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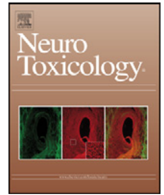

Review

Developmental neurotoxicity testing *in vitro*: Models for assessing chemical effects on neurite outgrowth

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ABSTRACT

In vitro models may be useful for the rapid toxicological screening of large numbers of chemicals for their potential to produce toxicity. Such screening could facilitate prioritization of resources needed for *in vivo* toxicity testing towards those chemicals most likely to result in adverse health effects. Cell cultures derived from nervous system tissue have proven to be powerful tools for elucidating cellular and molecular mechanisms of nervous system development and function, and have been used to understand the mechanism of action of neurotoxic chemicals. Recently, it has been suggested that *in vitro* models could be used to screen for chemical effects on critical cellular events of neurodevelopment, including differentiation and neurite growth. This review examines the use of neuronal cell cultures as an *in vitro* model of neurite outgrowth. Examples of the cell culture systems that are commonly used to examine the effects of chemicals on neurite outgrowth are provided, along with a description of the methods used to quantify this neurodevelopmental process *in vitro*. Issues relating to the relevance of the methods and models currently used to assess neurite outgrowth are discussed in the context of hazard identification and chemical screening. To demonstrate the utility of *in vitro* models of neurite outgrowth for the evaluation of large numbers of chemicals, efforts should be made to: (1) develop a set of reference chemicals that can be used as positive and negative controls for comparing neurite outgrowth between model systems, (2) focus on cell cultures of human origin, with emphasis on the emerging area of neural progenitor cells, and (3) use high-throughput methods to quantify endpoints of neurite outgrowth.

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1. Introduction

To control the risk of chemical effects on human health, recent legislation in both the United States and Europe (including the European Union and OECD Member Countries) prescribes increased testing of chemicals and chemical products to predict their potential hazards (Hengstler et al., 2006; Kimmel and Makris, 2001). Traditional paradigms for hazard identification and risk assessment are based on toxicity tests using *in vivo* animal models. These tests are designed to generate data relevant to specific adverse outcomes (e.g., cancer, reproductive toxicity, and neurotoxicity) or to known mechanisms of action (receptor interactions, enzyme inhibition, induction of metabolism, etc.). Traditional animal-based paradigms are impractical for screening, however, due to the resource requirements associated with testing large numbers of chemicals. New toxicity testing paradigms are being developed that make use of emerging screening technologies (Bhogal et al., 2005; Dix et al., 2007; NRC, 2007). These new paradigms integrate high-throughput *in silico* and *in vitro* procedures to screen large numbers of chemicals for toxicity. Results of these screening assays would be used to prioritize resources to those chemicals most likely to result in adverse health effects. Ultimately, new testing paradigms based on computational models and *in vitro* assays have the potential to eliminate the need for traditional animal tests (NRC, 2007).

The USEPA Developmental Neurotoxicity guideline for identification of chemicals that may pose a hazard to the developing nervous system is one example of a resource-intensive traditional toxicity test. This protocol recommends testing for neurobehavioral deficits and neuropathologic changes in rats after *in utero* and lactation exposure, and requires a substantial number of animals and time to complete (EPA, 1998). In order to facilitate the testing of large numbers of chemicals for their potential to result in developmental neurotoxicity (DNT), alternative methods for testing have been proposed including the use of *in vitro* cell culture systems and alternative species (Coecke et al., 2007; Lein et al., 2005, 2007). *In vitro* models can recapitulate the discrete cellular events of neurodevelopment (Cowan et al., 1997) and have been used to elucidate the molecular mechanisms underlying developmental processes, including neurite outgrowth (Craig and Banker, 1994). There are also numerous publications describing the effects of environmental and toxic chemicals on neurite outgrowth using *in vitro* models. Much of this work, however, focuses on the effect of individual chemicals and does not address the use of *in vitro* models for the purposes of screening large numbers of chemicals. This review is intended to provide an overview of the existing *in vitro* methods used to study chemical effects on neurite outgrowth. Methods for quantification of neurite outgrowth will be described, along with the neuronal culture systems that are commonly used as *in vitro* models. Examples of chemical effects on neurite outgrowth are provided in order to highlight the benefits and limitations of different model systems in the context of chemical screening and hazard identification.

In vitro cell preparations from nervous system tissue have been in use for many years, providing an important tool for the study of nervous system function at the molecular and cellular level. The use of cell culture models for assessing the neurotoxicity of chemicals has been reviewed (Costa, 1998; Gad, 2000; Harry et al., 1998; Tiffany-Castiglioni, 2004). *In vitro* cell culture systems are routinely used to test hypotheses regarding the mechanism of action of neurotoxic chemicals, and for identifying the molecular and cellular changes that are part of a critical pathway leading to neurotoxicity. More recently *in vitro* cell culture systems have been proposed for use in screening assays for chemical effects on specific neuronal or glial functions (Harry and Tiffany-Castiglioni, 2005). There are limits on their ability to model *in vivo* neurotoxicity, including diminished metabolic capability and the loss of interaction between cells (e.g., between pre- and post-synaptic neurons, between neurons and glia, between neural and non-neural cells). This may be particularly troublesome in applying cell culture techniques to the study of developmental neurotoxicity. Normal development of the nervous system is a dynamic process requiring the coordinated expression of molecular and cellular events in a time- and region-dependent manner (Rodier, 1994; Rice and Barone, 2000), and this coordination is largely lost in isolated preparations. However, the fundamental processes underlying nervous system development can be examined at the cellular level. Key cellular processes critical to normal brain development include proliferation, differentiation of precursor cells into neurons or glia, migration, elaboration of axons and dendrites, synapse formation, myelination, and programmed cell death (Cowan et al., 1997). The use of *in vitro* cell culture models to study developmental neurotoxicity is based on the premise that although neurotoxicants may act on a multitude of molecular targets, the final outcome will be reflected in alterations in one or more of these critical developmental events (Lein et al., 2005). Although there are limitations to all cell culture models as described above, at the cellular level neurons and glia in culture are remarkably similar to those *in vivo*. Many of the neurodevelopmental processes that occur *in vivo*, including cell differentiation, neurite outgrowth, and synaptogenesis, can be observed using *in vitro* neuronal systems, and a variety of *in vitro* preparations have been successfully developed and employed to study specific aspects of neuronal development and nervous system function (Boulton et al., 1999; Cestelli et al., 1992; Fedoroff and Richardson, 1997). Thus, cell cultures can provide simplified model systems to assess chemical effects on discrete neurodevelopmental endpoints using a battery of *in vitro* tests. The use of a cell-based system that focuses on developmental endpoints rather than biochemical or molecular endpoints will facilitate the screening of large numbers of chemicals for which there may be little available data on mechanism of action. Chemicals that affect one or more of these developmental processes *in vitro* would be flagged and recommended for further testing. Priority could be given to chemicals with high potency and effects *in vitro* that were selective for neurodevelopmental processes (compared to more general measures of cell health such as cell metabolism and viability).

Of all the methods used to evaluate the effects of chemicals on neurodevelopment *in vitro*, measurement of process outgrowth has received the most attention. This is not surprising since axonal and dendritic processes are a defining characteristic of neuronal morphology, and critical determinants of neuronal connectivity. In addition, neurite outgrowth can be assessed using a variety of methods including some that do not require sophisticated hardware or software. The term "neurite" refers collectively to axons and dendrites extended by primary cells growing in culture, or processes extended by neuronal cell lines, which are neither definitive axons, nor dendrites (Carmeliet, 2003). Because the extension of axonal and dendritic processes is a hallmark of differentiated cells, *in vitro* models of neurite outgrowth are also used to assess chemical effects on differentiation.

Neurite outgrowth is a process that occurs as a consequence of both the differentiation of precursor cells to a terminal neuronal phenotype and the initiation and development of broad, sheet-like extensions (lamellipodia) which subsequently condense into short processes (Craig and Banker, 1994). As the cells mature the processes will increase in length and complexity, and for some *in vitro* models including primary cell cultures, neurons will become polarized by developing a single long axon and several shorter dendrites (Fig. 1A). This has been described in detail by Dotti et al.

