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Research Article

Evaluation of Chemical Effects on Network Formation in Cortical Neurons Grown on Microelectrode Arrays

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ABSTRACT

Thousands of chemicals to which humans are potentially exposed have not been evaluated for potential developmental neurotoxicity (DNT), driving efforts to develop a battery of *in vitro* screening approaches for DNT hazard. Here, 136 unique chemicals were evaluated for potential DNT hazard using a network formation assay (NFA) in cortical cells grown on microelectrode arrays. The effects of chemical exposure from 2 h postplating through 12 days *in vitro* (DIV) on network formation were evaluated at DIV 5, 7, 9, and 12, with cell viability assessed at DIV 12. Only 82 chemicals altered at least 1 network development parameter. Assay results were reproducible; 10 chemicals tested as biological replicates yielded qualitative results that were 100% concordant, with consistent potency values. Toxicological tipping points were determined for 58 chemicals and were similar to or lower than the lowest 50% effect concentrations (EC_{50}) for all parameters. When EC_{50} and tipping point values from the NFA were compared to the range of potencies observed in ToxCast assays, the NFA EC_{50} values were less than the lower quartile for ToxCast assay potencies for a subset of chemicals, many of which are acutely neurotoxic *in vivo*. For 13 chemicals with available *in vivo* DNT data, estimated administered equivalent doses based on NFA results were similar to or lower than administered doses *in vivo*. Collectively, these results indicate that the NFA is sensitive to chemicals acting on nervous system function and will be a valuable contribution to an *in vitro* DNT screening battery.

Key words: developmental neurotoxicity; screening; microelectrode array; neural network formation.

It is now well recognized that humans are exposed to thousands of chemicals that have not been fully characterized for their toxicity (Judson *et al.*, 2009), including developmental neurotoxicity (DNT; Makris *et al.*, 2009). Because animal-based guideline studies are time-consuming, expensive, and utilize large number of animals (Crofton *et al.*, 2012), they cannot provide the data needed to evaluate the potential hazards of thousands of chemicals, including DNT hazard. To address this gap in knowledge, considerable effort has been made over the last decade to develop low-cost, higher throughput *in vitro* assays to test chemicals for the potential to cause DNT. As a result, assays have now been developed for many processes that are critical to nervous system development (Coecke *et al.*, 2007), including expression of neurodevelopmentally-regulated genes (Hogberg *et al.*, 2009, 2010; Krug *et al.*, 2013), neuroprogenitor cell proliferation (Baumann *et al.*, 2016; Breier *et al.*, 2008), differentiation (Hayess *et al.*, 2013; Zimmer *et al.*, 2011), migration (Zimmer *et al.*, 2014), apoptosis (Culbreth *et al.*, 2012; Druwe *et al.*, 2015),

neurite outgrowth (Harrill *et al.*, 2013; Radio *et al.*, 2008; Stiegler *et al.*, 2011), synaptogenesis (Harrill *et al.*, 2011), and neural network formation (Brown *et al.*, 2016; Frank *et al.*, 2017; Hogberg *et al.*, 2011). Ultimately, these assays will form the basis of an *in vitro* screening battery (Fritsche *et al.*, 2018) and contribute to integrated approaches to testing and assessment (IATAs) for DNT (Bal-Price *et al.*, 2018) that will facilitate decision-making for DNT risk.

Assessment of chemical effects on neural network formation using MEAs is a critical component of a DNT assay battery (Fritsche *et al.*, 2018) because it measures integrated neural function, whereas other assays in the proposed battery rely on structural or biochemical measurements. Previous studies in single well systems demonstrated that this approach is feasible (Hogberg *et al.*, 2011; Robinette *et al.*, 2011) and can be scaled up to higher throughput (48 well) formats (Brown *et al.*, 2016). Furthermore, the MEA-based network formation assay (NFA) positively identified over 70% of DNT chemicals (Frank *et al.*, 2017) in a set of 60 chemicals known to cause DNT in mammals (Mundy *et al.*, 2015). Recently, an assessment of the readiness of assays for DNT testing identified NFAs on MEAs as one of a set of assays that was ready for use (Bal-Price *et al.*, 2018). Because the NFA measures effects over a period of days, data from this assay can also be used to determine toxicological "tipping points" in network development that appear to be relevant to *in vivo* exposures that are associated with DNT in mammals (Frank *et al.*, 2018).

