Diverse neurotoxicants target the differentiation of embryonic neural stem cells into neuronal and glial phenotypes

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ABSTRACT

The large number of compounds that needs to be tested for developmental neurotoxicity drives the need to establish in vitro models to evaluate specific neurotoxic endpoints. We used neural stem cells derived from rat neuroepithelium on embryonic day 14 to evaluate the impact of diverse toxicants on their ability to differentiate into glia and neurons: a glucocorticoid (dexamethasone), organophosphate insecticides (chlorpyrifos, diazinon, parathion), insecticides targeting the GABA$_A$ receptor (dieldrin, fipronil), heavy metals (Ni^{2+}, Ag^+), nicotine and tobacco smoke extract. We found three broad groupings of effects. One diverse set of compounds, dexamethasone, the organophosphate pesticides, Ni^{2+} and nicotine, suppressed expression of the glial phenotype while having little or no effect on the neuronal phenotype. The second pattern was restricted to the pesticides acting on GABA$_A$ receptors. These compounds promoted the glial phenotype and suppressed the neuronal phenotype. Notably, the actions of compounds eliciting either of these differentiation patterns were clearly unrelated to deficits in cell numbers: dexamethasone, dieldrin and fipronil all reduced cell numbers, whereas organophosphates and Ni^{2+} had no effect. The third pattern, shared by Ag^+ and tobacco smoke extract, clearly delineated cytotoxicity, characterized by major cell loss with suppression of differentiation into both glial and neuronal phenotypes; but here again, there was some selectivity in that glia were suppressed more than neurons. Our results, from this survey with diverse compounds, point to convergence of neurotoxicant effects on a specific "decision node" that controls the emergence of neurons and glia from neural stem cells.

1. Introduction

The explosive rise in the incidence of neurodevelopmental disorders, termed a "silent pandemic," is due in large measure to the widespread increase in environmental exposures to neurotoxic chemicals (Grandjean and Landrigan, 2006, 2014). There are tens of thousands of chemicals in common industrial use, of which up to 25% are estimated to be neurotoxic (Boyes, 2001). However, few have been sufficiently tested for developmental neurotoxicity. It is not feasible to assess the potential of this huge number of compounds to disrupt nervous system development using classical in vivo approaches, reinforcing the need to develop higher-throughput in vitro systems to facilitate such evaluations. Currently, the most common endpoints studied for in vitro models typically relate to factors leading to deficits in the number of neurons: antimitotic activity, apoptosis and cytotoxicity (Bagchi et al., 1995; Culbreth et al., 2012; Druwe et al., 2015; Slotkin et al., 2007; Slotkin and Seidler, 2012). These outcomes readily lend themselves to high-throughput screening (Druwe et al., 2015). However, progress must be made to model more complex cellular processes than simple survival and proliferation. There are more subtle effects of neurotoxicants that are likely to be important at lower exposures that are not cytotoxic. As one prominent example, neuronotypic PC12 cells can be used to evaluate adverse effects on

neurite extension (Radio et al., 2008; Song et al., 1998) and differentiation into specific neurotransmitter phenotypes (Jameson et al., 2006b; Slotkin, 2005; Slotkin et al., 2007). For this reason, different cell types are needed in order to model the impact of chemicals on the various processes that control neural cell development (Coecke et al., 2007; Costa, 1998; van Thriel et al., 2012).

Our earlier work with the PC12 model showed how many of the adverse effects of organophosphate pesticides on brain development in vivo are exerted directly on neurodifferentiation at the cellular level, leading to impaired neurite formation and diversion of differentiation away from the acetylcholine phenotype and toward the dopamine phenotype (Jameson et al., 2006b; Slotkin, 2005; Slotkin et al., 2007). Additionally, we found that there are common endpoints for otherwise unrelated neurotoxicants, potentially explaining why diverse compounds can produce similar neurodevelopmental outcomes. In addition to the organophosphates, these included glucocorticoids (dexamethasone), insecticides targeting the GABA$_A$ receptor (dieldrin, fipronil), heavy metals (divalent nickel, monovalent silver), nicotine and tobacco smoke extract (TSE); all were antimitotic in undifferentiated PC12 cells and diverted the neurotransmitter phenotype in differentiating cells (Jameson et al., 2006a; Lassiter et al., 2009; Slotkin et al., 2007, 2014). These studies indicated that, rather than just classifying toxicants by chemical class (e.g. organophosphates), or by a single mechanism of systemic toxicity (e.g. acetylcholinesterase inhibition), it may be useful to group the toxicants by their common downstream effects on complex cellular processes ("mode of action").

