluripotency possessed by primitive progenitors from the medulla, which can differentiate into either chromaffin cells or sympathetic neurons, depending on the local microenvironment, such as the presence of NGF or glucocorticoids.[196] Representative phase-contrast images of PC12 cells under control conditions or after exposure to H_2O_2 are shown in Fig. 2.

PC12 cells may be also differentiated with the glucocorticoid dexamethasone, thus acquiring a chromaffin cells-like phenotype with increased Ca^{2+} current and increased synthesis, uptake and release of catecholamines.[197] Therefore, these characteristics allowed dexamethasone-differentiated PC12 to become a reference model for studying modulation of exocytosis at the single cell level in neurotoxicological studies.[27] Despite the existence of a complex interplay, NGF is known to enhance the effects of glucocorticoids on these cells. Specifically, dexamethasone in combination with NGF leads to increases in transmitter synthesis and vesicle size.[185] In addition, several other agents are described to differentiate the PC12 cell line to a more characteristically neuronal phenotype.

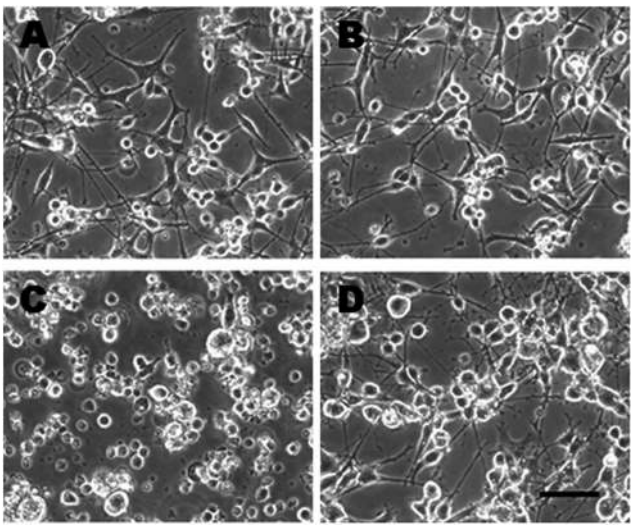

Fig. 2 Phase-contrast images of PC12 cells, stably transfected with an empty vector (A and C) or neuronal Ca^{2+} sensor-1 protein (B and D) and differentiated into neuron-like cells with 100 ng mL^{-1} nerve growth factor, were exposed to 0 (A and B) or 300 μM H_2O_2 for 3 days (C and D). Treatment with 300 μM of H_2O_2 for 3 days resulted in severe cellular damage in PC12 cells transfected with an empty vector; most cells were rounded up and detached from the substratum (C). In contrast, the same treatment caused only a little damage to cells overexpressing neuronal Ca^{2+} sensor-1 (D), indicating that the expression of neuronal Ca^{2+} sensor-1 renders PC12 cells more resistant to the H_2O_2 toxicity. Scale bar: 40 μm. Adapted with permission from *The Journal of Cell Biology* (T. Y. Nakamura, A. Jeromin, G. Smith, H. Kurushima, H. Koga, Y. Nakabeppu, *et al.*, Novel role of neuronal Ca^{2+} sensor-1 as a survival factor up-regulated in injured neurons. *J. Cell Biol.*, 2006, **172**, 1081–1091. DOI: 10.1083/jcb.200508156).[523] Copyright © 2006, The Rockefeller University Press.

Recently, it was shown that, like NGF, sodium nitrite induces neurite outgrowth and extension in this cell line, thus indicating a differentiation process.[186] Staurosporine is another agent known to induce neurite outgrowth in the PC12 cell line.[198,199] However, NGF constitutes the most studied and used agent to differentiate the PC12 cell line.

An important problem associated with the PC12 cell line includes its difficulty in adherence to tissue culture plates. However, on a collagen-coated surface or in cellulose filters, PC12 cells present increased adhesion to that surface, thus increasing neuronal differentiation.[200] Furthermore, coating of the tissue culture plates with L-3,4-dihydroxyphenylalanine, which is responsible for the adhesive property of mussel adhesive proteins, is a feasible strategy for such strong adhesion. In addition, polydopamine-modified surfaces enhance the cell adhesion and viability, and also promote the neuronal differentiation of NGF-stimulated PC12 cells, as evidenced by the elongation of neurites and expression of neuronal differentiation markers.[200] This constitutes a useful approach to increase cell differentiation into a more characteristically neuronal phenotype. Thus, the PC12 cell line provides a useful model for studying processes associated with neuronal differentiation, synthesis, storage, and release

of neurotransmitters, function and regulation of ion channels, and interactions of compounds with membrane-bound receptors.

The PC12 cell line has been extensively used to establish the neurotoxic potential of several compounds through mechanisms involving disruption in intracellular Ca^{2+} levels,[201–203] interference with ion channels,[201,204] changes in neurotransmitter uptake and release,[205,206] disruptions in neurite outgrowth,[202] or alteration in mitochondrial function[207] thus making it an important research tool in the field of neurotoxicology. Organic pollutants, by accumulation into the brain, cause behavioral symptoms and alterations in neurotransmission. Indeed, in this field, the effects of these pollutants on vesicular neurotransmitter release have been investigated in PC12 cells.[203,208] Therefore, since exocytosis strongly depends on the functionality of a large number of intracellular processes, exocytosis is likely among the most sensitive and relevant endpoints that can be determined *in vitro*, at least when measured with single vesicle resolution. In addition, the nature of the observed effects points to the underlying mechanisms, which could then be studied in more detail using other experimental tools. As such, amperometric recordings with PC12 cells could often be used for neurotoxicity assessment in order to determine effects of neurotoxicants on the neurotransmitter exocytosis and uptake.

5.1.2. Cell lines of glial origin

5.1.2.1. Glioma cell lines. Stable glioma cell lines can be generated by transfer of the biopsy material to tissue culture flasks and subsequent passaging. These cell lines possess many of the regulatory control mechanisms and differentiated properties of glial cells.[209]

Among the different glioma cell lines available for *in vitro* toxicological studies, the human U87-MG cell line[210] and the rat C6 cell line[211,212] have been frequently used. Glioma cell lines have been used in numerous studies of toxicity[210–212] and basic cellular mechanisms.[213,214] Probably the most widely used glial cell line is the rat C6 glioma cell line.

5.1.2.1.1. C6 cells: a rat glioma cell line

The C6 glioma cell line was developed in the late 1960s in Sweet's laboratory by repetitively administering methylnitrosourea to outbred Wistar rats over a period of approximately 8 months.[215]

The main characteristics of the C6 glioma cell line are summarized in Table 3. These cells present cancer stem cell-like characteristics, including self-renewal, the potential for multilineage differentiation *in vitro* and tumor formation *in vivo*.[216]

The C6 glioma cell line may be differentiated, after which it acquires a more characteristic glial phenotype. Upon differentiation with dBcAMP/theophylline C6 glioma cells have increased expression of the glial fibrillary acidic protein (GFAP).[217,218] Furthermore, after this differentiation protocol, cells present morphological alterations, including long projections or cellular processes.[217,218] Other agents are described to induce differentiation of this cell line. One example is the protein kinase A activator forskolin, which, by down-regulating cyclin D1 expression, represses cell growth, *via* cell cycle arrest in the G_0/G_1 phase, and induces cell differentiation characteristic with elongated processes and restoration of GFAP expression.[219] C6 glioma cells differentiated with cycloheximide present profound morphological transformations, cell-cycle arrest (stop to proliferate) and increased expression of GFAP.[220] Differentiation of C6 cells with cholera toxin is also associated with increased expression of GFAP.[221] Other agents, including bFGF,[222] platelet-derived growth factor (PDGF),[222] RA,[223,224] histone deacetylase inhibitors (trichostatin A, sodium butyrate or valproic acid)[225,226] or panaxydol,[227] are also described to induce differentiation of the C6 glioma cell line. In addition, differentiation of C6 glioma cells with bFGF plus PDGF generates both neurons and glia in culture.[222] Representative phase-contrast images of retinoic acid-differentiated C6 glioma cells under control conditions or after exposure to GLU are shown in Fig. 3.

This cell line has been established as a model in which several aspects of hormonal action previously observed *in vivo* or in primary brain cell cultures can be studied.[228] In addition, C6 glioma cells have been used to study the regulation and modulation of myelin specific genes.[229,230] Based upon the *in vivo* experimental studies and human case reports, suggesting that the major target sites of lead toxicity are myelinating cells, these cells have been used to examine lead toxicity.[231] Results suggest that lead has a selective inhibitory effect on an oligodendroglial function expressed in the C6 glial cell line. This was demonstrated by a dose-dependent inhibition of glucocorticoid-induced soluble cytoplasmic glycerol-3-phosphate dehydrogenase (GPDH), at the level of transcriptional processing. Since GPDH is a specific biochemical marker for the myelin-forming cells and oligodendrocytes and is believed to be involved in myelination, the selective inhibi-

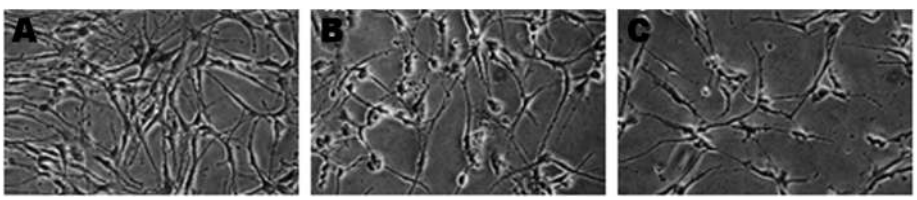

Fig. 3 Phase-contrast images of retinoic acid-differentiated C6 glioma cells under control conditions (A) or after exposure to 0.5 mM (B) or 1 mM (C) glutamate (GLU) for 24 h. Cells exposed to GLU for 24 h exhibited cell shrinkage and rounding. Adapted with permission from *PLoS ONE* (H. Kataria, R. Wadhwa, S. C. Kaul and G. Kaur, Water extract of the leaves of Withania somnifera protects RA differentiated C6 and IMR-32 cells against glutamate-induced excitotoxicity. *PLoS One*, 2012, **7**, e37080. DOI: 10.1371/journal.pone.0037080).[524] Copyright © 2012, PLoS Company.

tory effect of lead on GPDH induction is consistent with the *in vivo* observations of hypomyelination.[232] In addition, the C6 glioma cell line has also been used in experimental research to evaluate the neurotoxicity of other compounds, including tri-*ortho*-cresyl phosphate,[233] manganese[234] or methylmercury.[235] Therefore, this cell line constitutes another alternative model available for neurotoxicity research *in vitro*.

5.2. Primary cultures

5.2.1. Dissociated primary cultures. Dissociated primary cultures are, perhaps, the most widely used *in vitro* system in the field of neurotoxicity research. The dissociated primary cultures are prepared from suspensions of individual cells, obtained by dissociation of the brain tissue.[236–238] The plating efficiency is dependent on the dissociation technique, the type of substratum, the culture medium composition, and the type of tissue. The presence and amount of serum and trophic factors, oxygen tension, the composition of the substratum and the seeding density strongly affect the viability and differentiation of the cell culture (dedifferentiation, transdifferentiation, differentiation inhibition or induction of differentiation).[239] However, under favorable conditions, it is possible to maintain such cultures for long periods of time, during which cells acquire most properties of mature neurons. They develop distinct axons and dendrites, form synapses with other neurons, and express the receptors and ion channels characteristic of the corresponding cell type *in situ*.[32] Frequently, cells develop a considerable spontaneous electrical activity, including synaptic potentials. When co-cultured with Schwann cells[240,241] or oligodendrocytes,[242,243] axons become myelinated. Importantly, neurons obtained by dissociation appear to retain their individual identities, presumably as a result of their postmitotic nature and they are committed to their differentiation at the time when they are introduced in culture. In a few cases, it has been possible to demonstrate this directly by labeling specific populations of embryonic cells *in situ*, and then examining their properties in culture.[244] Therefore, in general, the morphological and physiological properties of the cell populations present in culture correspond closely to the cell characteristics *in vivo*.