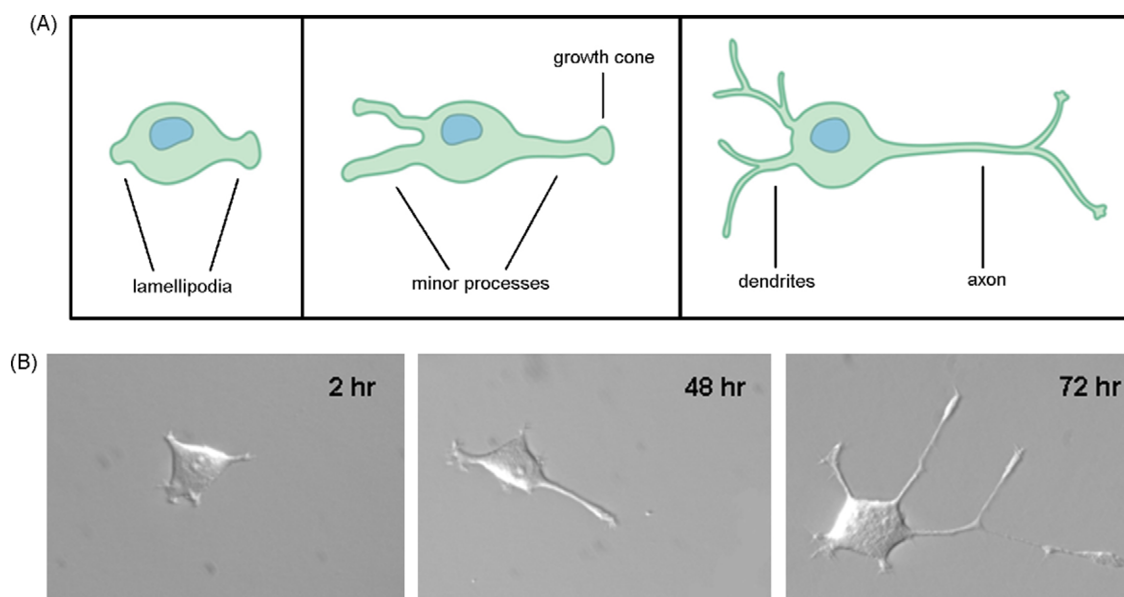

Fig. 1. Early events in neurite outgrowth. (A) Diagram of early events in neurite outgrowth illustrating a cell body with lamellipodia, the development of minor processes (tipped with a growth cone), and transformation of the processes into an axon and dendrites. (B) Hoffman modulation contrast photomicrographs of neurite outgrowth in PC12 cells 2, 48, and 72 h after treatment with 100 ng/ml nerve growth factor (NGF) to induce differentiation.

(1988) for hippocampal cells grown in culture. After plating, hippocampal neurons extend lamellipodia which develop into several short neurites. As the cells mature, one of the immature neurites grows rapidly in length and acquires axonal characteristics. Several days after formation of the axon, the remaining neurites elongate more slowly and become dendrites. Other models such as the widely used PC12 cell line exhibit extensive neurite outgrowth upon differentiation, but these neurites do not give rise to definitive axons or dendrites (Fig. 1B). Methods for culturing primary neurons and neuronal cell lines have been reviewed (Cohen and Wilkin, 1995; Fedoroff and Richardson, 1997). The extension of neurites *in vitro* is influenced by a number of factors (see below) including the surface coating on which the cells are grown. Substances commonly used to coat the surface of the plastic or glass culture vessel that enhance both cell adhesion and neurite growth include poly-lysine, laminin, and collagen (Edgar, 1985; Walsh et al., 2005). More recently, nanofiber surfaces have been developed to mimic the structural components of the extracellular matrix (Schindler et al., 2005) that promote neurite extension.

Initiation and elongation of neurites is controlled by both intrinsic and extrinsic factors, which ultimately regulate the assembly of cytoskeletal elements such as actin and tubulin into filaments and microtubules that determine the shape and length of the neurites (Raine, 1994). The intrinsic growth state of the neuron, defined as a phenotype expressed independent of the cell environment, appears to depend on transcriptional programming that “primes” the cell for growth by regulating the expression of many intracellular signaling pathways (Goldberg, 2004). Axon and dendrite growth is then supported by a set of extracellular cues. These extrinsic signals include trophic factors, extracellular matrix molecules, and activity-dependent depolarization (Arevalo and Chao, 2005; Kapfhammer and Schwab, 1992; Neely and Nicholls, 1995). Recent data suggests that these extracellular cues activate signaling cascades which converge on glycogen synthase kinase-3β (Yoshimura et al., 2006). The complex nature of the regulation of neurite outgrowth provides a wide range of potential targets for chemical perturbation of this process. Interference with gene expression, membrane receptors and ion channels or intracellular signaling can all result in altered neurite initiation and growth (Audesirk and Audesirk, 1998). Because of the many potential molecular targets, the use of a cell-based assay may be more suitable for hazard identification and screening for chemical-induced changes in neurite outgrowth when the site (or sites) of action are unknown.

2. Methods for assessment of neurite outgrowth

2.1. Semi-quantitative

A number of methods are used for assessing neurite outgrowth that can be considered semi-quantitative in the sense that they do not result in a calibrated measure of neurite length. Rather, the presence and/or length of processes emanating from a cell are scored or ranked based on predetermined criteria. Observations can be made directly from the microscope or from photomicrographs. These methods have the advantage of being rapid and simple to perform, do not require sophisticated equipment for imaging, or expensive software for analysis. Semi-quantitative methods can be subjective, however, with a risk of scoring bias. In many cases observations can be made on living cells, negating the need for fixation or staining. One of the simplest and most common endpoint assessed is the number of cells exhibiting neurites. In this case the presence or absence of a process of a predetermined length is scored for each cell. The length of a process that qualifies as a neurite is most often defined as being equal to or greater than one to two times the diameter of the cell body (Das et al., 2004; Morooka and Nishida, 1998; Nandi et al., 2006). Both the percentage of cells exhibiting neurites and the number of neurites per cell can be reported. Because the presence of a neurite is often used as a defining characteristic of differentiation, the data may be reported as percent differentiation. A variation of this method, which has been used with explanted cultures of peripheral ganglia, is to score the density of neurite outgrowth (Soderstrom and Ebendal, 1995) or the number of neurites crossing an annulus of a certain diameter (Bilsland et al., 1999; Blackman et al., 1993). Chemical effects on the morphology of primary neuronal cultures have also been scored based on developmental stage (Heidemann et al., 2001). In this study cells were determined by the observer to be either stage 1 (minimal change in the cell margin), stage 2 (two or more minor processes), or stage 3 (at least one process

undergoing elongation and identified as an axon) as described by Dotti et al. (1988) for hippocampal cultures.

2.2. Quantitative

Quantitative assessment of neurite outgrowth refers to those methods which provide, at a minimum, a calibrated measure for some aspect of neurite length (e.g., length of the longest neurite, total neurite length, average neurite length, and length of segments). In many cases a more complete assessment of neurite growth may be obtained including quantification of the number of neurites, the number of branch points per neurite, and measures of the complexity of neurite outgrowth. Quantitative morphometric analysis of neurite outgrowth is time-consuming and creates greater demands upon the observer compared to semi-quantitative analysis. Thus, it is almost always performed using microscopic imaging systems. As discussed below, a number of different tools have been developed, including semi-automated and fully automated image analysis programs, to facilitate data acquisition. Regardless of the tools employed to obtain measurements, images are associated with a calibration which provides a scale to relate length on the image (e.g., in pixels on a digital image) to length of the original object (e.g., in microns for neurite length). In order to obtain an accurate assessment of the morphology for individual neurons, cultures are generally grown under conditions which result in a cell density with minimal overlap of processes.

Fig. 2 provides an example of some of the morphologic features used to quantify neurite outgrowth. Length of the longest neurite is a common measure since it is relatively easy to delineate from photomicrographs. It has been assessed in both cell lines (Das et al., 2004) and in primary cultures (Bearer et al., 1999; Dey et al., 2006). In primary cultures, the longest neurite is sometimes designated as the axon, while the remaining neurites are by default designated as dendrites (Kern and Audesirk, 1995). Determining the total neurite length (sum of the lengths of all neurites emanating from a cell) requires considerably more effort and is often obtained using automated analyses. Total neurite length has been assessed in cell lines (Das and Barone, 1999) and primary cultures (Rosso et al., 2000; Vutskits et al., 2006). Neurite length may be reported separately for axons and dendrites. Differential assessment of total axonal and dendritic outgrowth can be obtained based on immunostaining for selective markers (Howard et al., 2005; Yanni and Lindsley, 2000). Data can also be reported as mean neurite (or axon and dendrite) length based on the number of processes per cell (Ferguson and Audesirk, 1995). Further, more detailed analysis of neuronal morphology can be performed in order to obtain a measure of the degree of complexity of process outgrowth. Neurites can be classified as primary (originating from the cell body) or as branches (originating from a primary neurite) and the number of primary neurites and branches determined (Dey et al., 2006). This approach was used by Vutskits et al. (2006) to demonstrate the effects of ketamine on dendrite growth in primary neurons. Ketamine decreased both total dendrite length and number of branches, suggesting a decrease in the complexity of the dendritic arbor. Similarly, a decrease in the number of branches indicated that organophosphates affected the complexity of neurite outgrowth in the PC12 cell line (Das and Barone, 1999).