Although several *in vitro* DNT assays have been established and small sets of chemicals have been tested in each of these, further work needs to be done to understand the utility of these assays. Testing of additional chemicals and comparing results across assays is critical to understanding further the added value of various *in vitro* assays. As such, the goal of the current study was to expand the number of chemicals that have been tested in the NFA, with the ultimate goal of comparing results across different assays. To that end, two sources were utilized to select a larger set of test chemicals.

Ninety-six chemicals were selected from the U.S. Environmental Protection Agency's Toxicity Forecaster (ToxCast) chemical library (Kavlock *et al.*, 2012; Richard *et al.*, 2016). The ToxCast program includes over 1000 assays covering a wide range of cellular responses and mapped to approximately 300 signaling pathways [https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data, Accessed March 18, 2019]. Currently, over 3800 unique chemicals have or are undergoing screening; the most complete assay coverage, to date, is available for approximately 1100 chemicals tested in the earliest phases of the program. Chemical categories represented within this approximately 1100 chemical portion of phase II test set include pesticides, pharmaceuticals, antimicrobials, flame retardants, food-additives, and "green" chemicals, among others. The second source of chemicals tested in the current study was provided by the National Toxicology Program (NTP) at the National Institutes for Environmental Health Sciences (NIEHS). The entire set consists of 91 chemicals ("NTP 91") and has been distributed to several commercial and academic institutions for testing using *in vitro* assays, including assays for events critical to neurodevelopment. To date, data on effects of these chemicals has been reported from other assays that may comprise an *in vitro* DNT assay battery, including neurite outgrowth (Ryan *et al.*, 2016), neural crest cell migration (Nyffeler *et al.*, 2017), and neuroprogenitor cell differentiation (Delp *et al.*, 2018). Thus, testing of the library chemicals in the NFA will allow for comparisons between assays that comprise a potential DNT screening battery, as well as to results from assays in other screening programs.

The chemicals tested in the present study cover a variety of classes including several different pesticide classes, flame retardants, polyaromatic hydrocarbons (PAHs), and metals. In addition, multiple chemicals from each of these classes are contained within the chemical set, which will provide an understanding of how the NFA responds not just to an individual chemical but to classes of chemicals. Finally, there will be a wealth of data from other *in vitro* assays to which the NFA results can be compared, which will be informative regarding the overall assay utility.

METHODS

Chemicals. All chemicals tested in the present study are listed in Table 1, along with the CAS number, concentration range tested and origin (ToxCast or NTP 91). Information has also been added to Table 1 regarding the category of the chemical and molecular targets (eg, receptors, ion channels) for the chemical, if well established in the literature. The NTP 91 chemical library (Behl *et al.*, 2019; Ryan *et al.*, 2016) consists of a combination of 87 unique (+4 replicates) environmental chemicals and drugs with reported DNT or neurotoxicity activity, as well as representatives of chemical classes of interest to the NTP but with limited or unknown neurotoxicity information (eg, organophosphate flame retardants). The NTP library was not tested in full in this manuscript, as 35 chemicals in the library have already been tested in this NFA (Frank *et al.*, 2017). However, 7 of those chemicals (hexachlorophene, TCEP, DEHP, permethrin, D-glucitol, BDE-47, and phenobarbital) were retested in the present study. In addition, 6 of the chemicals in the NTP 91 set were also in the set of 96 chemicals supplied by ToxCast (identified as "ToxCast [NTP 91]" in Table 1). Finally, toluene and *n*-hexane were not tested due to volatility and 2, 3, 7, 8-tetracholordibenzodioxin (TCDD) was not tested due to waste stream issues. This resulted in the 50 NTP chemicals tested in this study indicated in Table 1. For the NTP-91 library, stock solutions in dimethylsulfoxide (DMSO, Sigma, St Louis, MO) were prepared by MRIGlobal (Kansas City, MO) and stored at −20°C, and shipped to the US EPA for testing. Complete description of the library is found in Ryan *et al.* (2016) and associated supplemental materials. In addition to these chemicals, 96 chemicals were selected from the phase II library of EPA's ToxCast program. These chemicals were selected based on several factors, including also being on the NTP 91 list, evidence for potential DNT, based on Mundy *et al.* (2015), groups of chemicals from the same class (eg, pyrethroids), and suggestions from EPA's Office of Pesticides. ToxCast chemicals were supplied as 20mM aliquots in DMSO by EPA's ToxCast Chemical Contractor (Evotec, Princeton, NJ) in 50 µl aliquots in sealed, round-bottom 96 well plates. Upon arrival, 96-well plates were wrapped in parafilm and stored at −20°C until use. In total, 136 unique chemicals were tested in the present study; 10 chemicals (17β-estradiol, 3-iodo-2-propynyl-N-butylcarbamate, disulfiram, acetylsalicylic acid, bisphenol AF, captan, *p, p'*-DDT, parathion, phenanthrene, and rotenone) were selected as biological replicates, for a total of 146 chemicals tested. In addition, 8 of the chemicals tested were considered negative control chemicals as they are commonly used substances and/or drugs that are used during pregnancy without established adverse neurodevelopmental outcomes.