The main advantages and limitations in using dissociated primary cultures for neurotoxicological studies are summarized in Table 4. The most obvious advantage of the dissociated primary cultures is that they render accessibility to individual living cells. During the first few days in culture, before the neural network becomes too dense, individual neurons can be easily visualized in their entirety. This allows a direct observation of growing axons, their mode of branching and the behavior of their growth cones. Importantly, dissociated cells in culture allow a remarkably precise experimental analysis of these events. However, these cultures are less suited to traditional biochemical approaches, since the amount of biological material obtained from these cultures usually is limited. Another inherent limitation of the dissociated primary cultures is the lack of a homogeneous population of cells; primary cultures are as complex as the tissue from which they are originated. The heterogeneity of the culture also complicates studies using morphological and physiological techniques, since consistent results require the identification of specific cell populations. Nevertheless, the heterogeneity of primary cells can also be considered an advantage, as this represents a more complex cell model and is more similar to the intact nervous system. Developing approaches to deal with the heterogeneity of cell types is critical to the successful use of dissociated primary cultures. Such considerations begin with the choice of tissue to culture. Some regions of the nervous system are simply more complicated than others. There is also a general belief that cells in primary culture may be more sensitive to the effects of neurotoxicants. Though this is true at times, it is not always the case, because these differences of susceptibility may often be due to different culturing conditions.[32,98,134]

Table 4 Advantages and limitations in using dissociated primary cultures as *in vitro* models for neurotoxicological studies

Advantages
Individual living cells are accessible[98]
These cultures allow the monitoring of both morphological and electrophysiological features, which may be applied on a cell-by-cell basis[98]
Possibility of a direct observation of growing axons, their mode of branching and the behavior of their growth cones[28,98,134]
Possibility to obtain and correlate biochemical, morphological, electrophysiological and molecular data from a single cell[98]
Limitations
Heterogeneity of the cellular population[98]
The tissue organization is lost as a result of the dissociation procedure[98]
Requirement of an animal as a source of tissue for each preparation[28,98]
Limited amount of the biological material from each preparation[98]
Higher susceptibility to the effects of neurotoxicants[32,98]

5.2.1.1. Neuronal primary cultures. Much work has been focused on obtaining cultures representative of neuronal populations. Usually primary neuronal cultures are derived from many different brain regions, such as the hippocampus (HIP),[236,237] cortex,[88,90,238] striatum[245] or cerebellum,[89,246] or from the peripheral nervous system,[247] of rat[88,90,238] or mouse[236,245] fetal brain tissue. However, fetal brain tissue from humans[248] or chicken[247,249] has also been used. The use of fetal tissue is required, because neurons are much less susceptible to damage during dissociation, since their soma are still small, and they have not developed extensive axonal and dendritic arbors or become highly innervated. In addition, at early stages of development, neurons may also be less dependent on their target cells for trophic support.[98] Even within a brain region, such as the cerebellum, individual cell types require distinct donor ages. Although cerebellar granule cells are generally isolated from brains at postnatal days 6 to 8,[89,246] for successful Purkinje cell cultures, usually fetal tissue is used.[250] Therefore, as the age of the donor is critical for successful culturing of each cell type from different brain regions, it is important to maintain consistency in donor age between different experiments.

Different brain regions present different neuronal populations. Whereas primary cultures of cortical neurons are mainly constituted of gamma-aminobutyric acid (GABA)ergic and colinergic neurons, primary cultures of cerebellar granule cells are mainly constituted of glutamatergic neurons.[251] Thus, according to the endpoint of neurotoxicity that will be measured, there are brain areas that may be more appropriate than others.

On the other hand, it is difficult to obtain pure cultures of dissociated primary neurons, since these cultures are generally contaminated with glial cells. Thus it is difficult to discern pharmacological or toxicological phenomena that are specific for neurons.[252] To circumvent this limitation, cytosine arabinose may be added to primary cultures, generally 24 to 48 h after seeding, which, by inhibiting the glial proliferation, allows obtaining neuron-enriched primary cultures.[253–255]

In *in vitro* culture, neurons extend neurites rapidly with a minimal requirement for extracellular matrix proteins, neural cell adhesion molecules or NGF. Though brain tissue constitutes the most widely used source for culture of dissociated neuronal cells, cultures of sympathetic ganglion cells[256] and dorsal root ganglion cells[257,258] have also been prepared. In these cells, neurite outgrowth can be enhanced by the presence of various growth factors, including NGF. On the other hand, neurons in culture differ from neurons *in vivo* for a number of reasons, which must be considered when interpreting data from *in vitro* systems. Primary neurons in culture are metabolically, physiologically and morphologically stunted. Furthermore, they are not subject to normal excitatory or inhibitory inputs that are observed *in vivo*. Therefore, the susceptibility of these cells to toxic insult may be different. Another limitation of the use of primary neuronal culture is that these cells do not divide *in vitro* and survive for a limited time. In addition, it is difficult to obtain homogenous, well-defined and reproducible cultures.[41,134]

Despite all these limitations, primary neuronal cultures have proven to be useful models for measuring neurotoxicity *in vitro*. In fact, many studies have used primary neuronal cultures to study the neurotoxic potential of several compounds, namely drugs of abuse, such as MDMA,[237,238] amphetamine[259] or cocaine,[259] methylmercury,[260] lead,[261] organic pollutants,[260] neuropharmacological agents,[261] among other compounds. Several endpoints of neurotoxicity have been assessed, such as cell viability,[149,150,237,238] interference with neurite outgrowth[247,262] or disruption of the mitochondrial function.[259,261,263]

5.2.1.1.1. Primary cultures of cortical neurons

Derived from its size and intricate complexity, the cerebral cortex is constituted of a heterogeneous neuronal population of two broad classes of cortical neurons: projection neurons, which extend axons to distant intracortical, subcortical and subcerebral targets, and interneurons, in a large number, which make local connections.[264] Projection neurons (glutamatergic cells) are characterized by a typical pyramidal morphology and transmit information between different regions of the neocortex and to other brain regions. By contrast, interneurons present GABAergic and cholinergic features.[26,264]

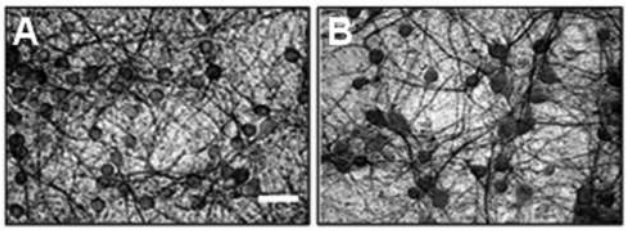

Fig. 4 Brightfield photomicrographs of primary cultures of mouse cortical neurons, detected by microtubule-associated protein 2 (MAP2) immunostaining, under control conditions (A) or after 24 h of exposure to 10 μM *N*-methyl-D-aspartate (NMDA) (B). Primary cultures of cortical neurons present increased excitotoxic neuronal death after exposure to NMDA. Scale bar: 100 μm. Adapted with permission from *Molecular Neurodegeneration* (A. Jullienne, A. Montagne, C. Orset, F. Lesept, D. Jane, D. Monaghan, *et al.*, Selective inhibition of GluN2D-containing *N*-methyl-D-aspartate receptors prevents tissue plasminogen activator-promoted neurotoxicity both *in vitro* and *in vivo*. *Mol. Neurodegener.*, 2011, **6**, 68. DOI: 10.1186/1750-1326-6-68).[525] Copyright © 2011, BioMed Central Ltd.

Thus, primary cultures of cortical neurons are mainly constituted of GABAergic and cholinergic neurons. Consistently, immunocytochemistry analyses for the GABA synthesizing enzyme glutamic acid decarboxylase, to identify GABAergic neurons, showed a high percentage of glutamic acid decarboxylase immunoreactive cells (39 ± 2%) in primary cultures of cortical neurons.[251] In contrast, immunofluorescence analyses using VAChT (cholinergic marker) antiserum revealed an extensive plexus of VAChT-immunoreactive fibers and cell bodies throughout all layers of rat's cerebral cortex.[265] Representative brightfield photomicrographs of primary cultures of cortical neurons, under control conditions or after exposure to *N*-methyl-D-aspartate (NMDA), are shown in Fig. 4 and the main features of interest that make these cultures useful for neurobiology and neurotoxicology studies are summarized in Table 5.

Generally, primary cultures of cortical neurons are prepared from E17 to E19 rat's embryos[85,86,88,238,266–269] or from mice's embryos at a comparable stage of development (E15 to E17).[262,270–273] At this stage, the neurogenesis, which takes place during the embryonic period, from E14 to E18 in the rat neocortex, is essentially complete.[274] Thus, the selection of this embryonic stage ensures that non-differentiated neurons, at an age close to the last mitotic division of the cells, are used for the preparation of primary cortical cultures.

In culture, cortical neurons might be classified as pyramidal-like, fusiform or multipolar cells. Pyramidal-like neurons present a triangular soma with one prominent apical dendrite, several somewhat shorter basilar dendrites and many spines along the dendrites. In contrast, neurons presenting dendritic processes emerging only from opposite poles of the soma are classified as fusiform, either bipolar or bitufted, depending on whether there are single or multiple dendrites, respectively. Furthermore, neurons with a fusiform morphology often have spherical soma and either spiny or aspinous dendrites. Lastly,

Table 5 Characteristics of primary cultures of cortical, hippocampal, cerebellar granule and sensory neurons

Primary cultures of cortical neurons
Usually prepared from rodent embryos, at embryonic day (E) 17 to E19 in rats,[85,86,88,238,266–269] or at E15 to E17 in mice[262,270–273]
Primary cortical neurons express functional GABA-A[270] and NMDA receptors[251]
According to morphological criteria, cultured cortical neurons might be classified as pyramidal-like, fusiform or multipolar cells[275]
A high amount of material can be taken from the cerebral cortex, compared to other brain regions like the hippocampus[251]
Constituted by a heterogeneous neuronal population of projection neurons and interneurons (major population)[264] that present the morphological features seen in their *in situ* environment[275]
Projection neurons are glutamatergic excitatory cells that use GLU as their major neurotransmitter and interneurons present GABAergic and cholinergic features.[26,264] Thus, primary cultures of cortical neurons are mainly constituted of GABAergic and cholinergic neurons[251,265]
The distinctions between pyramidal-like, fusiform and multipolar neurons are preserved from the earliest states of differentiation[275]
Primary cultures of hippocampal neurons
Usually prepared from rodent embryos, at E17 to E19 in rats,[237,288–291] or at E15 to E17 in mice[236,292–294]
Low content of interneurons and dentate granule cells[284,285]
Hippocampal pyramidal neurons are glutamatergic excitatory cells, though they also express GABA receptors.[286,287] Thus, primary hippocampal cultures are mainly constituted of glutamatergic neurons
Dendritic arbor is distinctive, consisting of a single long apical dendrite and several shorter basilar dendrites, all highly branched and studded with dendritic spines[284,514]
High content of pyramidal neurons[284,285,296]
The high content of pyramidal neurons, usually 85 to 90% of the total neuronal population,[284] allows one to obtain a more homogeneous experimental response[296]
Pyramidal neurons have a characteristic, well-defined shape, with a single axon and innumerous dendrites[284,514]
Pyramidal neurons establish direct connections with other neurons and with the population of endogenous interneurons[284,514]
Primary cultures of cerebellar granule neurons
Usually prepared from 7- to 8-day-old rodent pups[89,246,271,306,309,310,316]
Low content of other neuronal cells[304,305]
Cultured cerebellar granule neurons are glutamatergic excitatory cells[251,303,304,306,307] that express functional NMDA[309,313] and GABA-A[311,312] receptors. Thus, primary cultures of cerebellar granule cells are mainly constituted of glutamatergic neurons
Cerebellar granule neurons cultured for at least a week undergo extensive regrowth of neurite processes[303]
High content of granule neurons[303]
The high percentage of granule neurons in these cultures, usually over 95%,[303] allows one to obtain a more homogeneous experimental response
Cerebellar granule neurons establish direct connections with other neurons, such as Purkinje cells, and with the population of endogenous interneurons like type II Golgi interneurons[515]
A useful system for *in vitro* experimentation of excitotoxicity- and oxidative stress-related mechanisms[89,246,306,307,310,314,316]
Primary cultures of sensory neurons
Usually prepared from rodent embryos (approximately at E10–13 in mouse[257,318] and E9–16 in rat[257,258,319,320]), as well as from neonatal or adult animals[321–323]
Sensory neurons dissociated from adult animals can be cultured without trophic factors in defined media with vitamin supplement[321,328]
Sensory neurons in culture share many of the features of regenerating sensory neurons *in vivo*[516]
Robustly express molecules associated with axonal regeneration, such as growth associated protein 43, galanin and plasminogen activators[517]
Response properties change over time in culture in response to both the dissociation procedure and the altered environment[328]
Embryonic and neonatal preparations have higher cell yields and greater proportion of neurons, but are dependent on neurotrophins, for culture[321,328]
Composed of neurons with diverse response properties, target tissue innervation and growth factor responsiveness[328]
Sensory neurons express ion channels' receptors, neuropeptides and Ca^{2+}-binding proteins detected *in vivo* and respond to acute depolarization[328]
Respond to chemical,[319,518] thermal[519] and mechanical[520] stimuli

multipolar neurons have multiple processes of approximately equal lengths, arising from multiple sites around the cell body, and a wide range of spine densities on their dendrites.[275] The distinctions between pyramidal-like, fusiform and multipolar neurons are preserved from the earliest states of differentiation.[275]

Cortical neurons in culture exhibit the morphological features observed in their *in situ* environment.[275] Furthermore, cultured cortical neurons express GABA-A receptors, as demonstrated by the binding of specific radioligands.[270] Accordingly, [^{3}H]GABA uptake in primary cultures of cortical neurons is completely inhibited by guvacine and nipecotic acid.[276] Likewise, reports demonstrating an increase of intracellular Ca^{2+} caused by NMDA reveal the presence of functional NMDA receptors in these cultured neurons.[251] Compared to other brain regions, the relatively high amount of tissue that can be taken from the cerebral cortex constitutes another important feature that makes primary cultures of cortical neurons an useful system for extensive neurobiology and neurotoxicology studies.[251]

Primary cultures of cortical neurons have been successfully used to establish the neurotoxic potential of several compounds,[85,86,88,90,238,259,262,266,269,277–280] thus making them an important *in vitro* model in the field of neurotoxicology. Cultured cortical neurons have also been widely used for imaging protein or organelle dynamics[267,281,282] and for defining the molecular mechanisms underlying the development of neuronal polarity, dendritic growth, microtubules' dynamics and synapse function.[272,273,283] For morphological studies related to neuronal growth, culture conditions must allow the growth of cells at low density, to allow an easy observation of individual cells.