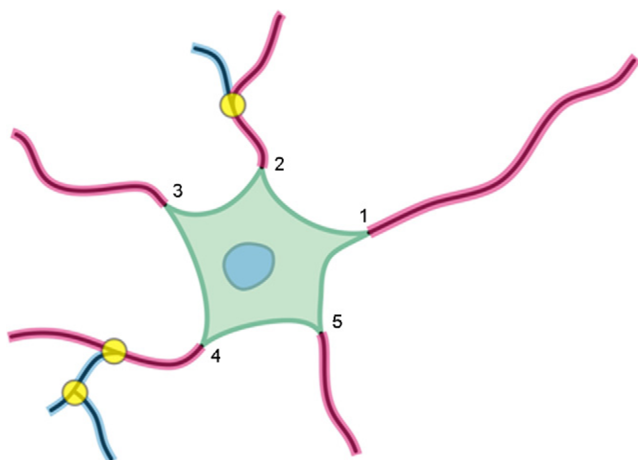

Fig. 2. Quantitative assessment of neurite outgrowth. The schematic illustrates several aspects of neuronal morphology that can be used to quantify neurite outgrowth. Identification of the nucleus (light blue) and or cell body (green) is often used as the basis for measurement. Primary neurites (red) are processes originating directly from the cell body and are labeled 1 through 5. The longest neurite is labeled as number 1. Secondary neurites (blue) originate from primary neurites. Branch points (yellow) indicate branching of primary or secondary neurites and can be a measure of the complexity of process outgrowth. Total neurite length is the sum of the lengths of all primary (red) and secondary (blue) neurites.

Measurements can be performed on images obtained from live cultures or cultures that have been fixed and stained. Imaging of live cultures using phase contrast or interference contrast optics can be performed without staining or manipulating the cells, thereby decreasing time and costs associated with fixation. In addition, changes in neurite outgrowth can be observed over time in the same culture. For example, Das et al. (2004) obtained multiple phase contrast images of the same PC12 cell culture over a 7-day period to quantify nerve growth factor (NGF) stimulated increases in neurite outgrowth. For some analysis software, however, images from phase or interference contrast microscopy may not provide sufficient contrast between background and neurites for analysis. Thus, cells are often fixed and processed using a variety of staining procedures (De Mey, 1983; Isaacs et al., 1998; Jockusch et al., 1979; Okazaki et al., 2003). Cell staining can help to delineate the neurites from the background and from other non-neurite containing cells in the culture that may interfere with analysis of the image. Fixed cells can also be processed using immunocytochemical techniques which selectively label different aspects of neuronal morphology, including axons and dendrites. This is useful in light of evidence that chemicals can have differential effects on the outgrowth of axons and dendrites. For example, based on immunocytochemical labeling of primary neurons, Howard et al. (2005) demonstrated that chlorpyrifos inhibited growth in processes identified as axons but enhanced growth in processes identified as dendrites. Disadvantages of fixation and labeling include increased reagent costs and processing time, and potential loss of cells due to the fixation and multiple wash steps associated with these procedures.

The advent of digital photography and computer-based image processing has greatly facilitated morphometric analysis of neurite growth. Many studies, however, report data derived from the labor-intensive process of manual tracing. A typical procedure includes: (1) observation and selection of a field of cells under phase contrast, bright field or fluorescent microscopy, (2) image capture with a digital camera, (3) image processing to enhance contrast, and (4) manual tracing of processes using image analysis software. Recent examples using this type of procedure include analysis of neurite outgrowth in neuroblastoma cells (Hong et al., 2003) and dendritic arborization in primary neurons (Vutskits et al., 2006). Although labor-intensive and time-consuming, the ability of a human observer to accurately discriminate a neurite from background artifacts makes manual tracing the gold standard by which automated methods are judged. A number of semi-automated techniques are now available which can provide assessments of neurite outgrowth with accuracy similar to that of manual tracing (Barone et al., 2003; Kawa et al., 1998; Meijering

et al., 2004; Sakai et al., 2000; Xiong et al., 2006). These semi-automated procedures still require manual image capture and interaction of the user with the image analysis software, but increase the throughput by automating the quantification of neurite outgrowth for each cell in the image.

In order to facilitate rapid assessment of neurite outgrowth, high-content screening (HCS) systems have been developed with automation of both image capture and image analysis (Smith and Eisenstein, 2005). These fully automated systems are designed to monitor phenotypic indicators in individual cells grown on multi-well plates, provide the hardware for image capture, and the software for image analysis. A number of such systems are now available for the assessment of chemical effects on neurite outgrowth (Hu et al., 2005; Liu et al., 2007; Price et al., 2006; Ramm et al., 2003; Simpson et al., 2001). HCS systems include automated stage movement for positioning, autofocusing and exposure adjustment for image capture, and software for image storage, image analysis, and data presentation. Cells are typically grown in 96- or 384-well plates, fixed and labeled with an antibody staining the cell body and neurites (e.g., β-tubulin), and counter-stained with a nuclear dye (e.g., Hoechst). In order to facilitate the automated acquisition and analysis of digital images, the conditions for culture (e.g., cell density) and cell labeling methods (e.g., antibody detection of cells with minimal background) are optimized for the cell model being used. Under the appropriate conditions, data from hundreds of cells per well can be obtained from 96-well in under an hour. Data on various parameters of neurite outgrowth (e.g., total neurite length and neurite count, branching) can be presented on a per cell basis or averaged over the entire well. An example of the images captured by an HCS system and the identification of neurites using image analysis software is presented in Fig. 3(A–C). Typical data obtained from a PC12 cell clone (NS-1 cells) in the absence and presence of NGF to induce differentiation are also presented (Fig. 3D–F). The data represents analysis of individual cell data from over 200 cells from a single well. Under optimal conditions, neurite outgrowth data obtained using a fully automated system is comparable to data obtained using manual methods (Ramm et al., 2003), although the throughput can be orders of magnitude greater. For example, manual assessment of neurite length on 200 cells (including image acquisition and image analysis) would take approximately 10 h of time for an individual investigator. This can be compared to only minutes required to obtain data for the multiple endpoints shown in Fig. 3.

It is clear that *in vivo*, even subtle alterations in process outgrowth can result in significant CNS dysfunction (Webb et al., 2001). In order to model subtle effects of chemicals *in vitro*, the more detailed analysis of neurite complexity described above may be required. There have not been systematic studies, however, to evaluate the sensitivity of the various measures of neurite outgrowth to chemical perturbation. The decision as to what measures to evaluate is often made on the basis of the methods available to the laboratory (e.g., manual vs. automated) or on the basis of a hypothesis regarding the mechanism of chemical effect (e.g., alteration in neurite length vs. neurite branching). The increased use of semi- and fully automated systems will facilitate assessment of multiple endpoints simultaneously. Comparison of chemical effects on multiple measures of neurite outgrowth in the same cell should be performed in order to determine which endpoints are most sensitive and appropriate for chemical screening.