Tissue culture. Primary cultures of cortical neurons were prepared as described in Brown *et al.* (2016). Briefly, frontal cortex from postnatal 0–1 day Long-Evans rat pups were removed and

Table 1. List of Chemicals Tested

PREFERRED_NAME	Common name/ Abbreviation	Casrn	DTXSID	Concentration Range (μM)	Source	Additional Information	Potential Target
17beta-Estradiol	Estrogen	50-28-2	DTXSID0020573	0.03–20	NTP 91	Other	Estrogen receptor
17beta-Estradiol		50-28-2	DTXSID0020573	0.03–20	ToxCast	Other	Estrogen Receptor
1-Ethyl-3-methylimidazolium diethylphosphate		848641-69-0	DTXSID9047889	0.03–20	NTP 91	Other	
1-Methyl-4-phenylpyridinium iodide	MPP+	36913-39-0	DTXSID40880040	0.01–10	NTP 91	DNT/NT[b]	
2, 2', 4, 4', 5, 5'-Hexabromodiphenyl ether	BDE-153	68631-49-2	DTXSID4030047	0.02–10	NTP 91	Flame retardant	
2, 2', 4, 4', 5-Pentabromodiphenyl ether	BDE-99	60348-60-9	DTXSID9030048	0.03–20	NTP 91	Flame retardant	
2, 2'4, 4'-Tetrabromodiphenyl ether	BDE-47	5436-43-1	DTXSID3030056	0.03–20	NTP 91	Flame retardant	
2, 2-Bis(4-hydroxyphenyl)-1, 1, 1-trichloroethane	HPTE	2971-36-0	DTXSID8022325	0.03–20	ToxCast	Organochlorine	VGSC
2-Ethylhexyl diphenyl phosphate	EHDP	1241-94-7	DTXSID1025300	0.03–20	NTP 91	Flame retardant	
2-Ethylhexyl 2, 3, 4, 5-tetrabromobenzoate	EH-TBB	183658-27-7	DTXSID9052686	0.03–20	NTP 91	Flame retardant	
2-Methoxyethanol		109-86-4	DTXSID5024182	0.03–20	NTP 91	DNT/NT	
3, 3'-Iminobispropanenitrile		111-94-4	DTXSID2041464	0.03–20	NTP 91	DNT/NT	
3-Iodo-2-propynyl-N-butylcarbamate	IPBC	55406-53-6	DTXSID0028038	0.01–20	ToxCast	Fungicide	Carbamate
3-Iodo-2-propynyl-N-butylcarbamate	IPBC	55406-53-6	DTXSID0028038	0.03–20	NTP 91	Fungicide	Carbamate
4H-Cyclopenta(def)phenanthrene		203-64-5	DTXSID1024887	0.03–20	NTP 91	PAH	
6-Propyl-2-thiouracil		51-52-5	DTXSID5021209	0.03–20	ToxCast (NTP 91)	Pharmaceutical	TPO
Oxidopamine hydrochloride	6OH-DA	28094-15-7	DTXSID0045838	0.03–20	NTP 91	DNT/NT	
6-Methyl-2-thiouracil		56-04-2	DTXSID2020890	0.03–20	ToxCast	Pharmaceutical	TPO
Abamectin[a]		71751-41-2	DTXSID8023892	0.0003–20	ToxCast	Mectin	GABAR
Acenaphthylene		208-96-8	DTXSID3023845	0.03–20	ToxCast (NTP 91)	PAH	
Acenaphthene		83-32-9	DTXSID3021774	0.03–20	ToxCast (NTP 91)	PAH	
Aspirin	Aspirin	50-78-2	DTXSID5020108	0.03–20	NTP 91	Negative control	
Aspirin		50-78-2	DTXSID5020108	0.03–20	ToxCast	Negative control	
Aldrin		309-00-2	DTXSID8020040	0.03–20	ToxCast	Organochlorine	GABAR
Allethrin		584-79-2	DTXSID8035180	0.03–20	ToxCast	Pyrethroid	VGSC
Anthracene		120-12-7	DTXSID0023878	0.03–20	NTP 91	PAH	
Atrazine		1912-24-9	DTXSID9020112	0.03–20	ToxCast	Herbicide	
Auramine hydrochloride		2465-27-2	DTXSID9020114	0.03–20	NTP 91	Other	
Azoxystrobin[a]		131860-33-8	DTXSID0032520	0.003–20	ToxCast	Fungicide	Strobilurn
Benz(a)anthracene		56-55-3	DTXSID5023902	0.03–20	ToxCast (NTP 91)	PAH	
Benzo(a)pyrene		50-32-8	DTXSID2020139	0.03–20	NTP 91	PAH	
Benzo(b)fluoranthene		205-99-2	DTXSID0023907	0.03–20	ToxCast (NTP 91)	PAH	
Benzo(e)pyrene		192-97-2	DTXSID3023764	0.03–20	NTP 91	PAH	
Benzo(k)fluoranthene		207-08-9	DTXSID0023909	0.0012–0.8	NTP 91	PAH	

