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Multi-well microelectrode array recordings detect neuroactivity of ToxCast compounds☆

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ABSTRACT

Spontaneous activity in neuronal cultures on microelectrode arrays (MEAs) is sensitive to effects of drugs, chemicals, and particles. Multi-well MEA (mwMEA) systems have increased throughput of MEAs, enabling their use for chemical screening. The present experiments examined a subset of EPA's ToxCast compounds for effects on spontaneous neuronal activity in primary cortical cultures using 48-well MEA plates. A first cohort of 68 compounds was selected from the ToxCast Phase I and II libraries; 37 were positive in one or more of 20 individual ToxCast Novascreen assays related to ion channels (NVS_IC), with the remainder selected based on known neuroactivity. A second cohort of 25 compounds was then tested with 20 originating from the ToxCast Phase I and II libraries (not hits in NVS_IC assays) and 5 known negatives from commercial vendors. Baseline activity (1 h) was recorded prior to exposing the networks to compounds for 1 h, and the weighted mean firing rate (wMFR) was determined in the absence and presence of each compound. Compounds that altered activity by greater than the weighted change of DMSO-treated wells plus 2SD were considered "hits". Of the first set of 68 compounds, 54 altered wMFR by more than the threshold, while in the second set, 13/25 compounds were hits. MEAs detected 30 of 37 (81.1%) compounds that were hits in NVS_IC assays, as well as detected known neurotoxicants that were negative in NVS_IC assays, primarily pyrethroids and $GABA_A$ receptor antagonists. Conversely, wMFR of cortical neuronal networks on MEAs was insensitive to nicotinic compounds, as only one neonicotinoid was detected by MEAs; this accounts for the bulk of non-concordant compounds between MEA and NVS_IC assays. These data demonstrate that mwMEAs can be used to screen chemicals efficiently for potential neurotoxicity, and that the results are concordant with predictions from ToxCast NVS_IC assays for interactions with ion channels.

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1. Introduction

Since the release of the National Academy's report entitled *Toxicity Testing the the 21st Century* (NRC, 2007), there has been an increased emphasis on the development of high-throughput/high content screens for the purpose of predicting the toxicity of chemicals and/or screening and prioritization of chemicals for subsequent testing. Adverse outcome pathways (AOPs) are a concept central to the use of data from in vitro screens to predict toxicity (Ankley et al., 2010; Watanabe et al., 2011), and describe scientific evidence to support the sequence of events linking a molecular initiating event (MIE) to the adverse outcome in vivo. Toxicity pathways are a subset of AOPs and describe the sequence of normal physiological responses from the MIE to the alterations in tissue function, that when sufficiently perturbed by chemicals, will lead to adverse responses (NRC, 2007).

The ToxCast program at the U.S. Environmental Protection Agency (Dix et al., 2007; Judson et al., 2010) includes ~500 in vitro assays used to profile the bioactivity of environmental

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compounds. Assays in the ToxCast suite include those for nuclear receptors, G protein-coupled receptors and ion channels, among others (Judson et al., 2010) and are potential MIEs that may lead to carcinogenesis, endocrine disruption, heptatic, renal, neuro- or other toxicities. Although previous investigations have begun to build putative AOPs related to developmental toxicity (Chandler et al., 2011; Sipes et al., 2011; Kleinstreuer et al., 2011a), vascular development and (Kleinstreuer et al., 2011b, 2013) and endocrine disruption (Rotroff et al., 2013), other potential toxicity pathways linking ToxCast endpoints to adverse outcomes have yet to be investigated, including those linked to neurotoxicity.

Adverse outcome pathways linked to neurotoxicity have not yet been formally described, with the exception of excitotoxicity linked to kainate-type glutamate receptors (Watanabe et al., 2011). However, it is well established that disruption of ion channel function can be an initiating event that leads to neurotoxicity following exposure to a broad variety of compounds (Coats, 1990; Narahashi, 2002; Tomizawa and Casida, 2005). Disruption of ion channel function by chemicals including insecticides such as the pyrethroids, organochlorines, and other chemical classes often leads to alterations in cellular excitability that ultimately result in perturbation of the functioning networks and pathways critical to nervous system homeostasis, resulting in neurotoxic signs and symptoms.

The ToxCast assay suite contains twenty binding assays (Novascreen (NVS_IC) assays) that measure the interaction of compounds with different ion channels. However, interference with ligand binding does not always equate to disruption of channel function, and the type of disruption produced (agonist, antagonist, modulator) cannot always be determined on the basis of binding alone. Thus, prediction of toxicity solely on the basis of binding results is unreliable (Lü and An, 2008). By contrast, rapid and efficient assays that allow assessment of ion channel function also have limitations (Lü and An, 2008). For example, high-throughput patch-clamp assays often require that ion channel subunits be expressed in non-neuronal cell models. This may lead to differences in responsiveness compared to native neurons and for toxicity screening where the target is unknown, is inefficient because multiple ion channels must be individually examined. Such approaches also do not allow for the measurement of activity in functionally connected networks of neurons, a prerequisite for nervous system function.