5.2.1.1.2. Primary cultures of hippocampal neurons

The HIP presents a population of neurons with well-characterized properties typical of neurons of the central nervous system, in general. Pyramidal neurons, the principal cell type in the HIP, have been estimated to account for 85 to 90% of the total neuronal population. Nevertheless, CA1 and CA3 regions of the HIP contain pyramidal neurons that differ from one another in some of their physiological properties and in some aspects of their connectivity, *i.e.* in their intrinsic characteristics, but all of them are classified as pyramidal neurons. Therefore, these neurons may be cultured separately. Despite this, they are similar in many fundamental aspects. A variety of interneurons have also been described in the HIP, though they are few in number, compared with pyramidal neurons. In addition, hippocampal primary cultures may present a low content of dentate granule cells.[284,285] Representative brightfield photomicrographs of primary cultures of mouse hippocampal neurons, under control conditions or after exposure to NMDA, are shown in Fig. 5 and the main features of interest that make these cultures useful for neurobiology and neurotoxicology studies are summarized in Table 5.

In the HIP, neurons of the try-synaptic circuit, granule and pyramidal cells are glutamatergic excitatory cells, whereas inhibitory interneurons are GABAergic cells. Despite this, hippocampal pyramidal neurons and dentate granule cells express GABA receptors, thus indicating that these cells may be modulated by GABA neurotransmission.[286,287] Therefore, primary hippocampal cultures are mainly constituted of glutamatergic neurons.

Generally, hippocampal primary cultures are prepared from E17 to E19 rat's embryos[237,288–291] or from mice's embryos at a comparable stage of development (E15 to E17).[236,292–295] At this stage, the generation of pyramidal neurons, which begins in the rat at about E15, is essentially complete, though the generation of dentate granule cells, which largely occurs postnatally, has scarcely begun.[296] Therefore, hippocampal primary cultures prepared from embryos at this stage are essentially constituted of pyramidal neurons. This characteristic allows obtaining a more homogeneous experimental response.

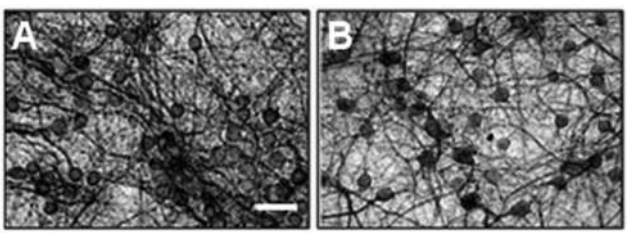

Fig. 5 Brightfield photomicrographs of primary cultures of mouse hippocampal neurons, detected by microtubule-associated protein 2 (MAP2) immunostaining, under control conditions (A) or after 24 h of exposure to 10 μM *N*-methyl-D-aspartate (NMDA) (B). Primary cultures of hippocampal neurons present increased excitotoxic neuronal death after exposure to NMDA. Scale bar: 100 μm. Adapted with permission from *Molecular Neurodegeneration* (A. Jullienne, A. Montagne, C. Orset, F. Lesept, D. Jane, D. Monaghan, *et al.*, Selective inhibition of GluN2D-containing *N*-methyl-D-aspartate receptors prevents tissue plasminogen activator-promoted neurotoxicity both *in vitro* and *in vivo*. *Mol. Neurodegener.*, 2011, **6**, 68. DOI: 10.1186/1750-1326-6-68).[525] Copyright © 2011, BioMed Central Ltd.

Hippocampal primary cultures have been extensively used to establish the neurotoxic potential of several compounds,[237,297,298] thus making them an important *in vitro* model in the field of neurotoxicology. In addition, hippocampal cultures have also been widely used for visualizing the subcellular location of endogenous or expressed proteins, for imaging protein or organelle trafficking[236,288,289,292–294] and for defining the molecular mechanisms underlying the development of neuronal polarity, dendritic growth and synapse formation.[272,273,290,291,295,299,300] For morphological studies, culture conditions must allow the growth of cells at low density, to allow easy observation of individual cells.

5.2.1.1.3. Primary cultures of cerebellar granule neurons

The cerebellum presents a relatively heterogeneous population of neuronal and non-neuronal cells. Cerebellar granule neurons, which have been implicated in motor learning[301] and in the control of information flow between cerebellar inputs and outputs,[301,302] represent the most numerous neuronal population (cerebellum comprises over 95% of this neuronal type).[303] Other neuronal cells are also identified in the cerebellum, namely Purkinje cells, unipolar brush cells, Golgi cells, Lugaro cells, candelabrum cells, basket cells, stellate cells and small neurons of the deep cerebellar nuclei.[304,305] However, since they are more difficult to identify by light microscopy in cerebellar cultures, it is more problematic to ascertain their relative numbers.[305] In addition, the cerebellar non-neuronal population comprises astrocytes, Bergmann glia (a specific type of astrocyte in the cerebellum), and oligodendrocytes. *In vivo*, these neurons and glial cells are arranged in a three-layer structure in the cerebellum, from superficial to deep: the molecular layer, Purkinje cell layer, and granule cell layer.[304]

In the cerebellum, granule neurons (such as unipolar brush cells) are excitatory neurons that use GLU as their major neurotransmitter (glutamatergic neurons). Alternatively, inhibitory neurons, including Purkinje cells, Golgi cells, Lugaro cells, candelabrum cells, basket cells, stellate cells, and small neurons of the deep cerebellar nuclei, use GABA and/or glycine as their major neurotransmitters (GABAergic neurons).[251,303,304,306,307] Thus, primary cultures of cerebellar granule cells are mainly constituted of glutamatergic neurons. Consistently, immunocytochemistry analyses for the GABA synthesizing enzyme, glutamic acid decarboxylase, to identify GABAergic neurons, showed a minority of glutamic acid decarboxylase-immunoreactive cells (6%) in primary cultures of cerebellar granule neurons.[308] Representative phase-contrast images of primary cultures of cerebellar granule neurons, under control conditions or after exposure to GLU, are shown in Fig. 6 and the main features of interest that make these cultures useful for neurobiology and neurotoxicology studies are summarized in Table 5.

Neurogenesis takes place postnatally from P0 to P15 for the granule cells in the rat cerebellum.[274] Tissue from the cerebel-

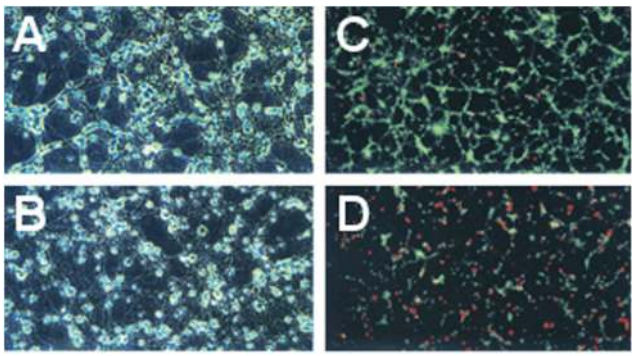

Fig. 6 Representative images of primary cultures of cerebellar granule neurons, photographed by phase-contrast microscopy (A and B) and after double staining with fluorescein diacetate and propidium iodide (C and D), under control conditions (A and C) or after exposure to 30 μM glutamate (GLU) for 24 h (B and D). Exposure to GLU greatly reduces the cell viability of primary cerebellar granule neurons. Magnification: 100×. Adapted with permission from *Proceedings of the National Academy of Sciences of the United States of America* (Y. Du, K. R. Bales, R. C. Dodel, E. Hamilton-Byrd, J. W. Horn, D. L. Czilli, *et al.*, Activation of a caspase 3-related cysteine protease is required for glutamate-mediated apoptosis of cultured cerebellar granule neurons. *Proc. Natl. Acad. Sci. U. S. A.*, 1997, **94**, 11657–11662).[526] Copyright (1997), The National Academy of Sciences, U.S.A.

lum is taken at defined developmental stages of neurogenesis to ensure that non-differentiated neurons, at an age close to the last mitotic division of the cells, are used for the preparation of primary neuronal cultures. Thus, primary cultures of cerebellar granule neurons are generally prepared from 7- to 8-day-old mouse[89,271,309] or rat[246,306,310] pups.

Cultured cerebellar granule neurons express GABA-A receptors, as has been demonstrated by the binding of the specific radioligands.[311,312] These cultured neurons also express functional NMDA receptors, as ascertained by the binding of [^{3}H] MK801 and immunolabeling and western blotting analysis for the NR1 subunit of the NMDA receptor.[309,313] Therefore, postsynaptic events in primary cultures of cerebellar granule neurons are mainly due to activation of ionotropic GABA and GLU receptors.

Primary cultures of cerebellar granule neurons have been extensively used to establish the neurotoxic potential of several compounds,[89,246,309,310,314,315] thus making them an important *in vitro* model in the field of neurotoxicology. Brief exposure of primary cultures of cerebellar granule cells to high concentrations of K^+ induces Ca^{2+}-dependent release of endogenous GLU. Thereafter, released GLU acts on postsynaptic NMDA receptors present in these cultures producing excitotoxic neuronal death, when extracellular levels of GLU rise up to micromolar concentrations.[316] As such, these cultures represent one of the most interesting and frequent models for *in vitro* experimentation of excitotoxicity and oxidative stress-related mechanisms.[89,246,306,307,309,310,314] Since neuronal degeneration and death might be an important excitotoxic component,[317] this system also constitutes a useful *in vitro* model for studying neuroprotective mechanisms and drugs against neurotoxic events.

5.2.1.1.4. Primary cultures of sensory neurons

Sensory neurons are located at the spinal ganglia, also called dorsal root ganglia (DRG), or in cranial sensory ganglia associated with cranial nerves V, VII, VIII, IX and X.

Usually, primary cultures of sensory neurons are prepared from DRG or trigeminal ganglia of mouse or rat embryos shortly after sensory neurons are generated (approximately on E10–13 in mice[257,318] and E9–16 in rats[257,258,319,320]), as well as from neonatal or adult animals.[321–323] Nevertheless, although sensory neurons from many different vertebrate species, such as frogs,[324] chicken[325] and even humans,[326] have been isolated and cultured, mice have a particular genetic advantage, given the many publicly available transgenic mouse lines in which genes have been deleted[257] or altered.[327] Embryonic and neonatal preparations have the advantage of higher cell yields and greater proportion of neurons. However, they are dependent on neurotrophins, especially nerve NGF, in the first week of culture. In contrast, DRG sensory neurons can be dissociated from adult animals maintained without trophic factors in defined media with vitamin supplement.[321,328] A substantial body of evidence has indicated that adult mammalian DRG neurons survive and regenerate in culture.[329] Representative phase-contrast images of cultured adult rat DRG neurons, under control conditions or after exposure to oxaliplatin, are shown in Fig. 7 and the main features of interest that make these cultures useful for neurobiology and neurotoxicology studies are summarized in Table 5.