(continued)

Table 1. (continued)

PREFERRED_NAME	Common name/ Abbreviation	Casrn	DTXSID	Concentration Range (μM)	Source	Additional Information	Potential Target
Benzo(g, h, i)perylene		191-24-2	DTXSID5023908	0.03–20	NTP 91	PAH	
Berberine chloride		633-65-8	DTXSID8024602	0.0001–0.1	NTP 91	Other	
Bifenthrin		82657-04-3	DTXSID9020160	0.03–20	ToxCast	Pyrethroid	VGSC
Bis(2-ethylhexyl) tetrabromophthalate	TBPH	26040-51-7	DTXSID7027887	0.03–20	NTP 91	Flame retardant	
Bisphenol AF		1478-61-1	DTXSID7037717	0.03–20	NTP 91	Plasticizer	Estrogen receptor
Bisphenol AF		1478-61-1	DTXSID7037717	0.03–20	ToxCast	Plasticizer	Estrogen receptor
Bisphenol B		77-40-7	DTXSID4022442	0.03–20	ToxCast	Plasticizer	Estrogen receptor
4, 4'-Sulfonyldiphenol		80-09-1	DTXSID3022409	0.03–20	NTP 91	Plasticizer	Estrogen receptor
Boric acid		10043-35-3	DTXSID1020194	0.03–20	ToxCast	Other	
Boscalid		188425-85-6	DTXSID6034392	0.03–20	ToxCast	Fungicide	Carboxamide
Busulfan		55-98-1	DTXSID3020910	0.03–20	ToxCast	Other	
Captan		133-06-2	DTXSID9020243	0.03–20	NTP 91	Fungicide	
Captan		133-06-2	DTXSID9020243	0.03–20	ToxCast	Fungicide	Phthalimide
Carbofuran		1563-66-2	DTXSID9020249	0.03–20	ToxCast	Fungicide	Carbamate
Cariporide mesylate		159138-81-5	DTXSID3047344	0.03–20	ToxCast	Pharmaceutical	VGSC
Chlordane		57-74-9	DTXSID7020267	0.03–20	ToxCast	Organochlorine	GABAR
Chlorendic acid		115-28-6	DTXSID2020268	0.03–20	ToxCast	Flame retardant	
Chrysene		218-01-9	DTXSID0022432	0.01–9.7	NTP 91	PAH	
Clodinafop-propargyl		105512-06-9	DTXSID6032354	0.03–20	ToxCast	Other	
Cloprop		101-10-0	DTXSID9034232	0.03–20	ToxCast	Herbicide	
Clothianidin		210880-92-5	DTXSID2034465	0.03–20	ToxCast	Neonicotinoid	nAChR
Clove leaf oil		8000-34-8	DTXSID8044175	0.03–20	ToxCast	Pharmaceutical	VGSC
CP-409092		194098-25-4	DTXSID2047276	0.03–20	ToxCast	Pharmaceutical	GABAR
CP-457920		220860-50-4	DTXSID4047254	0.03–20	ToxCast	Pharmaceutical	GABAR
Cyfluthrin		68359-37-5	DTXSID5035957	0.03–20	ToxCast	Pyrethroid	VGSC
Cymoxanil		57966-95-7	DTXSID6032358	0.03–20	ToxCast	Fungicide	
D-Glucitol		50-70-4	DTXSID5023588	0.03–20	NTP 91	Negative control	
Di(2-ethylhexyl) phthalate	DEHP	117-81-7	DTXSID5020607	0.03–20	NTP 91	DNT/NT	
Dibenz(a, h)anthracene		53-70-3	DTXSID9020409	0.02–10	NTP 91	PAH	
Dibenz[a, c]anthracene		215-58-7	DTXSID9049245	0.03–20	NTP 91	PAH	
Diphenhydramine hydrochloride	Diphenhydramine	147-24-0	DTXSID4020537	0.03–20	ToxCast	Pharmaceutical	H1 antagonist
Disulfiram[a]		97-77-8	DTXSID1021322	0.0003–20	ToxCast	Pharmaceutical	Acetaldehyde dehydrogenase inhibitor
Disulfiram[a]		97-77-8	DTXSID1021322	0.0001–0.1	NTP 91	DNT/NT	
Disulfoton		298-04-4	DTXSID0022018	0.03–20	ToxCast	Organophosphate	AcheE
D-Mannitol		69-65-8	DTXSID1023235	0.03–20	ToxCast	Negative control	
Enadoline		124378-77-4	DTXSID4047258	0.03–20	ToxCast	Pharmaceutical	OpoidR
Endosulfan		115-29-7	DTXSID1020560	0.03–20	ToxCast	Organochlorine	GABAR
Endrin		72-20-8	DTXSID6020561	0.03–20	ToxCast	Organochlorine	GABAR
EPTC		759-94-4	DTXSID1024091	0.03–20	ToxCast	Herbicide	Thiocarbamate herbicide
Erythromycin		114-07-8	DTXSID4022991	0.03–20	ToxCast	Negative control	