2.3. Biochemical

Neurite outgrowth has also been quantified using a variety of biochemical markers. These biomarkers correlate with both differentiation and increases in neurite length (Barone et al., 2003). Biochemical procedures including immunoblotting, ELISA, and proteomic techniques may be useful in higher throughput screening. As described below, changes in biochemical markers coincident with chemical-induced effects on neurite morphology

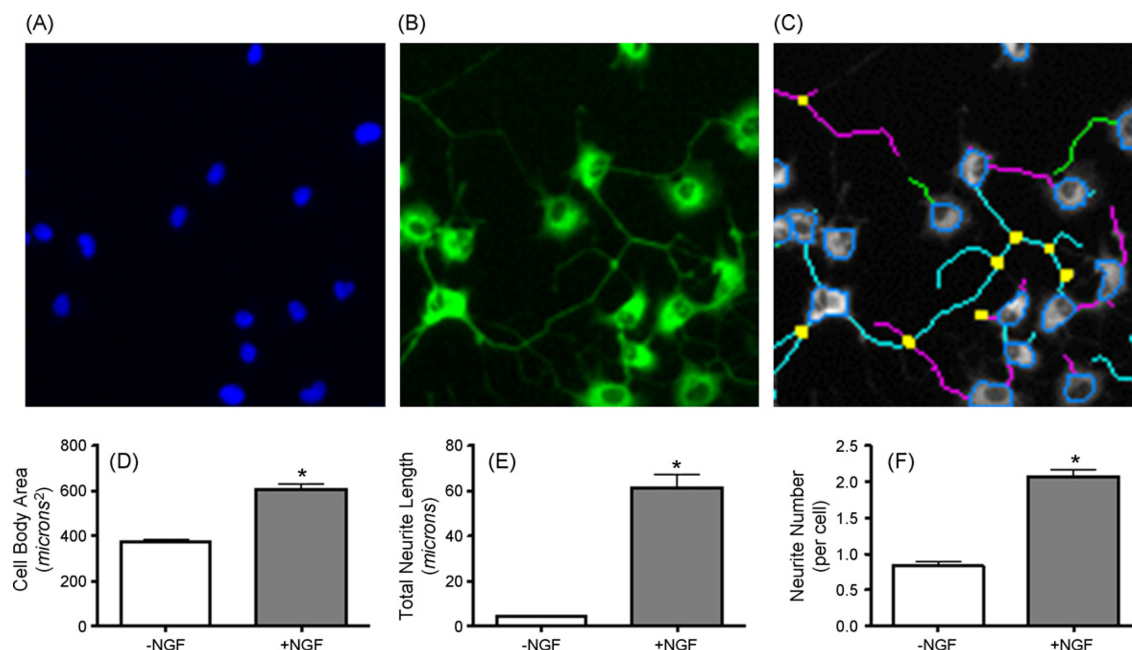

Fig. 3. High-content screening for neurite outgrowth. PC12 cells were grown in 96-well plates and treated with NGF (100 ng/ml) for 96 h. Images were automatically captured and analyzed using the Cellomics™ ArrayScan VTi®. (A) Nuclei are labeled blue on the basis of staining with Hoescht 33258 and are used to identify valid cells. (B) Cell bodies and processes are labeled green using anti-βIII tubulin primary antibody and Alexa 488-conjugated secondary antibody. (C) The image analysis algorithm identifies the cell body and measures a number of parameters including cell body size, neurite number, length, and branch points for each identified cell (D–F). Data from automated analysis of a single well of PC12 cells in the absence (open bars, n = 251 cells) and presence (closed bars, n = 205 cells) of NGF. Quantitative assessment of PC12 differentiation using high-content screening is consistent with manual and semi-automated procedures.

have been observed in a number of *in vitro* models. It is not clear however, which endpoint is more sensitive to chemical perturbation. In many studies the biochemical indices are not examined for their use as a biomarker for neurite outgrowth per se, but as potential sites of chemical action (Han et al., 2003; Sager and Matheson, 1988; Scortegagna et al., 1998). The studies outlined below are limited to those that have associated biochemical measures with morphological assessment of neurite outgrowth.

Neurotypic proteins associated with the cytoskeleton (including neurofilaments and microtubule-associated proteins) and the growth-associated protein GAP-43 have been used for the visualization of axons and dendrites (Brion et al., 1994; Dotti et al., 1987; Pennypacker et al., 1991; Ramakers et al., 1991). In addition, there are studies correlating their expression with neurite outgrowth during neuronal development *in vitro* as described below.

Neurofilaments belong to the class of intermediate filaments and are one of the most abundant structural proteins in axons (Lee and Cleveland, 1996). Neurofilament protein levels increase upon differentiation of cell lines (Yamazaki et al., 2005) and upon neurite outgrowth in primary cultures (Taniwaki and Schwartz, 1995). A correlation between chemical-induced reductions in neurite outgrowth and decreases in the level of neurofilament protein has been demonstrated in cell lines including NB41A3 cells (Abdulla and Campbell, 1993), SK-N-SH cells (Abdulla et al., 1995) and N2a cells (Flaskos et al., 2007). There may not be a direct relationship, however, between neurite length and neurofilament expression, as Cho and Tiffany-Castiglioni (2004) reported that decreased neurite length in SH-SY5Y cells in response to the pesticide exposure was associated with an upregulation of neurofilament protein.

Tau is a developmentally regulated microtubule-associated protein that is localized to the axon in neurons and is involved in axonal growth (Paglini et al., 2000). Studies in both cell lines (Rasouly et al., 1994) and primary cultures (Smith et al., 1995) show increases in tau protein coincident with neurite outgrowth. In primary cultures of hippocampal neurons, a concentration-dependent inhibition of neurite outgrowth after exposure to lithium correlated with a concentration-dependent decrease in tau expression (Takahashi et al., 1999).

GAP-43 is a growth-associated protein with a well-known role in growth cone formation, axonal elongation, and synaptic plasticity (Benowitz and Routtenberg, 1997). It is preferentially distributed in the growth cone and elongating axon in developing neurons, and levels increase dramatically coincident with neurite outgrowth in PC12 cells (Das et al., 2004; Jap Tjoen San et al., 1991) and primary cerebellar granule cells (Przyborski and Cambray-Deakin, 1994). In PC12 cells, chemical inhibition of neurite outgrowth has been-associated with a decrease in GAP-43 expression (Das et al., 2004; Jap Tjoen San et al., 1992).

3. Models of neurite outgrowth

3.1. Cell lines

Cell line is a general term applied to a defined population of cells that can be maintained in culture for an extended period of time. Cell lines are usually clonal, meaning that the entire population originated from a single common ancestor cell. A number of neuronal cell lines are available (James and Wood, 1992) and many have been used as *in vitro* models to examine neurite outgrowth. Table 1 lists the most commonly used cell lines and describe the associated phenotype and agents used to induce neurite outgrowth. References are provided for both their establishment and use for study of neurite outgrowth. Neuronal cell lines have been derived from tumors, including pheochromocytomas (Greene and Tischler, 1976) and neuroblastomas (Augusti-Tocco and Sato, 1969; Schubert et al., 1969). More recently, cell lines have been generated using oncogene-containing retroviruses (Bartlett et al., 1988). The introduction of oncogenes from the *myc* family into primary neural cells can result in neural cell lines that are immortalized while retaining many of the characteristics of the original cell population (Lendahl and McKay, 1990). Cell lines have a number of advantages that make them useful as *in vitro* models: (1) they provide a homogenous population of cells, (2) they are easy to obtain and can be stored indefinitely in liquid nitrogen, (3) they are relatively easy to grow using standard tissue culture plastic and media, and (4) they divide rapidly and can be continuously subcultured (passaged) to provide large numbers of cells in a short period of time. Neuronal cell lines can be induced to differentiate by adding different drugs or growth factors to the media, or the removal of serum from the media. Differentiation results in a non-dividing cell with many of the

Table 1
Cell lines used to assess neurite outgrowth *in vitro*

Cell Line	Source	Phenotype	Inducing agents	References
PC12	Rat pheochromocytoma	Adrenergic, Cholinergic, Dopaminergic	Nerve growth factor	Greene and Tischler (1976), Schubert et al. (1977), Greene (1977)
B50	Rat neuroblastoma	Cholinergic	Dibutyryl cyclic AMP, Serum removal	Schubert et al. (1974), Wu and Ledeen (1991), Audesirk et al. (1991)
NB2a	Mouse neuroblastoma	Adrenergic, Cholinergic, Dopaminergic	Dibutyryl cyclic AMP, Retinoic acid	Augusti-Tocco and Sato (1969), Shea et al. (1985)
N2a	Mouse neuroblastoma	Adrenergic, Dopaminergic	Dibutyryl cyclic AMP, Serum removal	Schubert et al. (1969), Flaskos et al. (1998), De Girolamo et al. (2000)
N1E-115	Mouse neuroblastoma	Adrenergic, Dopaminergic	Dimethyl sulfoxide, Prostaglandin E1 (PGE1), Serum removal	Amano et al. (1972), Kimhi et al. (1976), Audesirk et al. (1991)
SH-SY5Y	Human neuroblastoma	Adrenergic, Cholinergic, Dopaminergic	Retinoic acid, Dibutyryl cyclic AMP, Nerve growth factor	Ross and Biedler (1985), Pahlman et al. (1984), McLean et al. (1998), Perez-Polo et al. (1979), Ehrich (1995)
SK-N-SH	Human neuroblastoma	Dopaminergic	Retinoic acid, Nerve growth factor	Biedler et al. (1973), Perez-Polo et al. (1979), Preis et al. (1988)
IMR-32	Human neuroblastoma	Aminergic, Cholinergic	5-Bromo-deoxyuridine (BrdUr), Nerve growth factor	Tumilowicz et al. (1970), Gotti et al. (1987), Clementi et al. (1986), Reynolds and Perez-Polo (1981)
LA-N-5	Human neuroblastoma	Cholinergic	Retinoic acid	Haussler et al. (1983), Robson and Sidell (1985), Han et al. (1995)
NT2	Human embryonal carcinoma	Cholinergic	Retinoic acid	Fogh and Trempe (1975), Andrews et al. (1984), Zeller and Strauss (1995)

characteristics of a neuron, including the extension of neurites (Banker and Goslin, 1998). This ability to precisely control the timing and onset of differentiation has made cell lines an attractive model for screening chemical effects on neurite outgrowth.