The sensory ganglion composition is stereotyped from animal to animal. For example, for a particular ganglion, there

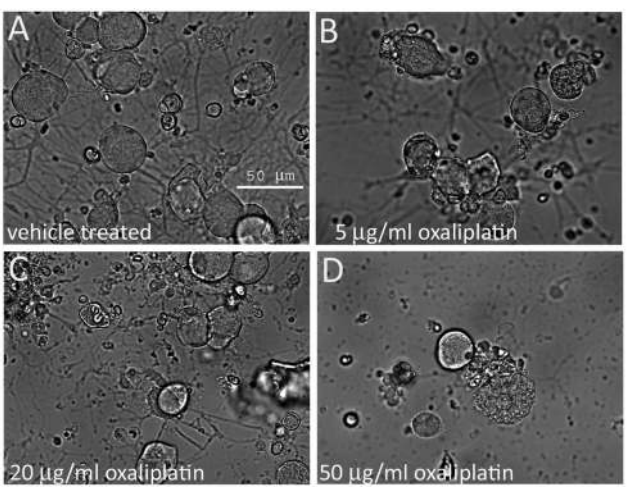

Fig. 7 Phase-contrast images of cultured adult rat dorsal root ganglion neurons under control conditions (vehicle treated; A) or after 48 h of exposure to 5 μg ml^{-1} (B), 20 μg ml^{-1} (C) or 50 μg ml^{-1} (D) oxaliplatin, showing a dose-dependent loss of neurite integrity and cell bodies, and accumulation of cell debris. Scale bar: 50 μm. Reproduced with permission from *Molecular Pain* (U. Anand, W. Otto, P. Anand, Sensitization of capsaicin and icilin responses in oxaliplatin treated adult rat DRG neurons. *Mol. Pain*, 2010, **6**, 82. DOI: 10.1186/1744-8069-6-82).[527] Copyright © 2010, BioMed Central Ltd.

is little variation between animals in terms of cell number, response properties and target innervation. Therefore, the same population of cells can be readily identified, removed and examined in parallel experiments from multiple animals. Each ganglion contains neurons with diverse response properties, target tissue innervation and growth factor responsiveness, making it possible to study the culture response to different stimuli.[328] In addition, many neurochemical markers that distinguish between restricted subsets of sensory neurons have been identified. For example, expression of the ion channel TRPV1 defines a specific subset of nociceptors, and the Ca^{2+}-binding protein parvalbumin is expressed primarily by proprioceptors.[330,331] The most significant limitation for the primary sensory neuron culture is the cell isolation procedure, which requires axotomy of both central and peripheral processes. As in other primary cultures, the presence of non-neuronal cells makes difficult the study of isolated neuronal effects, particularly when cells are cultured for extended periods (more than 3 days). To circumvent this apparent limitation, a variety of techniques have been developed to minimize the presence of non-neuronal cells in culture, including the pre-plating cell lysates to remove non-neuronal cells before culture,[332] the use of DNA topoisomerase inhibitors[333] and the use of mitotic inhibitors, such as 5-fluorodeoxyuridine.[334] Nevertheless, the use of cytosine arabinose is not recommended.[334]

Primary cultures of sensory neurons have been extensively used to establish the neurotoxic potential of several compounds,[319,320,322,335,336] thus making them an important *in vitro* model in the field of neurotoxicology. In addition, primary cultures of sensory neurons have been used for imaging of organelle trafficking[257,258] in a wide range of developmental studies, including the investigation of programmed cell death,[337] growth cone dynamics[338] and growth factor signaling pathways.[339] Compartmentalized culture systems separating cell bodies and processes have been used to discriminate location-specific signaling pathways.[340] In addition, these cultures have been successfully used to study processes important for both central and peripheral nervous system function, such as regeneration,[341,342] and the role of axonal translocation of proteins in modulating synaptic function.[343,344]

5.2.1.2. Glial primary cultures. A large number of techniques and media conditions exist for the generation of dissociated primary glial cell cultures. Usually primary glial cultures are derived from rat or mouse brain between birth and postnatal day 7.[345–348] Glial cultures are constituted of three phenotypically distinct glial cells, astrocytes, oligodendrocytes/Schwann cells, and microglial cells.[349] Although much information is available on each individual cell type in culture, only recently the interactions among the various glial cells have been studied. Depending on the postnatal age, cell cultures differ dramatically in the glial cell composition, maturation process and biochemical responsiveness. Therefore, for any interpretation of experimental data, it is critical to maintain the age of the animal within a well-characterized window.

Based upon differential cell adhesion, each cell type may be sub-cultured to a relatively enriched population.[350] Once a mixed glia culture has been established, which occurs after approximately 2 weeks when cultures are prepared from postnatal day 7 animals, culture flasks can be shaken to dislodge both microglial cells (within 2 h) and oligodendrocytes (18 h) from the underlying astrocyte monolayer. Since microglial cells remain in suspension, their culture is obtained by plating the media removed after 2 h of shaking. Cells are allowed to attach to tissue culture, after which the medium is removed and replaced with fresh medium. After shaking, astrocytes can be sub-cultured after enzymatic detachment, filtration and an plating period, which will allow removal of any remaining contaminating microglia. Additional steps must be taken to ensure removal of any contaminating microglia from the oligodendrocyte subculture.[350] Despite this, incubation of primary cultures with leucine methyl ester, which inhibits microglia proliferation, allows obtaining microglia-depleted cultures.[255,351]

To isolate Schwann cells, generally neonatal mice or rats from postnatal day 0 to 4 are used. The sciatic nerve is isolated away from the surrounding muscle and connective tissue, and then it is enzymatically and mechanically dissociated. These cultures initially contain both bipolar spindle-shape Schwann cells and fibroblasts.[346,348,352] The rapidly dividing fibroblasts may be removed by incubation with the antimitotic agent cytosine arabinose.[352] Alternatively, contaminating fibroblasts may be removed by antibody-mediated cytolysis.[346] In addition, the production of myelin-related proteins by Schwann cells is highly sensitive to axonal contact.[348]

Interactions between neurons and glial cells coordinate the intimate functional relationship that exists between these cells *in vivo*. Therefore, chemicals that disrupt these interactions are likely to be neurotoxic through functional isolation of these normally inter-dependent cells. Furthermore, toxicants that affect neuronal receptors or transduction systems might also collaterally impact their glial counterparts. In addition, growing evidence suggests that glial cells possess enzymes for xenobiotic biotransformation.[46,353] Thus, glial cells may prevent development of neurotoxicity through the metabolism of xenobiotics. However, these cells might also contribute to the metabolic bioactivation of protoxicants. MPTP represents a classical compound that is metabolized within astrocytes, by monoamine oxidase B to the reactive intermediate MPP^+, with subsequent propensity to selectively destroy nigrostriatal dopaminergic neurons.[44,50] Therefore, in this case, neuronal dysfunction depends on the astrocytic function. In addition, Schwann cells have been used to study the toxic effects caused by toxicants known to induce demyelinative peripheral polyneuropathy *in vivo*.[354] Therefore, as the interactions between neuronal and glial cells play an important role in determining the neurotoxicity of several compounds, the effects of xenobiotics on the function of the glial cells may be important in predicting their neurotoxic potential.

5.2.1.3. Neuron-glia sandwich co-cultures. The existence of a complex interdependence between neurons and glial cells is

well accepted (for a better understanding of the interdependence between neurons and glia see section 2.1.1.6.). Therefore, the use of mixed cultures of neurons and glia,[253,254,355] by reproducing *in vitro* the intercommunication among the different cell populations of the nervous system that occurs *in vivo*, allows the evaluation of this intercommunication in several physiological responses and/or propagation of the injury, and study the molecular mechanisms involved.[356] However, the existence of a mixed culture of neurons and glial cells makes it difficult to discern whether a toxic event is neuronal specific or whether glial toxicity also occurs.[252]

Consequently, to circumvent this limitation, another model was developed, the so-called neuron-glia sandwich co-culture.[252,357] A schematic representation of a neuron-glia sandwich co-culture is illustrated in Fig. 8. This constitutes a suitable *in vitro* cell system to evaluate cell-to-cell interactions occurring on the release of soluble factors. Furthermore, this system allows elucidation of the mechanisms involved in the development of a glia-dependent neurotoxic event. A sandwich co-culture is an *in vitro* cell system formed by two different cell populations growing on different surfaces, usually a coverslip and a petri dish or multiwell plates. These surfaces are separated by small paraffin dots at the edges of the coverslip, on which one of the cell populations is seeded. Alternatively, transwell chambers have also been used to generate neuron-glia sandwich co-cultures, in which one cell population grows into the plate and the other into the transwell insert.[358] In both cases, the two cell populations face each other without touching. Nevertheless, soluble substances can diffuse between them. The great advantage of this cell system, compared to the mixed culture of neuronal and glial cells, is the possibility of separating the two cell populations at any time, while retaining their integrity and organization. It is then possible to remove one cell population, retaining all trophic factors derived from the removed cells in the cell cultured medium. Furthermore, this physical separation allows manipulation of the cell types differently, thus providing information on the involvement of specific mediators or biochemical pathways.[359] In addition, this system allows one to perform different biochemical measurements on the two cell populations separately and evaluate the neuronal viability and activity in the presence or absence of a glial layer.[252]

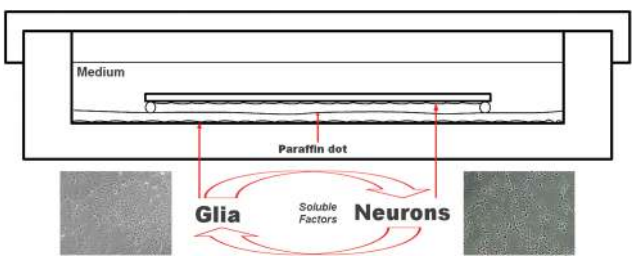

Fig. 8 Neuron–glia–neuron sandwich co-culture. Glial cells are seeded at the bottom of Petri dishes while neurons on the top of glass coverslips. Coverslips are inverted so that neurons face the glia monolayer. Paraffin dots create a narrow gap to separate neurons from glial cells. The two cell populations can be exposed together to the tested compounds and then separated at the end of the treatment to perform different studies.

6. Three-dimensional systems

The properties of neuronal cells are critically dependent on their extracellular environment, which is very difficult to mimic *in vitro*. Furthermore, neuronal cells *in vivo* live in a 3D environment, whereas in current *in vitro* models, neuronal cells are cultured in a less physiological two-dimensional (2D) environment. In a 2D environment, the culture of brain-derived cells presents significant challenges that obscure the fidelity of *in vitro* results, namely the lack of linkages between the neuronal cells in all directions and the positioning of the cells, which do not accurately represent *in vivo* conditions.[360] Therefore, to circumvent these important limitations of *in vitro* 2D neuronal models, *in vitro* models with a 3D environment have been developed.[361,362]

Aggregated primary cultures prepared from dissociated fetal cells emerged thus as an important *in vitro* neuronal system with a 3D environment.[363,364] This system was first described by Moscona.[365] The initial cell suspension, composed of neural stem cells, neural progenitor cells, immature postmitotic neurons and glial cells, is kept in a serum-free, chemically defined medium. In rotation-mediated culture, under rigorously controlled conditions, the isolated cells are able to reaggregate spontaneously and to form a large number of practically identical spheres, thus reconstituting spontaneously a brain architecture to reach a high level of structural and functional maturity.[363,364,366] Of note, aggregates may be maintained in suspension culture for several weeks.

The main advantages and limitations in using 3D systems as *in vitro* models for neurotoxicological studies are summarized in Table 6. As within the aggregates, the cells rearrange and mature, reproducing critical morphogenic events, aggregated primary cultures represent an alternative system that can be maintained under environmental control.[367] Furthermore, aggregated primary cultures can be grown routinely in batches

Table 6 Advantages and limitations in using 3D systems as *in vitro* models for neurotoxicological studies

Advantages
High reproducibility[363]
High degree of cellular organization and differentiation[363,364]
Possibility to study toxic effects at successive developmental stages[363,366]
Within the aggregates, cells rearrange and mature, reproducing critical morphogenic events (migration, proliferation, differentiation, synaptogenesis and myelination)[367]
Spatial complexity[367]
May be maintained in culture for several weeks[366,367]
The three-dimensional structure within spheres allows the establishment of cell–cell interactions[367]
Limitations
More difficult to obtain and maintain in culture[363,366]
Limited amount of biological material when prepared from dissociated primary cells, compared to the immortalized cell lines[363,366]

of up to 150 replicate cultures, each containing several thousand aggregates,[363,366] thus offering the possibility to study drug effects at successive developmental stages on identical cultures.