(continued)

Table 1. (continued)

PREFERRED_NAME	Common name/ Abbreviation	Casrn	DTXSID	Concentration Range (μM)	Source	Additional Information	Potential Target
Esfenvalerate		66230-04-4	DTXSID4032667	0.03–20	ToxCast	Pyrethroid	VGSC
Etofenprox		80844-07-1	DTXSID9032610	0.03–20	ToxCast	Pyrethroid	VGSC
Eugenol		97-53-0	DTXSID9020617	0.03–20	ToxCast	Pharmaceutical	VGSC
Fenamiphos		22224-92-6	DTXSID3024102	0.03–20	ToxCast	Organophosphate	AcheE
Fenitrothion		122-14-5	DTXSID4032613	0.03–20	ToxCast	Organophosphate	AcheE
Fenpropathrin		39515-41-8	DTXSID0024002	0.03–20	ToxCast	Pyrethroid	VGSC
Firemaster 550		860302-33-6	DTXSID70880073	0.03–20	NTP 91	Flame retardant	
Flufenacet		142459-58-3	DTXSID2032552	0.03–20	ToxCast	Organophosphate	AcheE
Fluorene		86-73-7	DTXSID8024105	0.03–20	ToxCast (NTP 91)	PAH	
Fluoxastrobin[a]		361377-29-9	DTXSID2034625	0.001–20	ToxCast	Fungicide	Strobilurn
Folic acid		59-30-3	DTXSID0022519	0.03–20	ToxCast	Negative control	
Difpas-pyrazole		151506-44-4	DTXSID6048175	0.03–20	ToxCast	Pharmaceutical	OpoidR
Glycerol		56-81-5	DTXSID9020663	0.03–20	ToxCast	Negative control	
Hexachlorophene		70-30-4	DTXSID6020690	0.03–20	NTP 91	DNT/NT	
Isodecyl diphenyl phosphate	IDDP	29761-21-5	DTXSID3025465	0.03–20	NTP 91	Flame retardant	
Kepone		143-50-0	DTXSID1020770	0.03–20	ToxCast	Organochlorine	GABAR
L-Ascorbic acid		50-81-7	DTXSID5020106	0.03–20	NTP 91	Negative control	
Lovastatin*		75330-75-5	DTXSID5020784	0.003–20	ToxCast	Pharmaceutical	Statin
Mancozeb		8018-01-7	DTXSID0034695	0.03–20	ToxCast	Fungicide	dithiocarbamate
Manganese(II) acetate	Mn2+	638-38-0	DTXSID5027279	0.03–20	NTP 91	Metal	
Mercuric chloride[a]		7487-94-7	DTXSID5020811	0.003–20	ToxCast	Metal	
Methadone hydrochloride	Methadone	1095-90-5	DTXSID2020501	0.03–20	ToxCast	Pharmaceutical	OpoidR
Methimazole		60-56-0	DTXSID4020820	0.03–20	ToxCast	Pharmaceutical	TPO
Methoxychlor		72-43-5	DTXSID9020827	0.03–20	ToxCast	Organochlorine	VGSC
Octylbicycloheptenedicarboximide		113-48-4	DTXSID6032562	0.03–20	ToxCast	Synergist	
Mirex		2385-85-5	DTXSID7020895	0.03–20	ToxCast	Organochlorine	GABAR