There are limitations to the use of cell lines as models for neurite outgrowth. Because these cells are tumor derived or virally transformed, they do not necessarily exhibit the same phenotype as the original primary cell. Thus, while expressing many of the general characteristics of neurons, cell lines may not accurately represent any specific neuronal subpopulation (Banker and Goslin, 1998). The mechanistic basis of induction of differentiation in cell lines is not always fully understood, and neurite outgrowth may be different from that occurring in primary neurons. For example, neurites elaborated by the PC12 cell line do not exhibit the properties of either axons or dendrites (Banker and Goslin, 1998). In addition, there can be phenotypic variability over time due to genetic instability with increasing passage number (Heumann et al., 1977; Tas et al., 1989). Phenotypic variability can be minimized by using a specific passage number. Cell lines have still proven to be useful, however, in understanding the basic biological processes involved in neuronal differentiation, and neuronal cell lines have been widely used to assess chemical effects on neurite outgrowth (Table 2).

A number of different experimental protocols have been used to investigate chemical effects on neurite outgrowth in cell lines. A common paradigm involves treating cells with the chemical of interest at the same time that differentiation is induced. Exposing the cells to the inducing factor and toxicant simultaneously allows the examination of both initiation of neurite growth and later events such as neurite extension. In order to selectively investigate chemical effects on neurite extension (as compared to neurite initiation), cell lines can be pre-treated with the inducing factor prior to exposure to the toxicant. Using this protocol, chemical effects can be examined in cells which have already elaborated neurites. A related protocol harvests cells after a period of differentiation such that existing neurites are sheared off. Upon re-plating, the cells elaborate a new network of neurites in an accelerated manner. Because the cells have already been differentiated to the neuronal phenotype, this protocol also focuses on alterations in neurite extension. Chemical effects on the regrowth of neurites from differentiated cells are described below in the "primed" model using PC12 cells.

The pheochromocytoma PC12 cell line has been widely used as a model for the study of neuronal differentiation and neurite outgrowth (Fujita et al., 1989). PC12 cells were derived from a rat adrenal medullary tumor (pheochromocytoma) and exhibit the phenotypic properties of their non-neoplastic counterparts, adrenal chromaffin cells (Greene and Tischler, 1976). PC12 cells respond to nerve growth factor (NGF) with a dramatic change in phenotype and acquire a number of properties of sympathetic neurons including the expression of catecholaminergic neurotransmitters, cholinergic receptors, and acetylcholinesterase (Fujita et al., 1989). NGF-treated PC12 cells cease proliferation, extend neurites and become electrically excitable (Greene and Tischler, 1982). The elaboration of neurites occurs within 24–48 h of NGF exposure and maximal neurite length is attained within a week (Das et al., 2004). In light of their widespread use in neurobiological studies of growth factor-induced differentiation, it is not surprising that many studies of toxicant effects on neurite outgrowth have used the PC12 cell model (Table 2). In particular, PC12 cells have been used to assess the involvement of NGF-activated signaling pathways (Parran et al., 2003; Williams et al., 2000) and cholinesterase inhibition (Das and Barone, 1999) as possible mechanisms by which toxicants effect differentiation and neurite outgrowth. Most studies use a protocol in which the cells are exposed to NGF and the toxicant concurrently. PC12 cells can also be "primed" by a previous exposure to NGF. In the primed model, PC12 cells are treated with NGF for a prolonged period of time (1 week) so that they become terminally differentiated and exhibit an extensive network of neurites. The cells are mechanically harvested and as a consequence lose their neurites. Upon re-plating in the presence of NGF they will rapidly (within 24 h) elaborate a new neurite network (Greene, 1977). This model allows for the rapid assessment of chemical effects on neurite growth, and can delineate effects on outgrowth from differentiation. It has been used to detect inhibition of neurite outgrowth following exposure to toxicants (Crumpton et al., 2001; Das and Barone, 1999; Jenkins et al., 2004; Parran et al., 2003). However, the regrowth of neurites after priming is not identical to the outgrowth of neurites in naïve PC12 cells. Cell damage resulting from the mechanical stress of re-plating has the potential to interact with chemical effects on neurite outgrowth.

Cell lines that can be differentiated to express the characteristics of neurons have been derived from rodent and human neuroblastomas (tumors arising from immature nerve cells). Neuroblastomas can be induced to differentiate and extend neurites after treatment or removal of the inducing agent. Of the rodent cell lines, the C1300 subclones NB2a and N2a have been extensively used for studies of chemical effects on neurite growth (Table 2). The N2a neuroblastoma cell line was derived from the mouse C1300 tumor and differentiates into a neuron-like cell that exhibits both cholinergic and adrenergic markers (Augusti-Tocco and Sato, 1969; Schubert et al., 1969). Upon the withdrawal of serum, N2a cells differentiate and elaborate neurites. NB2a cells are another C1300 clonal strain (Augusti-Tocco and Sato, 1969) that has been useful in the study of cytoskeletal dynamics leading to the elaboration of axons and dendrites. Differentiation of NB2a cells in the presence of dibutyryl cAMP results in a bipolar morphology where the neurites have properties of axons, while the addition of retinoic acid results in a more extensive network of branching neurites with the properties of dendrites (Shea et al., 1985; Shea et al., 1988). To date, systematic comparisons of dibutyryl cAMP and retinoic acid have not been performed to evaluate differential susceptibilities to axonal or dendritic development to chemical effects in this cell line.

The effect of chemical exposure upon neurite outgrowth is not uniformly observed in all rodent neuroblastoma lines. This is particularly evident in a study performed by Audesirk et al. (1991), in which B50 and N1E-115 cells were exposed to lead for 48 h. Lead exposure caused a decrease in neurite initiation in B50 cells, but an enhancement in both initiation and length in N1E-115 cells. The authors hypothesized that these opposing effects were due to differences in protein kinase C (PKC) isoforms in relation to neurite outgrowth across cell types. These observations illustrate the potential limitations in comparing chemical effects across different rodent neuroblastoma cells. To facilitate selection of a sensitive neuroblastoma cell model for use in screening, more studies are needed comparing the effect of the same chemicals in multiple cell lines to gain a better understanding of the extent of intraspecies variation on neurite outgrowth.

A number of human neuroblastoma cell lines including IMR-32, SH-SY5Y, and SK-N-SH cells have been used to evaluate toxicant effects on neurite outgrowth (Table 2). Because they are derived from human tissue, they may provide a species-specific response to chemical effects on neurite outgrowth, although currently there is not sufficient data to show that human cell lines are more accurate in predicting human developmental neurotoxicity. SK-N-SH cells and the subclone SH-SY5Y were derived from a metastatic neuroblastoma localized within the bone marrow (Biedler et al., 1973). They are an established model for studying the morphogenic action of retinoic acid (Pahlman et al.,

