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Defining toxicological tipping points in neuronal network development☆

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ABSTRACT

Measuring electrical activity of neural networks by microelectrode array (MEA) has recently shown promise for screening level assessments of chemical toxicity on network development and function. Important aspects of interneuronal communication can be quantified from a single MEA recording, including individual firing rates, coordinated bursting, and measures of network synchrony, providing rich datasets to evaluate chemical effects. Further, multiple recordings can be made from the same network, including during the formation of these networks *in vitro*. The ability to perform multiple recording sessions over the *in vitro* development of network activity may provide further insight into developmental effects of neurotoxicants. In the current study, a recently described MEA-based screen of 86 compounds in primary rat cortical cultures over 12 days *in vitro* was revisited to establish a framework that integrates all available primary measures of electrical activity from MEA recordings into a composite metric for deviation from normal activity (total scalar perturbation). Examining scalar perturbations over time and increasing concentration of compound allowed for definition of critical concentrations or "tipping points" at which the neural networks switched from recovery to non-recovery trajectories for 42 compounds. These tipping point concentrations occurred at predominantly lower concentrations than those causing overt cell viability loss or disrupting individual network parameters, suggesting tipping points may be a more sensitive measure of network functional loss. Comparing tipping points for six compounds with plasma concentrations known to cause developmental neurotoxicity *in vivo* demonstrated strong concordance and suggests there is potential for using tipping points for chemical prioritization.

1. Introduction

The lack of information on the potential for tens of thousands of chemicals in the environment to cause developmental neurotoxicity (DNT) is well documented (Grandjean and Landrigan, 2006, 2014). Because guideline DNT studies (US EPA, 1998; OECD, 2007) are expensive, time-consuming and animal intensive (Smirnova et al., 2014), it is now generally recognized that it will be necessary to use a battery of *in vitro* screening assays (Bal-Price et al., 2010, 2015; Fritsche et al., 2017) to screen thousands of compounds for their potential to cause DNT and thus prioritize them for additional testing. To this end, a wide variety of assays have been developed to determine the effects of compounds on processes critical to the development of the nervous system, including proliferation (Breier et al., 2008), neurite outgrowth (Radio and Mundy, 2008; Harrill et al., 2010; Stiegler et al., 2011), synaptogenesis (Harrill et al., 2011), transcriptomic responses (Krug et al., 2013), differentiation (Schmuck et al., 2017), and migration (Zimmer et al., 2014).

Formation of interconnected neural networks is critical to development of the nervous system. *In vivo* formation of networks occurs as the structures of the nervous system develop, and connections between different neurons form as axons and dendrites extend using chemical and electrical cues as guidance. Similar *in vitro* network activity arises spontaneously in primary monolayer cultures from many different brain regions, including the cortex, hippocampus, and brainstem as dissociated neurons (re)establish synaptic connections. Both in the brain and in primary cultures, this spontaneous electrical activity becomes patterned and coordinated over time, with synchronous bursting

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observed. *In vivo* synchronous bursting is associated with important nervous system processes including attention, learning, and memory (Buschman and Kastner, 2015; Korte and Schmitz, 2016; Salinas and Sejnowski, 2001). *In vitro*, such activity can be observed in networks of developing neurons using microelectrode array (MEA) recording techniques (Chiappalone et al., 2003; Van Pelt et al., 2004; Wagenaar et al., 2006; Cotterill et al., 2016; Brown et al., 2016). MEAs measure extracellular electrical activity associated with the generation and propagation of action potentials (spikes). Over time, patterned activity in the form of groups of spikes (bursts) from the same neuron, as well as coordinated bursting of multiple neurons across a network are readily observed. Measuring chemical effects on the development of spontaneous network activity has been proposed as a screening assay for DNT (Hogberg et al., 2011; Robinette et al., 2011; Dingemans et al., 2016), and our laboratory recently demonstrated that by using multi-well MEAs (mwMEAs) a medium-throughput assay is feasible (Brown et al., 2016; Frank et al., 2017).

Our laboratory recently reported results using this medium-throughput mwMEA-based DNT assay for 86 compounds, demonstrating that 49 of 60 (82%) compounds with evidence of developmental neurotoxicity in the published literature altered development of at least one network parameter (Frank et al., 2017). These data also were used to develop estimates of potency (effective concentration that caused a 50% change; EC_{50} value) for those compounds that altered network development (64/86). Although such information is useful for prioritization for additional testing, relating *in vitro* perturbations to *in vivo* adversity is a key challenge for 21st century toxicology (Keller et al., 2012). To address this issue, Shah et al. (2016) developed a novel systems toxicology approach to identify toxicological "tipping points" between adaptation and adversity.

