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Use of high content image analyses to detect chemical-mediated effects on neurite sub-populations in primary rat cortical neurons

Joshua A. Harrill [a,*], Brian L. Robinette [b], Theresa Freudenrich [b], William R. Mundy [b]

[a] Institute for Chemical Safety Sciences, The Hamner Institutes for Health Sciences, Research Triangle Park, NC, United States
[b] Integrated Systems Toxicology Division, National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC, United States

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ABSTRACT

Traditional developmental neurotoxicity tests performed *in vivo* are costly, time-consuming and utilize a large number of animals. In order to address these inefficiencies, *in vitro* models of neuronal development have been used in a first tier screening approach for developmental neurotoxicity hazard identification. One commonly used endpoint for assessing developmental neurotoxicity *in vitro* is measurement of neurite outgrowth. This biological process is amenable to high-throughput measurement using high content imaging (HCI) based methodologies. To date, a majority of HCI studies of neurite outgrowth have focused on measurements of total neurite outgrowth without examining whether stereotypic neuronal growth patterns are disrupted or whether specific sub-populations of neurites (*i.e.* axons or dendrites) are selectively affected. The present study describes the development and implementation of two HCI based analysis methods for assessing chemical effects on neuronal maturation. These methods utilize the stereotypical growth pattern of primary rat cortical neurons in culture (*i.e.* the Staging Method), as well as the differential cytoplasmic distribution of β_{III}-tubulin and MAP2 (*i.e.* the Subtraction Method), to quantify inhibition of neurite initiation, axon outgrowth and secondary neurite (or dendrite) outgrowth in response to chemical exposure. Results demonstrate that these distinct maturational processes are differentially affected by pharmacological compounds (K252a, Na_3VO_4, Bis-1) known to inhibit neurite outgrowth. Furthermore, a group of known developmental neurotoxicants also differentially affected the growth of axons and secondary neurites in primary cortical culture. This work improves upon previous HCI methods by providing a means in which to rapidly and specifically quantify chemical effects on the growth of axons and dendrites *in vitro*.

1. Introduction

Developmental neurotoxicity occurs when chemical exposures in early pre- or postnatal life result in deficits in nervous system function that persist in later life stages. There is emerging evidence that the interaction of exposure to environmental chemicals and genetic susceptibility factors may play a role in the increased incidences of neurodevelopmental disorders observed in industrialized countries (Grandjean and Landrigan, 2006; Winneke, 2011). This underscores the need for efficient methods to identify and characterize potential developmental neurotoxicant (DNT) hazards. Regulatory guideline studies for developmental neurotoxicity are costly, time-consuming, labor-intensive and require the use of many test animals (Lein et al., 2007; OECD, 2007; USEPA, 1998). Given these constraints, and the tens of thousands of chemicals in commerce for which there is no toxicity data (Hartung, 2010), a number of recent publications have recommended the use of cell-based *in vitro* test systems for screening and characterization of potential developmental neurotoxicants (Coecke et al., 2007; Crofton et al., 2011; Lein et al., 2007). Data from *in vitro* developmental neurotoxicity screening batteries could then be used to prioritize chemicals of concern and inform targeted *in vivo* developmental neurotoxicity tests, consistent with the tiered toxicity testing design outlined in the Toxicity Testing in the 21st Century research strategy document (NRC, 2007).

Changes in neuronal morphology and synaptic connectivity have been associated with developmental neurotoxicity (Alfano and Petit, 1982; Choi et al., 1981; Geschwind and Levitt, 2007; Hutsler and Zhang, 2010; Lee, 2009; McPartland et al., 2011). Many *in vitro* studies have been conducted in which changes in the

* Corresponding author at: The Hamner Institutes for Health Sciences, 6 Davis Drive, PO Box 12137, Research Triangle Park, NC 27709, United States. Tel.: +1 919 558 1254.
E-mail address: JHarrill@thehamner.org (J.A. Harrill).

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morphology of primary neurons and neural cells lines have been used as an endpoint of developmental neurotoxicity following exposure to test chemicals (Radio and Mundy, 2008). Traditionally, these studies have been conducted in a low throughput manner using various combinations of immunocytochemistry, phase contrast, fluorescence and confocal microscopy and either manual or semi-automated image analysis methods. However, advances in the field of automated imaging and high content image analysis (HCI) have the potential to increase the throughput of *in vitro* studies of neuronal morphology. Combining this technology with neuronal cultures prepared in multiwell microtiter plate format allows for efficient, cost–effective, simultaneous evaluations of large numbers of chemicals for effects on the morphological development of neurons *in vitro*. Currently, the most common neurodevelopmental process examined using a high throughput approach is neurite outgrowth. A variety of *in vitro* test systems have been used in neurite outgrowth chemical screening studies including tumor derived neural cell lines (*i.e.* PC12 cells), rodent primary cerebellar granule cells and cortical neurons and human neural stem cell-derived neurons (Anderl et al., 2009; Dragunow, 2008; Harrill et al., 2010, 2011a; Radio et al., 2008, 2010).

While neuronal cell lines have been demonstrated to be suitable tools for high throughput chemical screening for neurite outgrowth inhibition (Harrill et al., 2011a; Radio et al., 2010), primary cultures are likely to be a more accurate *in vitro* model of the maturation of post-mitotic neurons within the brain *in vivo*. Primary cortical and hippocampal neurons from late embryonic or early postnatal tissue rapidly extend neurites when prepared in a dissociated culture and develop distinct axons and dendrites consistent with the morphology of neurons observed in the brain *in vivo* (Caceres et al., 1986; de Lima et al., 1997; Dotti et al., 1988; Kosik and Finch, 1987). Because chemical exposure can selectively alter the growth of axons and dendrites (Heidemann et al., 2001; Howard et al., 2005; Ohtani-Kaneko et al., 2008; Sanchez et al., 2008), primary neuronal cultures may provide a more appropriate *in vitro* model for use in investigating chemical effects on these distinct developmental events.