Table 2
Chemical effects on neurite outgrowth in cell lines and primary cultures

Chemical	Model	Effect[a]	References
Metals			
Aluminum	IMR-32	−Initiation, −Length	Gotti et al. (1987)
	N2A	↑ Length	Uemura et al. (1992)
Cadmium	IMR-32	−Initiation, −Length	Gotti et al. (1987)
	N2A	−Initiation, −Length	Spoerri et al. (1990)
	Cerebellum	↓ Length	Sugawara et al. (1983)
Dibutyltin	PC12	↓ Length, ↓ Branching	Jenkins et al. (2004)
Dimethyltin	PC12	↓ Length, ↓ Branching	Jenkins et al. (2004)
Lead	PC12	↑ Length, ↑ Branching	Crumpton et al. (2001)
	PC12	↑ Initiation, ↑ −Length	Williams et al. (2000)
	B50	↓ Initiation, Length	Audesirk et al. (1991)
	N1E-115	↑ Initiation, ↑ Length	Audesirk et al. (1991)
	IMR-32	↓ Initiation, −Length	Gotti et al. (1987)
	Hippocampus	↓ Initiation, −Length, −Branching	Audesirk et al. (1991)
	Neocortex	↓ Initiation	Kern and Audesirk (1995)
	Neocortex	↑ Initiation, ↑ Length	Kern et al. (1993)
Lithium	PC12	↓ Length	Harada et al. (1996)
	PC12	↓ Initiation	Burstein et al. (1985)
Manganese	PC12	↑ Initiation	Lin et al. (1993)
Mercury	PC12	↓ Initiation, ↓ Length, −Branching	Parran et al. (2001)
	IMR-32	↓ Length	Stoiber et al. (2004)
Methylmercury	SH-SY5Y	↓ Length	Nordin-Anderson et al. (1998)
	Dorsal root ganglia	↓ Length	Soderstrom and Ebendal (1995)
	Sympathetic ganglia	↓ Length	Soderstrom and Ebendal (1995)
	PC12	↓ Length	Sakai et al. (2000)
	PC12	↓ Initiation, ↓ Length, ↓ Branching	Parran et al. (2001)
	PC12	↓ Length	Parran et al. (2003)
Monomethyltin	PC12	−Initiation, −Length, Branching	Jenkins et al. (2004)
Triethyltin chloride	SH-SY5Y	↓ Length	Nordin-Anderson et al. (1998)
Trimethyltin	PC12	↓ Initiation, ↓ Length, ↓ Branching	Jenkins et al. (2004)
Pesticides			
Aldicarb	SK-N-SH	↓ Length	Chang et al. (2006)
Benomyl	NB2a	↓ Length	McLean et al. (1998)
	SH-SY5Y	↓ Length	McLean et al. (1998)
Bifenthrin	PC12	↓ Initiation	Nandi et al. (2006)
Benzodioxophosphorin 2-oxide	PC12	↓ Initiation	Li and Casida (1998)
Carbaryl	SK-N-SH	↓ Length	Chang et al. (2006)
Chlorpyrifos	PC12	↓ Length	Das and Barone (1999)
	NB2a	↓ Length	Axelrad et al. (2002)
	N2a	↓ Initiation	Sachana et al. (2001)
	Superior cervical ganglia	−Initiation, ↓ Length	Howard et al. (2005)
Chlorpyrifos oxon	PC12	↓ Length	Das and Barone (1999)
	PC12	↓ Initiation	Li and Casida (1998)
	Superior cervical ganglia	−Initiation, ↓ Length	Howard et al. (2005)
Cypermethrin	N2a	−Initiation	Flaskos et al. (2007)
Diazinon	NB2a	↓ Length	Axelrad et al. (2002)
	NB2a	↓ Length	Axelrad et al. (2003)
	N2a	↓ Initiation	Flaskos et al. (2007)
Dichlorophenoxyacetic acid	Cerebellum	−Initiation, ↓ Length	Rosso et al. (2000)
Dichrolvos	PC12	↓ Length	Sakai et al. (2000)
2,4-Dinitrophenol	N2a	↑ Initiation, ↑ Length	Wasilewska-Sampaio et al. (2005)
Mipafox	SH-SY5Y	↓ Length	Hong et al. (2003)
Paraoxon	SH-SY5Y	Length	Hong et al. (2003)
Trichloropyridinol	PC12	↓ Length	Das and Barone (1999)
	Superior cervical ganglia	−Initiation, −Length	Howard et al. (2005)
Tricresyl phosphate	N2a	↓ Initiation	Flaskos et al. (1998)
	PC12	↓ Initiation	Flaskos et al. (1998)
Tri-ocresyl phosphate	SK-N-SH	↓ Length	Chang and Wu (2006)
Other neurotoxicants			
Acrylamide	SH-SY5Y	↓ Initiation	Nordin-Anderson et al. (1998)
	SH-SY5Y	↓ Initiation	Nordin-Andersson et al. (2003)
	N1E-115	↓ Initiation	Brat and Brimijoin (1993)
Cocaine	Locus coeruleus	↓ Initiation, ↓ Length	Snow et al. (2001)
	Locus coeruleus	↓ Initiation, ↓ Length	Dey et al. (2006)
	PC12	↓ Initiation	Zachor et al. (1996)
Ethanol	PC12	↑ Initiation	Messing et al. (1991)
	PC12	↑ Initiation	Wooten and Ewald (1991)
	LA-N-5	↓ Length	Saunders et al. (1995)

Table 2 (*Continued*)

Chemical	Model	Effect[a]	References
	Cerebellum	↓ Length	Bearer et al. (1999)
	Hippocampus	↓ Initiation, ↓ Length	Yanni and Lindsley (2000)
	Neocortex	↓ Length, ↓ Branching	Bingham et al. (2004)
	Dorsal root ganglion	↓ Initiation	Dow and Riopelle (1985)
Ketamine	Subventricular zone	↓ Initiation, ↓ Length, ↓ Branching	Vutskits et al. (2006)
Valproic acid	N1E115	↑ Initiation	Yamauchi et al. (2007)
	NT2	↓ Initiation	Skladchikova et al. (1998)
Vincristine	PC12	↓ Initiation	Geldof et al. (1998)

[a] ↑, increase; ↓, decrease; –, no effect. Initiation refers to appearance of neurite with length greater than 1 diameter of the cell body.

1984) but can also be induced to differentiate using nerve growth factor (Perez-Polo et al., 1979). Unlike PC12 cells, SH-SY5Y cells can form functional synapses (Pahlman et al., 1990). Differentiation with retinoic acid results in a relatively high expression of neurotoxic esterase and acetylcholinesterase and these cells have been widely used to investigate the neurotoxicity of cholinesterase inhibitors (Ehrich, 1995).

Overall there is a large literature on the effects of chemicals on neurite outgrowth in cell lines. This should not be construed to suggest that they are a more appropriate model than primary cells, but may be due, in part, to their widespread availability and ease of preparation. Of primary concern in their use for screening is the fact that cell lines do not replicate some of the more complex properties of neurite outgrowth observed in primary neurons. This may limit their predictive ability. They do, however, have a number of properties that are desirable for a model system for screening assays, including reproducibility and the ability to generate large numbers of cells. A comparison of human and rodent cell lines show both complimentary and opposing effects of chemicals on neurite outgrowth. For example, exposure to either acrylamide (Brat and Brimijoin, 1993; Nordin-Anderson et al., 1998; Nordin-Andersson et al., 2003) or benomyl (McLean et al., 1998) reduces neuronal initiation and outgrowth in cell lines from both species. In contrast, lead exposure in rodent N1E-115 cells facilitated neurite initiation and outgrowth (Audesirk et al., 1991), but at a similar concentration lead inhibited neurite initiation in human IMR-32 cells (Gotti et al., 1987). Differences in source of the chemical used, concentrations, and exposure conditions contribute to the difficulty in making these comparisons. In the absence of more definitive data, it is suggested that further research on the use of cell lines for screening of chemical effects on neurite outgrowth should focus on determining the predictive value of cell lines of human origin. Preference would be given to immortalized cell lines that retain the characteristics of the original primary neuron (Lendahl and McKay, 1990; Walsh et al., 2005). As other models with the advantages of self-renewal (such as neural stem cells and progenitor cells) become widely accessible, they should be considered in place of cell lines.

3.2. Primary cultures

Primary neuronal cell cultures consist of cells dissociated from peripheral or central nervous system tissue. These cells retain many of the morphological, neurochemical, and electrophysiological properties of neurons *in situ*. When maintained under the appropriate culture conditions, primary cells will acquire the properties of mature neurons and spontaneously elaborate neurites. Unlike cell lines, neurites from primary neurons in culture can be identified as distinct axons and dendrites. Although some preparations of primary neuronal cultures can contain a predominant neuronal cell type (e.g., sympathetic neurons from the superior cervical ganglia and granule cell cultures from post-natal cerebellum) many preparations are a mixture of different neuronal populations. Pure neuronal cultures can be obtained using defined media which prohibit the growth of glial cells or by adding an anti-mitotic agent to the medium during the first days in culture. A mixed glial/neuronal co-culture will result if these steps are not employed. A disadvantage of primary cultures is their limited lifespan (typically days to weeks). Because they are predominantly post-mitotic at the time of harvest, it is not possible to expand neuronal cell populations in culture. Thus, new cultures must be prepared from nervous system tissue on a regular basis, increasing the genetic variability of the model system across different cultures.