Toxicological "tipping points" are defined by Shah et al. as dose-dependent transitions in cells based on their inability to recover to normal (or basal) functions. To analyze tipping points they examined data on the effects of almost a thousand ToxCast chemicals in HepG2 cells using multiple high-content imaging (HCI) endpoints over time (0, 1, 24 and 72 h). They translated the temporal response of HepG2 cells to each chemical treatment as cell-state trajectories. Interestingly, all chemical effects on cells could be classified into three main groups: no effect, recovering and nonrecovering trajectories. Recovering trajectories were characterized by a return of cells to their normal (or basal) states. On the other hand, nonrecovering trajectories were assumed to be indicative of a loss of cellular homeostatic capacity. They defined the transition from recovering to nonrecovering trajectories as the tipping point, and the corresponding chemical concentration as the critical concentration. This approach for identifying tipping points developed by Shah et al. is quite general and can be readily applied to other *in vitro* models that involve temporal measurements.

In the present study, we used the data from Frank et al., 2017, to determine tipping points for chemical effects on *in vitro* neural network formation, and to estimate the corresponding critical chemical concentrations. The results indicate that by consideration of a unified framework for network formation, critical concentrations can be determined for many compounds that altered neural network development, and that in many cases, these concentrations are lower than the EC_{50} values for individual parameters describing neural network function or the composite values for effects on all parameters. Further, using high-throughput toxicokinetic estimates, we demonstrate that the tipping point concentrations for a small subset of chemicals are concordant with plasma concentrations *in vivo* that are associated with developmental neurotoxicity. The results indicate that such an analysis can provide a robust and sensitive determination of the point at which compounds begin to alter neural network development *in vitro*.

2. Methods

2.1. Compounds and methods

The present study represents additional analysis of the data presented in Frank et al., 2017. Briefly, in that study, concentration-dependent (typically 0.03 to 30 μM) effects of 86 compounds on neural network development were measured in primary cortical cultures prepared from day old rat pups using mwMEA recordings across 4 time points (Days *in vitro* (DIV) 5, 7, 9 and 12). Sixty of the tested compounds had at least limited literature evidence of DNT effects in humans or animal models (Mundy et al., 2015), 4 additional compounds had been previously tested in this assay (Brown et al., 2016) and found to impact network function (bisindolylmaleimide I, loperamide, mevastatin, and sodium orthovanadate), and 21 compounds were classified as unknowns with insufficient evidence for DNT effects in the published literature. Acetaminophen was used as a negative control. Cultures were plated at 150,000 cells/well in 48 well microelectrode array plates from Axion Biosystems (M768-KAP-48) using standardized culture and plating protocols (Cotterill et al., 2016; Brown et al., 2016), and chemical treatment commenced 2 h after plating of the cells and continued throughout the experiment. Chemicals were renewed when media was refreshed on DIV 5, and 9. As described in complete detail in Brown et al., 2016, electrical activity in the cultures was recorded for 15 min on DIV 5, 7, 9 and 12 using an Axion Maestro amplifier, Middleman A/D conversion, and AXiS 1.9 or later software. Axion raw (*.raw) and spike (*.spk) files were saved to a redundant physical hard drive for later analysis. Culture viability was assessed following the final DIV12 recording using total LDH release and CellTiter-Blue assays as described in Frank et al., 2017.

2.2. Initial data processing and normalization

Axion spikelist files with time-stamped spikes were combined with comma-separated plate layout data for conversion to hierarchical data format (.h5) files. Hierarchical data format files were used as input to the 'meadq' (https://github.com/dianaransomhall/meadq) and 'sjemea' (https://github.com/sje30/sjemea) R packages, which together generated a set of 16 network measures per well that were used for downstream analyses with date and plate ID tracking information attached. An additional network parameter, normalized mutual information (Ball et al., 2017), a measure of shared information in the network, was computed for each recording separately. Nine of the 17 network parameters (mean firing rate (MFR), number of active electrodes (#AE), burst rate (BR), number of actively bursting electrodes (#ABE), percent of spikes in bursts (%SiB), number of network spikes (#NS), percent of spikes in network spikes (%SiNS), Pearson's correlation (r) between activity on electrodes, and normalized mutual information (MI)) were selected for incorporation into the critical concentration analysis.

To reduce the impact of any batch effects, raw network parameter values were normalized by dividing by the median of untreated control well values located on the same plate and on the same DIV (5, 7, 9, or 12). If the median of same-plate untreated control wells was zero, all values for that parameter on that plate were set to 1 (no change from controls). The fold-change from control median values were then log-transformed to reduce the impact of outlier values. The mean and standard deviation of log-transformed fold change values of all untreated control wells on the same DIV were then used to z-score scale all fold-change values. This resulted in values centered at zero that indicate the number standard deviations away from the untreated control mean (positive or negative).

2.3. Tipping point analysis

We used the methods described previously (Shah et al., 2016) to identify tipping points by calculating the following four quantities from the data: system perturbation (X), total scalar perturbation ($|X|$), velocity (V) and derivative of the velocity with respect to concentration (∂V_c). A conceptual overview of these quantities is provided in Fig. 1. The scripts used for the analysis of tipping points are available at the