In vitro, primary cortical and hippocampal neurons follow a stereotypic pattern of neuronal maturation (de Lima et al., 1997; Dotti et al., 1988). A study by Dotti et al. (1988) characterized the growth pattern of hippocampal neurons in terms of developmental stages. Stage 1 neurons are rounded cells that have not yet extended neurites, but may have lammelipodial protrusions. Stage 2 neurons have developed one or more immature neurites which have not yet differentiated into either an axon or dendrite. The transition from Stage 2 to Stage 3 occurs when one of the immature neurites undergoes a rapid period of outgrowth, eventually becoming an axon. Similarly, the transition from Stage 3 to Stage 4 is denoted by the maturation of secondary neurites into more mature dendrites. Stage 5 denotes a mature neuron with synaptic connections. In early stages of neuronal culture (*i.e.* Stage 3), developing axons are easily recognizable as being many times longer than secondary neurites emerging from the same neuron. In more mature cultures (Stage 4 and Stage 5) axons and dendrites can also be distinguished morphologically. Axons are comparatively smaller in diameter and tend to have a more uniform diameter throughout the entire axon length. Dendrites are comparatively larger in diameter proximal to the cell body with a tapered (sometimes branched) morphology distal to the cell body. In addition, the cytoplasmic distribution of microtubule-associated proteins and neurofilaments can be used to distinguish axons from dendrites. For example, the localization of microtubule associated protein 2 (MAP2) is restricted to dendrites and the cell body of mature primary neurons. Likewise, the localization of the microtubule associated protein Tau is restricted to axons (Caceres et al., 1986; Dotti et al., 1987; Kosik and Finch, 1987).

Both stereotypic growth patterns (*i.e.* neuron staging) and the cytoplasmic localization of axon and dendrite marker proteins have been used to evaluate the effects of chemicals on different subpopulations of neurites *in vitro* (Heidemann et al., 2001; Howard et al., 2005; Ohtani-Kaneko et al., 2008; Sanchez et al., 2008). While informative, these types of studies are limited by the time-consuming tasks of manual image acquisition, manual neurite measurement, observer-biased sampling and by the low number of neurons and treatments which can be measured within a reasonable time frame. The goal of the present study was to refine previously published methods for high content image analysis (HCI) of neurite outgrowth in primary cultures. The utility of neuronal staging and fluorescent co-labeling methods in assessing chemical effects on the specific processes of axon and dendrite development is explored in conjunction with "standard" neurite outgrowth measurements. Two HCI methods were developed and compared. The first method (*e.g.* the Staging Method) automatically quantifies the percentage of Stage 1, Stage 2 and Stage 3 neurons in a relatively immature (1 DIV) low density culture. The second method (*e.g.* the Subtraction Method) utilizes the relative distribution of the neuronal markers β_{III}-tubulin and MAP2 to measure axon and dendrite lengths in higher-density, more complex cultures. The results show that high content imaging can be used to assess the effects of chemicals on axon and dendrite outgrowth in primary neurons cultured *in vitro*.

2. Methods

2.1. Materials

Bisbenzamide H 33258 fluorochrome (Hoechst stain), bisindolylmaleimide I (Bis-1), CalbioChem® Insolution™ K252a and anti-microtubule associated protein 2 (MAP2) mouse monoclonal antibody (AB3418) were purchased from EMD Millipore, Inc. (Bellerica, MA). Sodium orthovanadate (Na_3VO_4), dexamethasone, *t*-retinoic acid, cadmium chloride ($CdCl_2$), methylmercury chloride (MeHg), dimethyl sulfoxide (DMSO) and poly-D-lysine were purchased from Sigma–Aldrich, Inc. (St Louis, MO). Anti-β_{III} tubulin rabbit polyclonal antibody (PRB-435P) and anti-pan axonal neurofilament (pNF) mouse monoclonal antibody (SMI-312R) were purchased from Convance, Inc. (Princeton, NJ). Invitrogen™ Alexa Fluor®-conjugated goat anti-mouse IgG and goat-anti-rabbit IgG secondary antibodies were purchased from Life Technologies, Inc. (Grand Island, NY). Polystyrene 96-well tissue culture plates were purchased from Corning, Inc. (Corning, NY). MicroAmp™ optical adhesive film was purchased from Applied Biosystems, Inc. (Foster City, CA).

2.2. Cell culture

Pregnant Long–Evans rat dams (Charles River Inc., Wilmington, MA) were received and housed in polycarbonate hanging cages containing heat-sterilized wood chips for 6 days prior to parturition. Dams were maintained on a 12 h: 12 h (light:dark) photoperiod with free access to food and water. The animal facility was approved by the American Association for Accreditation of Laboratory Animal Care (AAALAC) and all animal care procedures were approved in advance by the US EPA, NHEERL Animal Care and Use Committee. Cultures of primary cortical neurons were prepared from post-natal day 0 (PND0) Long–Evans rat pups as described (Harrill et al., 2011b). Briefly, cortical cells were dissociated and resuspended in DMEM containing (in mM): GlutaMAX™-I (2), D-glucose (25), sodium pyruvate (1), HEPES (10), plus penicillin (100 U/mL), streptomycin (0.1 mg/mL) and 10% HyClone horse serum. Cultures were plated in Corning® 96-well polystyrene cell culture plates coated with poly-L-lysine. Cells