In recent years, 3D brain models have advanced considerably, with the introduction of new tools to induce the formation of aggregates, thus allowing the generation of a highly organized 3D environment.[361,362] This 3D environment may be created by using concave microwells or by seeding the cells on biomatrices, such as alginate,[368] fibrin,[369,370] agarose,[371,372] collagen,[373] peptide-based hydrogels,[374] aragonite[362] or bacterial nanocellulose,[361] which allow mimicking of the properties of the extracellular matrix.[375] In the development of the CNS, the extracellular matrix plays a crucial role in neuronal cell adhesion, proliferation, differentiation, migration and neurite outgrowth, as well as in providing the optimal environment for supporting neuronal morphological and electrophysiological properties. In addition, the extracellular matrix also regulates the differentiation of other cells in the CNS, such as glia and oligodendrocytes.[376,377] These tools make it possible to obtain 3D systems derived either from dissociated fetal cells[360,362] or from immortalized cell lines.[361]

By favoring a more natural growth and differentiation of cells by improving the flow of oxygen, nutrients, waste products and growth factors, 3D cultures allow generation of uniformly sized neurospheres as a neural tissue model.[360,361] Representative scanning electron microscopy (SEM) images of neurospheres are shown in Fig. 9. In 3D cultures, cells present a smaller size and a round shape, whereas cells on the 2D culture plate present a flatter and more outstretched morphology.[361,378] Furthermore, in the 3D system, neuronal cells also exhibit an increased ability to differentiate and form aggregates.[379] In accordance with these features, neural cells cultured within 3D polymer networks create their own cellular microenvironment to survive, proliferate and differentiate and form neurons and glia that are electrophysiologically responsive to neurotransmitters. Furthermore, rat hippocampal cells cultured in a 3D environment created by aragonite biomatrices showed an increased viability and higher neuron/astrocyte ratio, as compared with their 2D counterparts.[362] In addition, 3D cultures have a higher cell–cell and cell–matrix interaction than 2D monolayer culture. In a 3D environment, cells contact with other cells in all directions, whereas in 2D monolayer cultures, cell–cell connections exist in only one plane.[361] Cell–cell contacts and their topology are known to have significant roles in mechanical signaling for determining cell fates. Therefore, since 2D and 3D cell culture environments lead to quite different cell–cell contacts, different mechanical signals are passed between the cells.[380]

Despite this, 3D cultures offer additional advantages for neurotoxicological research. The high number of aggregates available, combined with the excellent reproducibility of the cultures, facilitates routine test procedures.[363] The 3D structure within spheres allows the establishment of cell–cell interactions, a process known to play an important role in signaling pathways.[367] Furthermore, similar to the monolayer cultures (*e.g.* dissociated primary cultures and immortalized cell lines), the effects of exogenously added compounds on the aggregate cultures can be studied directly in the absence of possible confounding factors observed *in vivo*, such as the bioavailability and systemic toxicity. Another important feature of aggregate cultures is the possibility to introduce modified

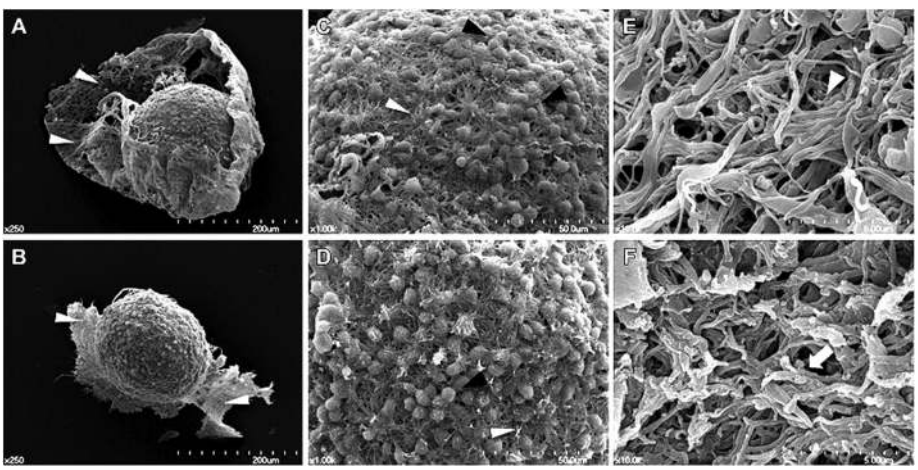

Fig. 9 Ultrastructure of neurospheres of Group 1 (control neurospheres – neurons were cultured for 10 days *in vitro* (DIV) in normal medium) and Group 2 (β-amyloid protein-exposed neurospheres – neurons were cultured for the first 7DIV in normal medium and for the next 3DIV in medium containing 5 μM β-amyloid protein). (A, B) Scanning electron microscopy (SEM) images of Group 1 (A) and Group 2 (B) neurospheres at 10DIV. White triangular arrows indicate neurites extended out from the microwells. Scale bar: 200 μm. (C, D) Magnified SEM images of Group 1 (C) and Group 2 (D) neurospheres. Black triangular arrows indicate soma of neurons and white triangular arrows indicate neurites of neurons. Scale bar: 50 μm. (E, F) Surface of Group 1 (E) and Group 2 (F) neurospheres. White triangular arrows indicate robust neurites, while white arrows indicate damaged neurites. Scale bar: 5 μm. Reproduced with permission from *Biomaterials* (Y. J. Choi, J. S. Park and S. H. Lee, Size-controllable networked neurospheres as 3D neuronal tissue models for Alzheimer's disease studies. *Biomaterials*, 2013, **34**, 2938–2946. DOI: 10.1016/j.biomaterials.2013.01.038).[360] Copyright © 2013, Elsevier Ltd.

cells into the seeding content of the system.[381] In addition, the cellular composition of the aggregates can be manipulated to specifically increase or decrease the proportion of selected cell types.[367]

The high degree of cellular organization and differentiation makes 3D systems useful models for neurotoxicity testing, both during the development and at advanced cellular differentiation. Indeed, this system may be useful to evaluate potentially teratogenic compounds, which act at early states of cell proliferation and differentiation, as well as compounds that present cell-specific toxicity (*e.g.* neurotoxicity, the toxic effect on astrocytes or oligodendrocytes and demyelinating effects), which are observed at a more advanced developmental stage.[363,364] In addition, with these cultures it is also possible to reproduce critical steps of brain development, since the morphological and biochemical features of this system mimic several morphogenetic events occurring *in vivo*.[366]

Though limited in scope, several studies have used 3D *in vitro* models to address deleterious effects of some toxicants on the nervous system. Among these toxicants are mercury and methylmercury,[382] β-amyloid protein,[360] polybrominated diphenyl ethers,[383] lithium[384] or acrylamide.[384] Neurospheres have also been used to study the effect of some compounds on neurogenesis or gliogenesis processes. Using this model, it was demonstrated that high concentrations of ethanol (50 mM) affect cell proliferation, neurogenesis and, more deeply, gliogenesis processes.[385] Therefore, 3D neuronal models also allow studying alterations in intrinsic cellular mechanisms of stem cell fate choices contributing to altered neurogenesis and gliogenesis during nervous system maturation, which might in part be responsible for defective astroglial and neuronal functions observed in some neuropathological or toxicological conditions.

Therefore, considering the high yield and reproducibility of the cultures and the possibility to perform multiparametric endpoint analyses[364], 3D cell systems constitute a promising approach for well-organized neurotoxicity studies in *in vivo*-mimicking environments.[364,386–388]

Besides their application in the neurotoxicity field, the 3D neurospheres' model can also be used in the study of neurodegenerative diseases. Furthermore, neurospheres can also be used as building blocks to produce organ-like structures.[360]

7. Organotypic cultures

Organotypic cultures include organ/explant and slice cultures. These cultures are usually obtained from early postnatal, or, less frequently, from the embryonic material. However, early postnatal periods (P0 to P7) are ideally suited for culturing because the cytoarchitectonic fundamentals are already established in most areas, the brain is larger and easier to manipulate, and nerve cells are more likely to survive.[389] Therefore, the goal of this culture technique is to obtain a preparation with a high degree of cellular maturation and differentiation and with an organotypic organization with the ability to assess individual neurons.

Table 7 Advantages and limitations in using organotypic cultures as *in vitro* models for neurotoxicological studies

Advantages
Allow the study of heterogeneous populations of cells as they are found *in vivo*[389,391]
Retain the morphology and functionality of the tissue[389,391]
Allow a more reliable distribution of extracellular signaling molecules, similar to the *in vivo* scenario[392]
Allow to study individual cells in their milieu of heterogeneous cells[389,391]
Allow the study of neuron–glia interactions[389,391]
Maintain intact many of the cellular dynamics among the multiple cell types and intercellular matrices that are lost in cultures of isolated cells[391]
Limitations
Difficult to maintain for long periods[404]
Nutrient supply to the cells within the explant/slice is highly dependent on the tissue thickness[401]
Difficulty in quantifying small changes in differentiation or viability[389,391]

Since their introduction, these culture systems have become an attractive tool for neurobiological and neurotoxicological studies of cell proliferation and death *in vitro*.[390] The main advantages and limitations in using organotypic cultures as *in vitro* models for neurotoxicological studies are summarized in Table 7. In contrast to primary cultures, organotypic cultures are cultured as a whole, without dissociation, allowing the *in vitro* study of heterogeneous populations of cells as they are found *in vivo*. Furthermore, in organotypic cultures, the morphology and functionality of the organ is temporarily retained, as a result of their three-dimensional organization, being that the cellular modifications observed may be comparable to the *in vivo* conditions. Therefore, this is the only cell culture system that allows for individual cells to be studied in their milieu of heterogeneous cells.[389,391]

In organotypic cultures the distribution of extracellular signaling molecules may be replicated more reliably, since local concentrations may be elevated in the intercellular spaces within the tissue, as opposed to dissociated cultures where these molecules diffuse away when freely released. Moreover, as the organotypic cultures survive from weeks to months, they are useful for long-lasting studies, such as those studying recovery following excitotoxicity.[392] In addition, organotypic culture systems provide a better alternative to animal experimentation than cellular cultures composed of only a single cell type, as they maintain many of the cellular dynamics among the multiple cell types and intercellular matrices that are lost in cultures of isolated cells.[391]

Since the goal of this culture technique is to obtain a preparation with a high degree of cellular maturation and differentiation, and with an organotypic organization with the ability to assess individual neurons, these systems can be used to study processes involved in nervous system development, such as the specificity of axonal growth and the mechanisms involved in target contact. However, given the massive denervation and cell death caused by the preparation procedures and the novel influences of the tissue culture environments, it cannot be assumed that the properties of organotypic cultures are identical to their *in vivo* counterparts.