Primary neuronal cultures give rise to axons and dendrites that have the characteristics of those from the corresponding cells *in situ*, and they have provided an important model for neurobiological studies of the process of axonal and dendritic outgrowth (Craig and Banker, 1994). Cultured primary neurons are committed to a specific lineage and retain many properties of the cells present in the region of origin (Banker and Goslin, 1988). This allows the study of chemical effects on specific populations of cells. Primary neuronal cultures derived from different regions of the nervous system have been used as models of neurite outgrowth (Table 3). While the ability to culture regional subpopulations of neurons may be useful in addressing specific

Table 3
Primary cell cultures used to assess neurite outgrowth *in vitro*

Region	Source	Principle cell types	References
Peripheral nervous system			
Superior cervical ganglia	Rodent	Sympathetic	Freschi (1982), Obata and Tanaka (1980)
Dorsal root ganglia	Rodent, Avian	Sensory	Okun (1972), Kimpinski and Mearow (2001), He and Baas (2003)
Sympathetic ganglia	Avian	Catecholaminergic	Bruckenstein and Higgins (1988a), Bruckenstein and Higgins (1988b), Skaper et al. (1983)
Central nervous system			
Locus coeruleus	Rodent	Noradrenergic	Amaral and Sinnamon (1977), Masuko et al. (1986)
Substantia nigra	Rodent	Dopaminergic	O'Malley et al. (1991), Poltorak et al. (1992)
Cerebellum	Rodent	Granule, Purkinje	Altman (1972), Dou and Levine (1995), Kingsbury et al. (1985)
Hippocampus	Rodent	Pyramidal, Granule	Banker and Cowan (1977), Kern and Audesirk (1995)
Neocortex	Rodent	Pryamidal, Stellate	Dichter (1978), Mattson and Rychlik (1991)
Subventricular zone	Rodent	GABAergic interneurons	Van Eden et al. (1989), Gascon et al. (2006)

hypotheses, it may be problematic when using primary cultures as *in vitro* models for chemical screening. Differences in neuronal cell types contained within regions can result in a regional specificity for neurotoxic effects, including neurite outgrowth. An example of a regional difference in neurite outgrowth in response to a toxicant was demonstrated in a study by Dey et al. (2006). *In vitro* exposure to cocaine inhibited differentiation and neurite outgrowth in primary neuronal cultures prepared from the locus coeruleus, but had no effect on cultures prepared from the substantia nigra. The basis for this difference was not identified, but likely related to the different phenotypes expressed by noradrenergic locus coeruleus neurons and dopaminergic substantia nigra neurons (Dey et al., 2006).

Primary cultures of neurons from the peripheral nervous system, including the superior cervical ganglion and dorsal root ganglion, have played a key role in studies of the control of neuronal differentiation by trophic factors and extracellular matrix proteins (Higgins et al., 1997). These cells are readily obtained from rodent and avian species and grown as explants or dissociated cultures (Mahanthappa and Patterson, 1998; Smith, 1998). Neurite outgrowth can be enhanced by the presence of various growth factors including NGF. Cultures prepared from the cervical ganglion are easily dissected and contain a relatively homogenous population of sympathetic neurons. Upon plating these cells will elaborate a single axon-like process but can be induced to extend multiple dendrites by treatment with bone morphogenetic protein (Lein et al., 1995). Dorsal root ganglion cultures also respond to growth factor containing media by extending axons. Both cultures have been used to examine the effects of toxicants on neurite growth (Table 2). The usefulness of a primary neuronal culture in which axons and dendrites can be distinguished from one another was demonstrated by Howard et al. (2005). In this study, exposure of sympathetic neurons from the superior cervical ganglion to chlorpyrifos and its active metabolite resulted in a significant reduction in total axon length per neuron, but enhanced dendritic growth. If only total neurite length had been assessed, changes in neurite outgrowth may have been obscured by the opposing effects of chlorpyrifos on axonal and dendritic length.

Primary neuronal cultures derived from many different regions of the central nervous system including the hippocampus, neocortex, cerebellum, midbrain and subventricular zone have been used to examine toxicant effects on neurite outgrowth (Table 2). These cultures are usually prepared using tissue from rodents but can also be prepared from avian species such as the chicken (Heidemann et al., 2001). Primary neurons prepared from the central nervous system provide a model that can be used to distinguish chemical effects on more complex aspects of axonal and dendritic outgrowth. For example, Yanni and Lindsley (2000) used primary hippocampal cultures to examine the effect of ethanol on dendrite development. Ethanol decreased not only total dendritic length, but also the number of dendrites per cell and the length of individual dendrites. Similarly, Vutskits et al. (2006) showed that the anesthetic ketamine decreased total dendritic length, the number of primary dendrites and dendrite branch points in primary cultures of GABAergic neurons. Because they resemble neurons *in situ*, primary neurons in culture are often used to investigate cellular mechanisms of developmental neurotoxicity observed after *in vivo* chemical exposure. In some cases effects observed *in vivo* are replicated in the *in vitro* model. For example, developmental exposure of rats to lead can result in ultrastructural changes in the hippocampus associated with decreased axonal and dendritic outgrowth (Alfano and Petit, 1982), and inhibitory effects of lead on neurite outgrowth have been observed in primary cultures of rat hippocampal neurons (Kern and Audesirk, 1995; Kern et al., 1993). A similar correlation was observed between the inhibitory effects of ethanol on dendritic arbor size in the hippocampus *in vivo* (Davies and Smith, 1981) and in primary hippocampal neurons *in vitro* (Yanni and Lindsley, 2000).

3.3. Chemical effects between neuronal cell lines and primary neuronal cultures

Neuronal cell lines and primary neuronal cultures both have unique attributes that make them useful as *in vitro* models of neurite growth. Cell lines provide an almost unlimited supply of a homogenous population of neurons that can be induced to differentiate at a specified time. Primary cultures provide neurons that elaborate axons and dendrites characteristic of those observed *in vivo*. The biology underlying these attributes may contribute to differences in their response to chemical perturbation. There has not been a systematic evaluation of the response of cell lines and primary cells to toxicant exposure, and it is difficult to make generalizations regarding their relative utility as models of neurite outgrowth. Even for studies which have used the same chemical, comparisons are difficult due to differences in experimental protocols including differentiation paradigms, time of exposure and endpoints measured. As can be seen in the data summarized in Table 2, the same chemical can alter neurite outgrowth in different ways depending upon the cell culture used. For example, the effects of ethanol have been examined in both primary neurons in culture and in several neuronal cell lines. Concentrations of 50–100 mM ethanol inhibited some aspect of neurite outgrowth in a majority of studies using primary neuronal cultures including rat cerebellum (Bearer et al., 1999), cortex (Barclay et al., 2005), hippocampus (Yanni and Lindsley, 2000), and chick dorsal root ganglia (Dow and Riopelle, 1985). In a study using primary rat cerebellum neurons, however, ethanol enhanced neurite outgrowth (Zou et al., 1993). In PC12 cells, ethanol enhanced NGF-stimulated neurite outgrowth (Messing et al., 1991; Wooten and Ewald, 1991). In contrast, ethanol inhibited neurite outgrowth stimulated by retinoic acid in the LA-N-5 neuroblastoma cell line (Saunders et al., 1995). While the direction of ethanol effects differed both within and between the different model systems, in all cases a change in neurite outgrowth was observed at similar concentrations, suggesting that all models would be capable of detecting a chemical-induced change in the context of screening. The organophosphorus insecticide chlorpyrifos has been examined in a number of different cell lines and in one primary culture. For every cell line tested, chlorpyrifos inhibited neurite outgrowth at concentrations ranging from 1 to 50 μM (Axelrad et al., 2003; Das and Barone, 1999; Sachana et al., 2001). In primary cultures of the superior cervical ganglia, however, the effect of chlorpyrifos depended upon the process measured. Chlorpyrifos inhibited axonal growth at low concentrations (1 nM), and enhanced dendritic growth at a higher concentration of 1 uM (Howard et al., 2005). In this case, a primary neuronal culture was more sensitive to perturbations of neurite outgrowth compared to neuronal cell lines.

Although primary cultures provide cells that correspond closely to those *in situ*, they may not be as useful for chemical screening due to the need to continually prepare new cultures from animal tissue, the potential for variability within and between laboratories, and differences in response to chemicals as described above for cultures prepared from different regions. However, because of there similarity to neurons *in vivo* and the potential to be more sensitive to chemical effects, primary cultures could be considered as a baseline to develop other models that may be useful for screening. Effects on neurite outgrowth could be examined in primary cell models for those chemicals that will be used as

positive and negative controls in further studies comparing models systems for screening assays.