Nevertheless, there are two distinct limitations of the PC12 model: it is a transformed cell line, and it is already committed to a neuronal phenotype. Accordingly, the current study addresses the issue of whether the ability of diverse neurotoxicants to redirect neurodifferentiation extends to earlier stages of development, at the point where neural stem cells (NSCs) have the choice to become neurons or glia, and whether such diversion occurs in nontransformed cells. We used NSCs derived from rat neuroepithelium on embryonic day 14, a stage in brain development where the phenotypic derivation of neurons and glia is first determined (Dotan et al., 2010; Rodier, 1988), and focused on the ability of otherwise unrelated neurotoxicants to alter differentiation fate. Most studies of developmental neurotoxicity focus on neurons, but glia are also a target for toxicant effects (Aschner, 2000; Aschner et al., 1999; Garcia et al., 2005). Glia provide critical nutritional, structural and homeostatic support that are essential to architectural modeling of the brain and to the establishment and maintenance of synaptic function (Aschner et al., 1999); glia provide antioxidant activity that protects neurons from toxicants (Sagara et al., 1993), a potentially important factor in protection from organophosphates (Garcia et al., 2001; Pizzurro et al., 2014) or glucocorticoids (Chou, 1998). For neurotoxicity in the adult, effects on the glia/neuron ratio can be used as an index of neurotoxicity (O'Callaghan, 1983, 1988). Our studies were modeled on our previous work with PC12 cells, encompassing the same diverse classes of neurotoxicants, focusing on their ability to alter differentiation fate in embryonic NSCs, and specifically distinguishing whether diversion of phenotype is independent of the ability of the agents to affect cell numbers.

2. Materials and methods

2.1. Cell cultures and treatments

Primary neural stem cells (passage zero) were isolated from rat cortical neuroepithelium on embryonic day 14 and were shipped frozen in DMEM/F-12 medium with N2 supplement (see Materials, below) and 10% dimethylsulfoxide (DMSO). Cells were thawed and plated at 35,000 cells/cm^2 on 12 mm coverslips pre-coated with poly-L-ornithine, contained in 24-well culture plates. The culture medium consisted of DMEM/F-12, GlutaMAX^{TM} with N2 Supplement, 20 ng/ml human fibroblast growth factor and 20 ng/ml epidermal growth factor. Cultures were maintained in a humidified incubator at 37 °C with 5% CO$_2$. Twenty four hours later, the medium was changed to initiate spontaneous differentiation by eliminating the two growth factors, with the addition of 200 μM ascorbic acid and the test compounds. Due to the limited water solubility of chlorpyrifos, diazinon, parathion, dieldrin, fipronil and TSE, they were dissolved in DMSO to achieve a final vehicle concentration in the medium of 0.05%, or in the case of the high TSE concentration (see Results), 0.2%. We performed preliminary studies with those concentrations of DMSO and found no statistically significant changes in any of the parameters, although the higher DMSO concentration tended ($0.05 < p < 0.1$) to decrease the percentage of cells differentiating into neurons (data not shown); likewise, although we did not use it in the ensuing studies, we evaluated 0.1% methanol as an alternative vehicle for lipophilic substances, and found no adverse effects. For experiments with agents dissolved in DMSO, the vehicle was also added to the controls at the appropriate concentration. Exposures were conducted for a period of 3 or 6 days as indicated; after 3 days, half the medium was replaced, including the indicated treatment agents.

At the end of the exposure period, the medium was removed and the coverslips washed with Dulbecco's phosphate-buffered saline, fixed with 4% paraformaldehyde and washed three times with Dulbecco's phosphate-buffered saline containing additional Ca^{2+} and Mg^{2+} (see Materials, below). Cells were permeabilized for 30 min in phosphate buffered saline containing 0.2% Triton X-100, washed three times with phosphate buffered saline (without Triton), followed by a 30 min incubation in BlockAid^{TM} solution. Cells expressing a neuronal or astroglial phenotype were identified by immunocytochemistry according to manufacturers' instructions, using microtubule-associated protein 2 (MAP2) for neurons and glial fibrillary acidic protein (GFAP) for astroglia. After permeabilization, the coverslips were incubated for 1 h at room temperature using rabbit anti-MAP2 (1:200) and rat anti-GFAP (1:20) in BlockAid^{TM}. Coverslips were rinsed four times with phosphate-buffered saline and then incubated for 1 h at room temperature with the appropriate fluor-conjugated secondary antibodies (donkey anti-rabbit IgG Alexa Fluor 647 and goat anti-rat IgG Alexa Fluor 555) diluted 1:400 in BlockAid^{TM}. After an additional five rinses with phosphate buffered saline, coverslips were incubated for 5 min with 300 nM DAPI nucleic acid stain to label individual cells. Coverslips were rinsed three times with phosphate buffered saline and mounted onto glass slides using ProLong Diamond Antifade mountant.

Images of 3–4 fields/slide (each field = 3.22×10^5 μm^2) were captured using a Zeiss Axio Imager widefield fluorescence microscope with $200\times$ magnification and quantified for total cells (DAPI-positive stain for nuclei); across the multiple fields in a given culture, thousands of cells were counted. Each cell was then examined to see if it expressed a neuronal phenotype (MAP2-positive) or a glial phenotype (GFAP-positive). A cell was counted as positive only when the stain for a given phenotype coincided with a DAPI-stained nucleus. Values were averaged across the fields to render a single value for each culture. Representative images are shown in Fig. 1.

We did not evaluate the third potential differentiation phenotype, oligodendrocytes, because they represent a much smaller proportion than neurons or glia when NSCs are derived at this early stage of embryonic brain development (Dotan et al., 2010).