7.1. Slice cultures

Brain slice preparations are well-established models for a wide spectrum of *in vitro* investigations in the field of neuroscience and neurotoxicology. Over the years, slice culture systems have been successfully established from a variety of brain regions, including the HIP,[393–395] striatum,[396,397] cortex,[398,399] spinal cord[400] and cerebellum,[244] as models to study the mechanisms involved in xenobiotic-induced neurotoxicity.[393,395,397,399]

The slice culture preparations maintain the 3D organization of the brain and allow extensive experimental manipulation and measurement, as well as the study of mechanisms of intercellular interactions.[392] These tissue slices are maintained in an artificial environment, as intact functioning units, in which the interconnections between neurons and glial cells are maintained. Therefore, *in vitro* brain slice studies have provided great insights into CNS functioning.[396,400]

Brain slice preparations are advantageous since they preserve the local network and anatomical features of the brain, allow access for microscopic and electrophysiological approaches, and allow for alterations in solutions bathing the slices, while *in vivo* brain studies require live animals.[401] Therefore, this balance between the intactness of *in vivo* preparations and the tractability of dissociated cultures makes cultured slices a useful tool to study synaptic function and dysfunction in the brain, using many approaches, including electrophysiological measurements.[402,403]

Though slice cultures display a high degree of neuronal differentiation, this system does not ensure that all cell types survive or that all important properties, such as receptor sensitivity or neuronal connections, are established.[404] In addition, one of the fundamental limitations of the slice cultures is their thickness. As oxygen delivery is exclusively due to diffusion, there will be very large differences in the oxygen tension throughout the slice or more intact isolated tissue. Therefore, since neuronal function is highly dependent on the tissue oxygen partial pressure, oxygen gradients are acceptable, as long as the oxygen partial pressure at the center of the tissue is sufficient to meet its metabolic demand.[401]

Generally, brain slice cultures derive from early postnatal (P0–P7) rats[394,395] or mice.[405] However, cultures of adolescent or adult brain tissue have also been prepared.[393,396,399,406] Brain tissues from these young animals show a high degree of plasticity and resistance to mechanical trauma during the slice preparation, which is important in obtaining viable and healthy cultures routinely. In addition, at the P0–P7 age, basic synaptic connections have been grown, but mature synapses have not yet been established in the brain, which normally develop in the following 2–3 weeks *in vivo*. On the other hand, brain slices from neonates are highly viable during culture, though gradually their morphological characteristics are lost, thus being unsuitable for long-term culture.[404]

Acute brain slices are often used for recording immediately after slicing.[401] The technical advantages of these brain slices in conducting electrophysiological experiments are many, but mostly center around the exceptional accessibility, with landmarks such as cell body layers visible, and the possibility of drug application. However, acute slices also have some limitations. The simple act of cutting brain tissue into slices damages numerous axonal connections and kills many cells near the surface of the slice. In addition, while the electrochemical function of these slices remains intact for several hours, this decline in viability makes it difficult to use them for more than half a day, therefore limiting the study of neurological processes that take place over days, such as neurogenesis and synaptogenesis.[407]

To overcome the mentioned limitations, new tools for brain slice culture were developed to maintain the slice viability for weeks to months. One method is simply to culture the brain slice in an interface chamber, thus maintaining the slice viable for a few weeks. In this method, excitatory and inhibitory synaptic potentials can easily be analyzed.[408] Furthermore, brain tissue maintained in interface-style chambers, where oxygen delivery is increased by only immersing one side of the slice in the artificial cerebrospinal fluid (CSF), often displays *in vivo*-like oscillations. On the other hand, in the interface configuration, the visualization is compromised and the recordings have to be performed in a blind fashion, thus making observation by the researcher impossible.[409] In addition, submerged slice chambers, in which the brain slice exchanges oxygen, nutrients, and waste products through fluid superfusing the slice, constitute another method for culturing brain slices *in vitro*.

Another method for culturing brain slices, which increases their lifespan in culture, is the roller tube system. When cultured in this system, brain slices form thin monolayers that maintain the overall organization of the source tissue.[407] The roller-tube, half-filled with media, is inclined so that the slice is partially immersed in artificial CSF and partially exposed to oxygen. As the system that holds the tubes rotates, the slice maintains alternative contacts with oxygen and media periodically to ensure thorough exchange with both.[401] Therefore, slices may be maintained in this system for several weeks or more, thus allowing long-lasting experiments.[407] Co-culture of slice cultures has also been used to establish an *in vitro* analog of *in vivo* axonal connectivity among various brain regions, thus allowing the study of trophic factor interactions, axonal targeting, and synaptic transmission.[405,410]

A number of features of the slice culture make it attractive for determining the effects of various pharmacological and toxic agents and potential protective pathways. Perhaps, most importantly, and in clear contrast to dissociated neurons, slice cultures routinely respond to a variety of neurotoxic insults in a manner closely mimicking the regional specificity and selective vulnerability of neuronal subtypes following toxicant exposure *in vivo*. In fact, studies on brain slices have provided important insights into the mechanistic basis involved in brain injury mediated by MDMA[403,406] and other classical neurotoxicants like ethanol,[411] organophosphate pesticides,[397] methylmercury[393] and trimethyltin.[395] In addition, this model has provided valuable insights for screening the neurotoxic potential of several compounds.[391,402]

7.2. Organ/explant cultures

Organ/explant culture can be defined as an *in vitro* system that maintains whole or portions of organs in culture using specialized media and substrates. Large parts of the spinal cord may be maintained as organ cultures.[412] Organ culture offers several advantages, which include the preservation of the histotypic relationships among cells of an organ without any disturbance of the cellular or tissue architecture that may be caused by the enzymes, chemicals or proper mechanical separation of the tissue. Therefore, the preservation of cell–cell contacts constitutes a main benefit of organ/explant cultures. On the other hand, and in contrast to the culture of isolated cells, which implies the relatively homogeneous isolation and culture of specific types of cells in defined media and under controlled conditions, organ/explant cultures use whole or small segments of organs or tissues, which contain multiple cell types. Therefore, these *in vitro* systems mimic the *in vivo* situation most accurately by preserving the histological architecture, as well as the ratio of neuronal and glial cells.[413] Furthermore, in this system, it may be assumed that the concentration of the tested compound at the target cells is comparable to the *in vivo* situation (whether the concentration in the perfusate is the same as that in the CSF). A further advantage is that electrophysiological effects can be measured without knowing the target cell type.[412]

Explants have also been used in co-culture with other cells to establish a more suitable *in vitro* model for neurobiological or neurotoxicological studies. To this end, explants are cultured in a culture disk, which allows the migration of the cells of interest by selection of media and the substrate, and co-cultured with other dissociated cells. Thus, it is possible to study the influence of proteins expressed by the dissociated cell system on the development of neuronal structures or interconnections.[414] For example, using explants of brain tissue co-cultured with HEK293 cells over-expressing netrin-1 (chemotrophic factor involved in axon guidance), the involvement of this protein in the chemoattraction of migrating neurons from the lower rhombic lip was studied.[414] In addition, as many human *in vivo* toxicological studies are ethically impossible, these systems are especially suitable for screening neurotoxic compounds[415] and experimental pharmacological chemicals.[416]

8. Neural stem cells

The topic of neural stem cells has been discussed in innumerous reviews.[37,135,417–421] Human embryonic stem cells (hESCs) are derived from the inner cell mass of fresh or frozen embryos at the blastocyst stage of development.[422] Most importantly, hESCs self-renew to allow for indefinite maintenance of the undifferentiated state *in vitro* and thereby retain the ability to differentiate into derivatives of the three embryonic germ layers that subsequently form all the tissues of a developing fetus. Following the ethical problems associated with hESC derivation, efforts were made in order to generate human induced pluripotent stem cells (hiPSCs) from human somatic cells[423,424] (for a more comprehensive review about special considerations and technical challenges relative to hiPSC generation and maintenance see ref. 419 and 425). Thus, in contrast to hESCs, hiPSCs are derived by reprogramming somatic cells to a pluripotent state through the overexpression of a key set of transcription factors. In addition, since the techniques for hiPSC derivation are easily applicable to adult somatic cell types, cell lines can be easily derived from a variety of genetic backgrounds.[426] Despite this, both hESCs and hiPSCs share the important properties of self-renewal and pluripotency.[426,427] A more comprehensive review on hESCs and iPSCs can be found in ref. 426 and 427.

Differentiating hESCs represent an interesting new model system to study early neural development and allow the generation of pure populations of early neuroectoderm CNS progenitors[428] and neural crest cells, progenitors of the PNS.[429,430] At this level, studies derived from Marcel Leist's Lab, using neural crest cells generated from hESCs, have also been employed for the screening of environmental toxicants and in defining toxicity pathways.[431–433] Thus, the availability of human neural crest cells offers new opportunities for studies of neural crest development and for efforts to model and treat neural crest-related disorders.[429] In addition, models based on human neural crest cells can help in revealing dysfunction of neural crest cell migration by developmental toxicants with good sensitivity and specificity.[431,433]

The human nervous system is known to be particularly vulnerable to toxic compounds during development, with the sensitivity to exogenous substances that varies with the developmental stage at exposure.[434] Developmental neurotoxicity has been defined as adverse effects of xenobiotics on the nervous system associated with exposure during development, which may result in adverse effects on symmetric stem cell division, proliferation of neural progenitor cells, neuronal and glial cell differentiation, cell migration, synaptogenesis, cell death, development of neurotransmitter systems and receptors, axonal connections and myelination.[435] Therefore, stem cell-based systems may provide a new tool for improved understanding of drug-induced adverse reactions during the development stage. In fact, a number of recent reviews have described potential applications of hESC and hiPSC technology to toxicology, pharmacology and the study of human diseases that have environmental contributions to their etiology.[419,425,436–443]

Neural stem cells (NSCs), also designed as neural progenitor cells, are the most primitive cells in the CNS and it is generally agreed that they exist in a variety of developmental stages residing in designated stem cell niches that provide a controlled environment for proliferation and differentiation.[444] The main characteristics of NSCs *in vitro* are summarized in Table 8. NSCs are characterized by their ability for self-renewal and the ability to generate the three major cell types of the nervous system, neurons, astrocytes and oligodendrocytes.[30] Since most mature neural cells, with particular reference to neurons, are specialized cells and are quite sensitive to

Table 8 Main characteristics of NSC *in vitro*

Retain the ability to self-renew[420]
Can generate both neuronal and glial cells[420]
Can divide and differentiate in the absence of a niche that controls their activity *in vivo*[421]
Present asymmetrical and symmetrical cell division[421]
Human adult NSCs do not show telomerase activity and have limited proliferation capacity for passaging *in vitro*[521]
Only partial functional maturation can be achieved[421]
Although, *in vivo*, a clear separation between NSCs and transient amplifying progenitors can be drawn, *in vitro* transient amplifying progenitors can acquire a NSC identity[420]
NSCs that have undergone long-term expansion mainly have a single radial glia-like identity[421]
NSCs that have undergone long-term expansion tend to lose the codes of transcription factors that determine positional identity[421]
NSCs that have undergone long-term *in vitro* expansion can give rise to limited assortments of specialized neuronal progeny[421]
The temporal evolution is partially recapitulated during embryonic stem cell neuralization processes. NSCs that have undergone long-term expansion mainly have a single radial glia-like identity[421]

environmental changes, such as oxygen conditions or excitotoxic molecules, the importance of NSCs in sustaining the development and homeostasis of nervous tissue is essential.[444] In addition, in the developing brain, NSCs are also present in discrete neurogenic areas of the brain, where they represent a unique cell population with the neurogenerative ability.[445] The use of human neural tissue may also be valuable for the extrapolation of data derived from animal tissue to the human situation.[32] Therefore, NSCs may be relevant for neurotoxicity studies and hazard identification, not only in relation to the developing brain, but also in relation to the adult nervous system.[445] Actually, a number of neural stem cell lines, commercialized by different companies, are available for research proposals.[446] One of the most important advantages of neural stem cell lines is their non-tumoral origin, as compared to other cell lines, such as neuroblastoma, pheocromocytoma or glioma cell lines, which are tumor-derived systems. Representative images of NSCs from the mouse brain subventricular zone are shown in Fig. 10.

Exciting work from Leonora Buzanska's lab, with NSCs derived from human umbilical cord blood, initially suggested as a promising model for studying neurotoxicity,[447] has been performed to detect the adverse effects of neurotoxic compounds.[435,448] These studies used human umbilical cord blood-NSCs (HUCB-NSC), which is a non-transformed cell line obtained in Leonora Buzanska's lab,[449] with a stable growth rate, and the ability to self-renew and to differentiate into neuronal, astrocytic and oligodendroglial lineages. This line can be maintained at different stages of neural progenitor development in the presence of trophic factors, mitogens and neuromorphogens in culture media. Enhanced neuronal differentiation induced by dBcAMP treatment was accompanied by expression of several functional proteins including glutamatergic, GABAergic, DA, serotonin and Ach receptors.[450] Thus HUCB-NSC represents a promising model for studying the interactions between the microenvironment (*e.g.*, matrix adhesive biomolecules, the culture soluble factors and possible toxic compounds) and the neural stem cells in order to investigate cellular responses to the varying niche composition.[435] Elegant work from other laboratories, including Ellen Fritsche's,[451,452] William R. Mundy's[32,453,454] and Timothy J. Shafer's[72,135,455] laboratories, has also provided notable considerations in *in vitro* neurotoxicity testing using neural stem cell-based systems as experimental models.