Neuronal networks grown on microelectrode arrays (MEAs) have been proposed as a suitable approach for neurotoxicity screening that is sensitive to different classes of compounds (Johnstone et al., 2010), reproducible across laboratories (Novellino et al., 2011) and provides high sensitivity (correct identification of known positive compounds) and specificity (correct identification/rejection of known negative compounds) for training sets containing 20–30 compounds (DeFranchi et al., 2011; McConnell et al., 2012). Recently, it has been demonstrated that mwMEAs can provide sufficient throughput for compound screening while retaining the other desirable qualities of single well MEAs (McConnell et al., 2012). Thus, neuronal networks grown on mwMEAs provide a means to evaluate further the potential neurotoxicity of ToxCast compounds. However, previously tested training sets (DeFranchi et al., 2011; McConnell et al., 2012) have been limited in the compound space that was examined, as well as in the number of compounds from a particular class of compounds that were represented. Furthermore, these previous studies have not used compounds for which there are other screening data available, nor did they attempt to place results into the context of adverse outcome or toxicity pathways. Finally, while networks of cortical neurons grown on MEAs are sensitive to many classes of compounds, these initial studies (DeFranchi et al., 2011; McConnell et al., 2012) indicated that changes in firing rate of the network may be insensitive to compounds acting via the nicotinic acetylcholine receptor (nAChR). Thus, the "fit-for-purpose" of the assay needs further investigation in this regard.

The present studies were designed to test subsets of compounds from the ToxCast phase I and II inventories in primary cultures of cortical neuronal networks grown on mwMEAs. The compounds tested were selected based on their activity (or lack thereof) in NVS_IC ion channel assays in ToxCast, or their known neurotoxicity in vivo. This allowed for the testing of two separate but related hypotheses: (1) compounds testing positive in NVS_IC ion channel assays will alter network mean firing rate; and (2) compounds that alter firing rate will be neuroactive or neurotoxic in vivo. Because the NVS_IC assays consider only a subset of all potential MIEs that could alter network firing rate, it is not reasonable to expect that compounds that are negative in these assays will necessarily be without effects on network firing rate measured using MEAs. In total, 92 unique compounds were examined using MEAs (saccharin was included in both cohorts), including 6 inhibitors of acetylcholinesterase (AChE), 10 compounds active on $GABA_A$ receptors, 9 compounds that alter voltage-gated sodium channel (VGSC) function and 7 compounds known to act on nicotinic acetylcholine receptors. This dataset will provide additional information on the "fit-for-purpose" of cortical neuronal networks as a screening tool, and in combination with the ToxCast data, allow for the proposal of putative toxicity pathways that may contribute to neurotoxicity.

2. Materials and methods

2.1. Compounds

Eighty-eight ToxCast compounds (the five negatives in the second cohort were not from ToxCast) were evaluated in two separate cohorts. The first cohort contained 68 ToxCast compounds and included two negative control compounds: acetaminophen and saccharin (McConnell et al., 2012) without activity in vitro or in vivo. In this cohort, compounds were selected on the basis of (1) known neurotoxicity/activity (based on the authors' review of the literature) and/or (2) positive result in one of 20 Novascreen (NVS_IC) assays in ToxCast. The second cohort contained 20 ToxCast compounds as well as five additional negative controls: amoxicillin, glyphosate, saccharin, salicylic acid, and sorbitol. The saccharin used in the first cohort was sourced from ToxCast while in the second it was purchased directly from Sigma. The second cohort selected compounds that were not positive in ToxCast NVS_IC assays but were active in other ToxCast Assays. Additionally, some compounds in this set were selected because they belonged to the same class as compounds in the first cohort (e.g. conazoles and tetramethrin), which were active in MEAs in the first set. Compound name, CAS #, class, target and action (when known), NVS_IC hit count (the number of NVS_IC assays with a positive result), and molecular initiating event are listed in Table 1. The first cohort included several "failed" pharmaceutical compounds for which limited information is available; these are referred to as "pharma 1–6" in the present manuscript. The 88 ToxCast compounds were received in as 20 mM (unless otherwise noted in Table 1) stock solutions in dimethyl sulfoxide (DMSO). The five additional negative compounds included in the second set were obtained from Sigma (St. Louis, MO) and prepared as 50 mM stock solutions in DMSO (glyphosate was prepared in H_2O). DMSO (Sigma), pancreatin (Sigma), and EtOH (Pharmaco-Aapr, Brookfield, CT) were obtained from commercial vendors.