3.4. Neural stem cells

Neural stem cells (also referred to as neural progenitor cells) can be defined by the capacity for self-renewal and the ability to generate the three major cell types of the nervous system: neurons, astrocytes and oligodendrocytes. They can be derived from pluripotent embryonic stem cells or multipotent adult progenitor cells from multiple species including rodents and humans (Seaberg and van der Kooy, 2003). Recently, neural stem cells of human origin have been immortalized to create clonal neural stem cell lines (Cacci et al., 2007) and several human neural stem cell cultures have become available commercially (Krathwohl and Kaiser, 2004). Neural stem cells are an emerging model that may be extremely useful for toxicity screening (Klemm and Schrattenholz, 2004). They have a potentially unlimited capacity for proliferation and can generate multiple cell types. Human neural stem cells can provide a readily available source of cells that can differentiate into neurons and glia identical to those *in vivo*. The use of human neural tissue can also decrease one source of uncertainty in toxicity testing, the extrapolation of data derived from animal tissue to humans.

The culture of neural stem cells in an undifferentiated and proliferative state can be performed as free-floating neurospheres or as a monolayer and requires the presence of basic fibroblast growth factor (Kornblum, 2007). Removal of the growth factor from the media induces a relatively prolonged state of differentiation resulting in a mixed population of neurons and glia (Kornblum, 2007). Although neurons from differentiated stem cells can elaborate axons and dendrites (Salero and Hatten, 2007), to date there have not been any studies on the effects of chemicals on neurite outgrowth using this model. However, a number of studies have examined the effect of neurotoxicants on neural stem cell differentiation. In most of these studies stem cells are differentiated in the presence of the chemical, and the resulting population of neurons and glia are compared to the control condition. Using this paradigm, lead (Huang and Schneider, 2004), ethanol (Tateno et al., 2004), PCBs (Fritsche et al., 2005), methylmercury (Tamm et al., 2006) and cocaine (Hu et al., 2006) have all been shown to disrupt neural stem cell differentiation. In these studies, stem cells proved to be a relatively sensitive model system, and could distinguish between chemical effects in cells prepared from different brain regions. A major benefit of using neural stem cells, as with all cell lines, in the context of screening chemicals for effects on neurite outgrowth is the capacity for self-renewal (which provides a large numbers of cells of identical origin). In addition, stem cells derived from humans provide a model that closely resembles the developing human neuron *in situ*. Future research should directly compare stem cells derived from humans and rodents to determine the ability of each model to predict chemical-induced changes in neurite outgrowth. In the absence of differences in sensitivity, stem cells derived from humans should be used in order to decrease the uncertainty associated with extrapolating toxicity across species.

4. Conclusions

The outgrowth of axonal and dendritic processes is a hallmark of neuronal differentiation and maturation. The elongation and branching of neurites are key events during brain development as they underlie the formation of a properly wired neuronal network. The relationship between abnormal neurite outgrowth during development and impaired behavior has been reviewed (Berger-Sweeney and Hohmann, 1997), and even subtle alterations in this process can lead to CNS dysfunction in humans (Ramakers, 2002; Webb et al., 2001). Accordingly, chemicals that affect the process of neurite outgrowth have the potential to cause developmental neurotoxicity. Although it has been shown that neurite outgrowth is vulnerable to chemical insult during development, morphological assessment of this endpoint *in vivo* is labor-intensive and requires the use of advanced techniques including immunocytochemical staining, dye injection and advanced microscopy (Alfano and Petit, 1982; Granato and Van Pelt, 2003). The *in vitro* studies cited in this review demonstrate the ability of cell culture models to provide a relatively simple system for monitoring the complex morphological changes which accompany process outgrowth. The level of analysis can range from simple scoring of cells exhibiting neurite growth to the determination of the complexity of dendritic arbors in primary cultures. Thus, with the appropriate cell model and level of analysis relatively subtle changes in cellular morphology (such as the number of branches or length of secondary dendrites) can be assessed. Cell culture models also facilitate assessment of the specificity of chemical effects on neurite outgrowth. The concentration–response relationship for chemical effects on neurite outgrowth can be directly compared to other, more general measures of cell health measured in the same culture including changes in size of the cell soma or nucleus, cell metabolism, and cell viability.

The extrapolation of *in vitro* findings to the prediction of adverse effects *in vivo* depends in part, upon a plausible relationship between the endpoint measured and normal biological function, and validation against data obtained in animals and (ideally) humans. *In vitro* models described in this review can recapitulate many aspects of axonal and dendritic outgrowth that are observed *in vivo*, and alterations in neurite outgrowth are clearly related to CNS dysfunction in both animals and humans (Berger-Sweeney and Hohmann, 1997; Ramakers, 2002). The relevance of the *in vitro* models is supported by the available *in vivo* animal studies, although the data is limited. Out of the 35 chemicals listed in Table 2, references for changes in axonal or dendritic outgrowth after *in vivo* exposure were noted for only four. Changes in axonal or dendritic morphology have been observed after developmental exposure to the neurotoxicants lead (Alfano and Petit, 1982; Campbell et al., 1982; Reuhl et al., 1989), ethanol (Burrows et al., 1995; Granato and Van Pelt, 2003; Smith and Davies, 1990), cocaine (Harvey et al., 2001; Stanwood et al., 2001) and methyl mercury (Choi et al., 1981; Stoltenburg-Didinger and Markwort, 1990). While it would be desirable to increase the data set to better understand the relationship between chemical effects on neurite outgrowth *in vitro* and *in vivo*, a one-to-one correspondence may not be necessary in the context of chemical screening. The use of *in vitro* assays of developmental processes for screening and hazard identification is based on the premise that chemicals that have an effect *in vitro* have the potential to alter these processes *in vivo*. The application of *in vitro* data directly to risk assessment will require a more complete understanding of the mechanisms underlying the expression of toxicity *in vivo*, as well as the ability to extrapolate from the effective concentrations obtained *in vitro* to likely target tissue levels *in vivo*.

This review has provided an overview of the methods and models that have been used previously to assess chemical effects on neurite outgrowth, and suggests a number of directions for research that would facilitate the development of a protocol useful for chemical screening. Assays for neurite outgrowth will need to incorporate high-throughput technology. Recent advances in automated imaging platforms for HCS make the assessment of neuronal outgrowth a practical method for the rapid screening of large numbers of chemicals. The use of imaged-based assays, which monitor developmental processes at the cellular level, casts a wide net that can detect chemical-induced toxicity regardless of

the mechanism of action. In addition, automated image analysis systems can assess multiple endpoints of neurite outgrowth in the same cell, facilitating the determination of those endpoints that are most sensitive and predictive of a chemical effect.

A major roadblock to understanding which neuronal model may be most appropriate for screening is the fact that very few chemicals have been tested in multiple systems. Thus, in order to make meaningful comparisons between model systems, a standard set of chemicals should be tested in all models. This "reference chemical" set should include compounds known to inhibit neurite outgrowth, as well as compounds that are non-toxic. As many different chemical classes as possible should be included. In addition, the reference chemicals should be tested over a large concentration range in order to get a measure of the sensitivity of each model system. In order to demonstrate the dynamic range of neurite outgrowth, both positive and negative controls (e.g., differentiated cells and non-differentiated cells) should be included in every experiment.

Tables 1 and 3 show that chemical effects on neurite outgrowth have been assessed in at least 19 *in vitro* models, and many more are available. In the absence of testing the reference chemical set in every model, how do we select the most sensitive model system for screening? There are a number of desirable traits that should be considered. These include a model system that closely recapitulates the process of neurite outgrowth as observed *in vivo*, is widely available to the research community, and provides reproducible data between laboratories. Primary cells provide a model of outgrowth that closely corresponds to neurons *in situ*, but the need to continually prepare new cultures and the potential for variability make it unlikely that they will be widely used for screening. Primary neuronal cultures could be considered, however, as a standard with which to compare other, less complex model systems such as cell lines. Neuronal cell lines are widely available and have been used extensively to assess neurite outgrowth. Because neuronal cell lines can provide homogenous population of cells in large numbers, they are likely to be examined as a model for screening. If this is the case, the focus should be on cell lines of human origin. Preference could be given to immortalized cell lines that retain the properties of the neuronal cell of origin. The increasing availability of neural stem cells (including immortalized neural stem cell lines) will provide the opportunity to examine what may be the most relevant model system: self-renewing human neural progenitors that differentiate into fully functional cells almost identical to that found in the human nervous system. Efforts should be made to characterize neurite outgrowth in neural stem cell models, and determine their suitability for use in chemical screening.

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