A large number of studies have also examined the effect of neurotoxicants on neural stem cell differentiation. In most of these studies, stem cells are differentiated in the presence of a chemical. Using this paradigm, well-known neurotoxicants, such as ethanol,[456] polychlorinated biphenyls,[457] methylmercury[458] and cocaine,[459] have all been shown to disrupt neural stem cell differentiation. In addition, a large number of studies have examined the effects of several neurotoxicants on the proliferation and survival of neural stem cells. It was recently demonstrated that methylmercury, by inhibiting the protein kinase B isoform 1/mammalian target of the rapamycin signaling pathway, induces caspase-dependent apoptosis and autophagy in HB1.F3 human NSCs.[458] In the same way,

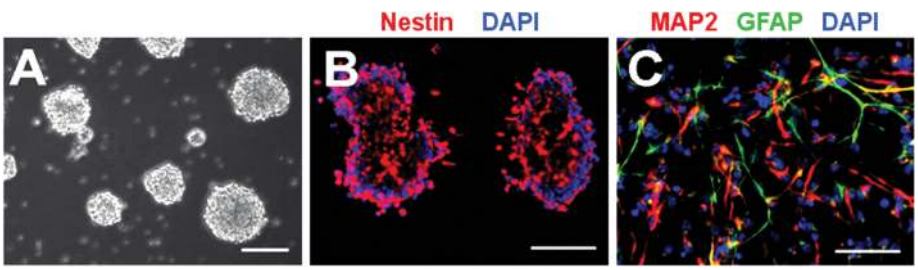

Fig. 10 Cell culture and characterization of neural stem cells (NSCs) from the mouse brain subventricular zone. (A) Cells isolated from the mouse brain subventricular zone formed neurospheres after 7 days *in vitro* (DIV). (B) Many cells in neurospheres were neural progenitor marker nestin (red) positive. DAPI (blue) staining shows all cells. (C) 12DIV after neuronal differentiation [5 μM retinoic acid, in Dulbecco's modified Eagle medium/F12 (1 : 1) supplemented with 2% B27, for 6DIV, followed by 20 ng mL^{-1} nerve growth factor, in neurobasal medium supplement with 2% B27, for additional 6 DIV], many cells became positive to the neuronal marker microtubule-associated protein 2 (MAP2; red) or astrocyte marker glial fibrillary acidic protein (GFAP; green). Nuclei are stained with DAPI. Scale bar: 100 μm. Adapted with permission from *International Journal of Physiology, Pathophysiology and Pharmacology* (H. Gu, S. P. Yu, C. A. Gutekunst, R. Gross and L. Wei, Inhibition of the Rho signaling pathway improves neurite outgrowth and neuronal differentiation of mouse neural stem cells. *Int. J. Physiol. Pathophysiol. Pharmacol.*, 2013, **5**, 11–20).[462] Copyright © 2013, e-Century Publishing Corporation.

β-amyloid oligomer$_{25–35}$ was shown to decrease viability proliferation and mobilization of rat's NSCs, and increase the free radical levels.[460] Similarly, exposure of NSCs to polybrominate-diphenyl ethers altered the expression of several proteins, including vimentin, a neurofilaments' protein, and cofilin1, an intracellular actin-modulating protein,[461] thus supporting the usefulness of this model in addressing the effects of neurotoxicants.

Neurite outgrowth is a process that occurs as a consequence of the differentiation of precursor cells to a terminal neuronal phenotype. Thus, neurons from differentiated stem cells can elaborate axons and dendrites.[462] As such, NSCs have been used to evaluate the effects of neurotoxicants on neurite outgrowth,[453,454] providing additional information in understanding the role of the neurite outgrowth impairment in the neurotoxic effects mediated by several compounds.

9. Blood–brain barrier models

BBB is a term used to describe a series of unique properties associated with the blood vessels in the brain, which tightly regulate the movement of molecules, ions, and cells between the blood and the neural tissue. The highly regulated BBB provides stringent control of the extracellular environment in neural tissues, which is critical for proper neuronal function that requires precise ionic concentrations in the surroundings. In addition, the BBB protects the CNS from injury and disease by limiting the entry of toxins, pathogens, and the body's own immune system into the neural tissue.[71] Most of the properties of the BBB in limiting the passage of compounds to the neural tissue are derived from the endothelial cells, as they form highly polarized cells held together by tight junctions and greatly limit the movement of molecules and ions between cells. Consequently, the passage of molecules through the BBB generally requires the existence of active transport systems. Furthermore, astrocytes have long been demonstrated to play a major role in regulating junctional and transport properties of the BBB in capillary endothelial cells, both *in vivo* and *in vitro*.[71,463] Therefore, neurotoxicants may disrupt the BBB either by damaging the endothelial cells or by interfering in the interdependence of astrocyte and endothelial cells. In addition, increasing evidence is pointing to a similarly important role for pericytes in barrier development and maintenance.[464]

Many neurotoxicants, such as lead,[465] aluminum,[466] MDMA[467] or methamphetamine,[468] are described to disrupt the BBB function. Furthermore, excitotoxins, such as GLU, NMDA, ibotenate or kainate, are known to induce neuronal demyelination, which is thought to occur as a consequence of the BBB breakdown.[469]

Since many neurotoxins gain access to the brain parenchyma by disrupting the BBB *in vivo*, attempts have been made to develop *in vitro* models of the BBB. The existing *in vitro* models of BBB can represent various degrees of complexity, depending upon their use.

In vitro models of the BBB started to emerge in the early 1990s as potential new research tools. A schematic representation of different BBB models is shown in Fig. 11. The most important advantage of BBB models based on monolayers of endothelial cells (Fig. 11A) is their simple preparation. The artificial environment provided by these *in vitro* models allows the study and manipulation of the BBB function easily, without the interference of other variables observed *in vivo*, thus allowing testing of a broad range of experimental paradigms. Nevertheless, given their simplicity, these models are

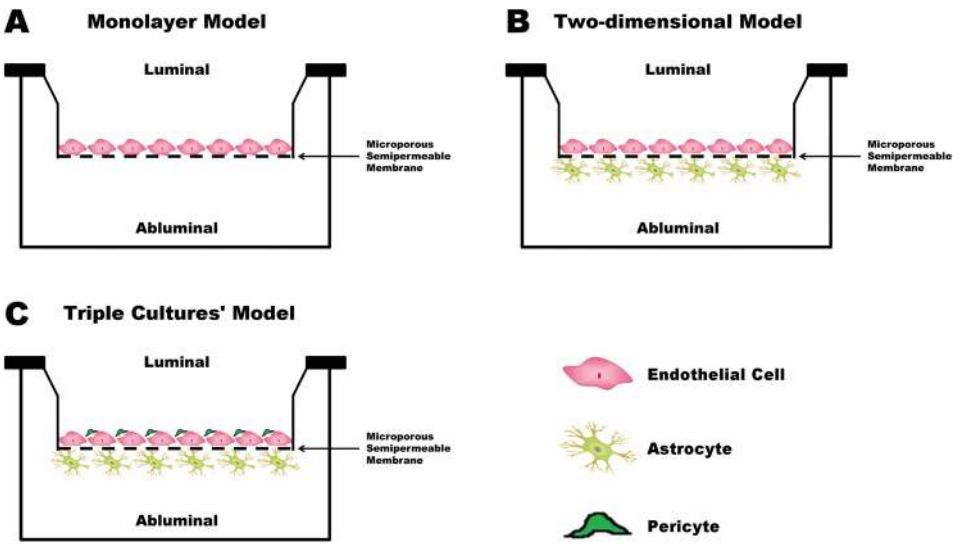

Fig. 11 Schematic representation of different BBB models. (A) *In vitro* monolayer models are constructed by seeding various endothelial cells on the upper side of the microporous semipermeable membrane. (B) *In vitro* primary cell culture barrier models have progressed from simple cultures of endothelial cells to more complex co-culture models, in which endothelial cells are grown on porous cell culture inserts and co-cultured with primary astrocytes. (C) Recently, increasingly complex co-culture models, such as triple cultures of endothelial cells with astrocytes and pericytes, have been developed.

associated with some limitations. Cells cultured in an artificial environment may undergo dedifferentiation as a result of the lack of exposure to physiological factors. Thus, this may limit the relevance of *in vitro* findings to the *in vivo* scenario.[470]

In addition, the utility of the NSCs in reproducing the BBB properties *in vitro* has also been demonstrated, which appear to be representative of *in vivo* BBB development.[471]

9.1. Monocultures of endothelial cells

The most common and widely utilized BBB model was developed based on a simplified view of the BBB, which was represented as a monolayer of highly specialized brain microvascular endothelial cells (Fig. 11A).

The main advantages and limitations in using monocultures of endothelial cells as *in vitro* blood–brain barrier models for neurotoxicological studies are summarized in Table 9.

Several cell culture models in monolayers have been used to screen the drug permeability across BBB. They include BBB primary endothelial cell culture models with cell lines, such as the bovine brain microvascular endothelial cell line (BMEC) or the rat brain microvascular endothelial cell line (RBE4),[472] primary cultures, such as human, rat, mouse, bovine or pig brain microvascular endothelial cells or rat or mouse spinal cord endothelial cells,[463] or, alternatively, non-cerebral models, such as the Caco-2 cell line.[473] This system, also known as the transwell apparatus, is a vertical side-by-side diffusion system, across a microporous semipermeable membrane, thus separating the luminal and the abluminal compartments. Thus, microvascular endothelial cells grow to confluence on the upper (luminal) surface of the membrane immersed in their specific growth media.

Like the Caco-2 cell line, other non-cerebral cell lines, namely Mandin Darby Canine Kidney (MDCK) cells and human umbilical vein endothelia (ECV304), have also been employed as models of BBB.[474] Nevertheless, since these cells are morphologically different from brain endothelial cells, they express different transport systems and present different metabolic and growth properties,[474] so they should be used only when practice with brain-derived cells is difficult.

9.2. Two-dimensional models of the BBB: co-culture of endothelial cells with glia

In vitro primary cell culture barrier models have progressed from simple cultures of brain endothelial cells to more

Table 9 Advantages and limitations in using monocultures of endothelial cells, two-dimensional models or dynamic models of the blood–brain barrier for *in vitro* neurotoxicological studies

Monocultures of endothelial cells
Advantages
Pure cell populations[470,483]
Allow an easy study and manipulation of the BBB function without the interference of other variables observed *in vivo*[470]
The use of the microporous membrane interface allows the nutrient exchange and the passage of cell-derived and exogenous substances[470,483]
An extensive number of well-documented methods to obtain purified brain capillaries[483]
Limitations
Limited viability of the vascular endothelium, possibly resulting from metabolic deficiencies during vessel isolation[483]
Cells cultured in an artificial environment may undergo dedifferentiation as a result of the lack of exposure to physiological factors[483]
The absence of inter-cell interactions, not allowing the maintenance of intrinsic properties and function of the BBB[483]
Lack of antimitotic activity, which leads to irregular patterns of cell adhesion and irregular/uncontrolled multilayer cellular growth[483]
Two-dimensional models
Advantages
Simple preparation[483]
Allow an easy study and manipulation of the BBB function without the interference of other variables observed *in vivo*[470]
The presence of a well-differentiated cerebral endothelium displaying most of the features observed *in vivo*[480,481]
Limitations
Do not allow high throughput pharmaceutical studies[483]
Require more time, skill and costs to be established, as compared to the monocultures of endothelial cells[483]
Dynamic models
Advantages
Allow the concomitant circulation of blood cells[491]
Allow an easy study and manipulation of the BBB function without the interference of other variables observed *in vivo*[470]
Mimic *in situ* BBB conditions, both anatomically and functionally[485]
Make possible the study and characterization of the BBB role in the pathogenesis of major neuro-inflammatory diseases[488] and rheology-related impairments[489,490]
Limitations
Do not allow high throughput pharmaceutical studies[483]
Probable alteration of morphological and physiological characteristics of the cells[483]
Limited visibility of the intraluminal compartment, thus making it difficult to study the morphological/phenotypic changes of the vascular endothelium[470]
Particularly labor-intensive, require skills and are expensive to establish, as compared to the conventional BBB models[483,496]
Endothelial cells are not exposed to perivascular modulatory factors, not allowing their complete differentiation into a BBB phenotype[483]
An high initial cell load is required to establish this model, thus making it relatively inconvenient, particularly if primary cultures of cells obtained from animals are used[470]

complex co-culture models in which endothelial cells (derived from the adult brain of rodents) are grown on porous cell culture inserts and co-cultured with postnatal rodent astrocytes (Fig. 11B).[463]

The main advantages and limitations in using two-dimensional models of the blood–brain barrier for *in vitro* neurotoxicological studies are summarized in Table 9. Increasing evidence suggests that astrocyte interactions within the brain endothelium play a major role in determining BBB function, morphology and protein expression.[475,476] The presence of glial cells and the establishment of glial–endothelial interactions have been shown to increase the expression of transporters (*e.g.* P-glycoprotein), tight junctions and brain endothelial marker enzymes (*e.g.* Na^+- and K^+-activated adenosine 5′-triphosphatase).[477–479] In addition, the transendothelial electrical resistance, which measures the ionic conductance across the BBB, is typically higher in endothelial cell-glia co-culture systems than in monocultures of endothelial cells, and a limited cell polarity is inducible in endothelial cells when co-cultured with glia or in the presence of glial conditioned medium.[480,481] Therefore, one of the major advantages of this model is the presence of a well-differentiated cerebral endothelium displaying most of the features observed *in vivo*.

Thus, astrocytes may be plated either into the bottom of a multiwell dish, in which the insert is placed or grown on the underside of the insert itself in so-called back-to-back contact co-culture models.[463]

9.3. Dynamic models of the BBB

Following the initial models of the BBB, based on monolayers of endothelial cells or the models based on co-culture of endothelial cells with astrocytes, new dynamic systems of the BBB have been developed.

The main advantages and limitations in using dynamic models of the blood–brain barrier for *in vitro* neurotoxicological studies are summarized in Table 9. One of these models is called cone-plate apparatus, when the shear stress is created by a continuously rotating cone and the level of endothelial cells is determined using the cone angle and angular velocity. The endothelial cell layer is cultured on the bottom of the plate and continuously exposed to the shear stress generated by the rotating cone. The shear force is transmitted to the vascular endothelium through the culture media spinning in the direction of the rotating cone. The cone angle and the angular velocity regulate the shear stress generated, which is not entirely uniform along the radius of the plate supporting the endothelial cells' monolayer. This cone-plate apparatus, initially described by Bussolari and coworkers,[482] was designed to study the effects of laminar or turbulent fluid shear stress on cells and the relative contribution of the fluid viscosity, the time of exposure, and other physiological variables. Nevertheless, in this system, endothelial cells are not exposed to perivascular modulatory factors, not allowing complete differentiation of endothelial cells into the BBB phenotype.[483]

Artificial capillary-like structures, including hollow fibers, generally made of thermoplastic polymers like polypropylene, have also been adapted to model hollow organ-like structures, such as the BBB.[484] This *in vitro* system allows brain microvascular endothelial cells (both primary cultures and cell lines) to be co-cultured with abluminal astrocytes. Furthermore, cells are exposed to a quasi-physiological pulsatile laminar shear stress, thus allowing the formation of a BBB model that closely mimics *in situ* BBB conditions, both anatomically and functionally.[485] Glial cells from the perivascular side of brain microcapillaries are seeded in juxtaposition to the endothelial cells on the outer surface of hollow fibers. The exchange of oxygen and carbon dioxide takes place using artificial capillaries connected to a media reservoir through a gas permeable tubing system. A physiologically comparable shear stress level and intraluminal pressure can be obtained by regulating the intraluminal flow through a servo-controlled variable speed pulsatile pump.[486,487]

This model allows the study and characterization of the role of BBB in the pathogenesis of major neuro-inflammatory diseases, including multiple sclerosis,[488] and the study of rheology-related impairment, such as brain edema[489] or cerebral ischemia.[490] In addition, it reproduces the concomitant circulation of blood cells.[491]

Since the *in situ* microenvironment plays a critical role in modulating the BBB functions, the 3D culturing systems represent a good approach to mimic the BBB *in vivo*.[492,493] Thus, a new generation of *in vitro* BBB dynamic models, based on self-polymerizing extracellular matrix protein (*e.g.* laminin, collagen or fibronectin) scaffolds, was developed, providing an alternative model to the artificial capillary-like structure supports. This dynamic model of BBB enables close inter-cell interactions, when the quasi-physiological biochemical gradient exposure is needed for efficient cell-to-cell communication and cross-signaling. Thus, this model, allied to advanced microscopy studies, such as confocal microscopy and tomography, allows one to appraise dynamic changes in the cultured 3D microenvironment mediated by pathological stimuli like harmful xenobiotics.[483] Despite this, since this model is an uniform matrix, it lacks the discontinuities or gaps observed under *in vivo* conditions. Nevertheless, this limitation may be partially addressed by using cell-derived matrices, which maintain some of the original gaps allowing cell accommodation.[494,495]

Recently, increasingly complex co-culture models, such as triple cultures of endothelial cells with astrocytes and pericytes have been developed (Fig. 11C).[496,497] However, though these models display good barrier phenotypes *in vitro*, they are particularly labor-intensive, require skills and are expensive to establish.[496]

In studies of neurotoxicity screening, *in vitro* models of the BBB are useful in determining whether the tested compounds enter into the CNS. Furthermore, with these BBB models, it is possible to assess whether xenobiotics have a direct effect on barrier components, causing potential alterations or damage, or whether they modify the BBB function, and, consequently, induce neurotoxicity.[33]

10. Other approaches

10.1. Neurons generated from non-neural lineages

Recent studies have identified a combination of transcription factors that are capable of directly converting fibroblasts into neurons.[498,499] Expression of the single factor Ascl1 in mouse fibroblasts is sufficient to induce immature neuronal features. Nevertheless, the additional expression of Brn2 and Myt1l generates mature functional neurons with efficiencies of up to 19.5%.[499] These cells display functional neuronal properties, such as the generation of trains of action potentials and synapse formation. Furthermore, fibroblast-derived neurons generated with this approach are excitatory and express markers of cortical identity. A low proportion of these neurons also expressed markers of GABAergic neurons.[499] Using the same approach, human fibroblasts can also be converted into neurons. These cells display typical neuronal morphologies and express multiple neuronal markers, even after down-regulation of exogenous transcription factors.[498] The generation of neurons with this approach is fast, efficient and devoid of tumorigenic pluripotent stem cells, enabling robust generation of human neurons for *in vitro* experimentation. Recently, emerging studies have also demonstrated a direct conversion of human fibroblasts into neurons by expression of brain-enriched microRNAs.[500–502] These findings highlight the potential of exploiting the synergism between microRNAs and transcription factors to generate specific neuronal subtypes. Therefore, neurons generated from fibroblasts can provide a novel and powerful system for studying cellular identity and plasticity, neurotoxicological phenomena, neurological disease-modeling, drug discovery, and regenerative medicine.

11. Concluding remarks

Current *in vivo* animal procedures are, sometimes, not ideal predictors of human neurotoxicity. There is a consensus that *in vitro* methods can provide useful information concerning basic biological processes underlying neurotoxicity, and specific information about the mechanisms of action of neurotoxicants. At this stage of method development and validation, *in vitro* techniques can provide data that complement established *in vivo* testing approaches. However, *in vitro* models are not yet able to reproduce the *in vivo* situation, because they neither reflect the *in vivo* complexity of the nervous system nor assess the full range of neurobiological processes observed *in vivo*, such as cognition, motor coordination, and sensory processing and integration. Furthermore, *in vitro* systems are not still viable means of predicting neurotoxicity where the site of action is unclear or not known. Results from *in vitro* studies must be interpreted within the context of the integrated nervous system. However, it is important to ensure that the procedures used in *in vitro* neurotoxicology are valid. The results of *in vitro* procedures should also be interpretable within the context of plausible biological responsiveness to toxicological exposure, and possess the ability to predict the neurotoxic risk in humans. *In vitro* models show higher potential in experiments in which mechanistic hypotheses are tested. In addition, *in vitro* approaches could be used in several target sites where there is a significant understanding of basic biological processes including chemical-induced effects on the neuronal structure and function.

12. Future perspectives

The need to reduce the use of *in vivo* models for laboratorial experimentation, as a result of ethical considerations and costs, has led to the increased development of *in vitro* models for assessment of brain functioning and toxicity. However, due to the nervous system complexity, both functionally and structurally, and the multiplicity of potential targets, the development of *in vitro* models and methods for neurotoxicity assessment is an issue that requires continuous investigation.

Even though neurotoxicity is commonly defined solely in terms of cell level response, the timing and duration of exposure may be critical in developing neurotoxic effects. Furthermore, usually, *in vitro* experiments often use a high concentration approach, as compared to the expected brain levels, in order to appraise more easily the mechanistic basis of a neurotoxic event. Despite this, some requirements, such as high predictability, reduced costs and facility in extrapolating to the *in vivo* scenario, are critical in selecting a suitable *in vitro* neuronal model for predicting neurotoxicity.

Although simple *in vitro* models like isolated mitochondria or synaptosomes are useful in addressing some signals of brain dysfunction, the predictability of drugs' neurotoxicity is more accurate through the use of systems in which the relative contribution of active transport systems and metabolic transformation to drugs' bioavailability and, consequently, to their toxicity is taken into account. Nevertheless, actually cell-based *in vitro* models do not provide an accurate approximation to the drugs' distribution into the brain, though advanced cell-based models reproducing some aspects of the *in vivo* situation can provide more accurate results. Therefore, most efforts are needed in developing new models, with increased complexity, combining neuronal and metabolic competent cells in a heterogenic system, to easily address the influence of metabolism on drug's neurotoxicity.

On the other hand, one of the remaining obstacles in incorporating *in vitro* data for predicting *in vivo* neurotoxicological phenomena relies on the limited number of neurotoxic endpoints that may be addressed. Indeed, the brain development and functioning count on a highly diverse array of general cell functions, such as energy metabolism, glucose uptake, Ca^{2+} homeostasis, and specific processes like electrical activity, neuronal–glial interactions and synaptogenesis, which can potentially be targets for neurotoxic compounds. The increased employment of 3D systems in predicting drugs' neurotoxicity, in order to mimic the conditions observed in the whole animal, has added notable progress to the *in vitro* neurotoxicological experimentation. Despite this, new artificial

in vitro systems, with increased complexity, are required to make possible the incorporation of automatisms to monitor a variety of physiological and toxicological parameters. The introduction of automatic systems will greatly minimize time-consuming routine maintenance and monitoring, and facilitate operator efforts.

The introduction of new cell culture support materials may add additional advances to *in vitro* neurotoxicological experimentation. For example, models incorporating in their matrix construction the desired anchoring molecules, for cell adhesion or to stimulate specific cellular responses, may allow a higher degree of cell response and interaction. As such, efforts must continue to develop culture conditions for nerve cells that avoid loss of their biochemical characteristics.

Alternatively, with the increased knowledge about the mechanisms of neurotoxicity and the identification of endpoints that may be addressed in this context, the development of easy and rapid screening methods based on representative and well-organized neuronal models may help in the screening of putative toxic interactions of xenobiotics with neuronal cells. These methods should be based on endpoints with particular relevance to the processes of nervous system dysfunction and comparable to the *in vivo* situation, in what concerns the toxicants' exposure.

Otherwise, promising tools for neurotoxicity assessment based on the measurement of neuronal electrical activity using micro-electrode arrays have advanced neurotoxicity testing. In addition, the suppliers of micro-electrode array chips have lately been producing chips with an increased number of recording chambers (multi-chambers), which will allow testing of a higher amount of chemicals leading not only to high content but also to high throughput screening.

Lastly, the possibility of generating neurons from human fibroblasts[498] might constitute an important approach particularly useful in evaluating the neurotoxic potential of compounds in the human scenario.

In summary, it is tempting to consider that the development of new models and approaches for addressing neurotoxicological phenomena is critical for successful identification of neurotoxicants, as well as in addressing the brain targets and mechanisms involved in the neurotoxic effects. In addition, these models should be representative of *in vivo* brain functioning for good extrapolation to the *in vivo* scenario.

Abbreviations

2D	Two-dimensional
3D	Three-dimensional
ACh	Acetylcholine
AChE	Acetylcholinesterase
ADR	Adrenaline
BBB	Blood–brain barrier
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
ChAT	Choline acetyl transferase
CNS	Central nervous system
CSF	Cerebrospinal fluid
DA	Dopamine
DAT	Dopamine transporter
dBcAMP	Dibutyryl cyclic adenosine 5′-monophosphate
DRG	Dorsal root ganglia
E	Embryonic day
GABA	Gamma-aminobutyric acid
GFAP	Glial fibrillary acidic protein
GPDH	Glycerol-3-phosphate dehydrogenase
GLU	Glutamate
hESC	Human embryonic stem cells
hiPSCs	Human induced pluripotent stem cells
HIP	Hippocampus
IGF	Insulin-like growth factor
MDMA	3,4-Methylenedioxymethamphetamine or "ecstasy"
MPP^+	*N*-Methyl-4-phenylpyridinium ion
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NA	Noradrenaline
NFs	Neurofilaments or intermediate filaments
NGF	Nerve growth factor
NMDA	*N*-Methyl-D-aspartate
NPY	Neuropeptide Y
NSCs	Neural stem cells
NSE	Neuron-specific enolase
PDGF	Platelet-derived growth factor
RA	Retinoic acid
TH	Tyrosine hydroxylase
TPA	12-*O*-Tetradecanoylphorbol-13-acetate
VAChT	Vesicular acetylcholine transporter

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