(Methylcyclopentadienyl) tricarbonylmanganese	MMT	12108-13-3	DTXSID9027738	0.03–20	NTP 91	Metal	
Molinate		2212-67-1	DTXSID6024206	0.03–20	ToxCast	Herbicide	
Naphthalene		91-20-3	DTXSID8020913	0.03–20	NTP 91	PAH	
o, p'-DDT		789-02-6	DTXSID6022345	0.03–20	ToxCast	Organochlorine	VGSC
p, p'-DDD		72-54-8	DTXSID4020373	0.03–20	ToxCast	Organochlorine	VGSC
p, p'-DDE		72-55-9	DTXSID9020374	0.03–20	ToxCast	Organochlorine	VGSC
Dichlorodiphenyltrichloroethane	p, p'-DDT	50-29-3	DTXSID4020375	0.03–20	NTP 91	Organochlorine	VGSC
Dichlorodiphenyltrichloroethane	p, p'-DDT	50-29-3	DTXSID4020375	0.03–20	ToxCast	Organochlorine	VGSC
Parathion		56-38-2	DTXSID7021100	0.03–20	ToxCast	Organophosphate	AcheE
Parathion		56-38-2	DTXSID7021100	0.03–20	NTP 91	Organophosphate	AcheE
Permethrin		52645-53-1	DTXSID8022292	0.03–20	NTP 91	Pyrethroid	VGSC
PharmaGSID_47330		NOCAS_47330	DTXSID9047330	0.03–20	ToxCast	Pharmaceutical	GABAR
Phenanthrene		85-01-8	DTXSID6024254	0.03–20	NTP 91	PAH	
Phenanthrene		85-01-8	DTXSID6024254	0.03–20	ToxCast	PAH	
Phenobarbital		50-06-6	DTXSID5021122	0.03–20	NTP 91	DNT/NT	
Triphenyl phosphates isopropylated	IPP	68937-41-7	DTXSID4028880	0.03–20	NTP 91	Flame retardant	
Phenylmercuric acetate[a]		62-38-4	DTXSID7021150	0.0003–20	ToxCast	Metal	

(continued)

Table 1. (continued)

PREFERRED_NAME	Common name/ Abbreviation	Casrn	DTXSID	Concentration Range (μM)	Source	Additional Information	Potential Target
Picoxystrobin[a]		117428-22-5	DTXSID9047542	0.0003–20	ToxCast	Fungicide	Strobilurin
Piperonyl butoxide		51-03-6	DTXSID1021166	0.03–20	ToxCast	Synergist	
Prallethrin		23031-36-9	DTXSID0032572	0.03–20	ToxCast	Pyrethroid	VGSC
Pravastatin sodium		81131-70-6	DTXSID6047525	0.03–20	ToxCast	Pharmaceutical	Statin
Pyraclostrobin[a]		175013-18-0	DTXSID7032638	0.0003–20	ToxCast	Fungicide	Strobilurin
Pyrene		129-00-0	DTXSID3024289	0.03–20	NTP 91	PAH	
Reserpine		50-55-5	DTXSID7021237	0.03–20	ToxCast	Other	VMAT
Resmethrin		10453-86-8	DTXSID7022253	0.03–20	ToxCast	Pyrethroid	VGSC
Rotenone[a]		83-79-4	DTXSID6021248	0.00003–0.03	NTP 91	DNT/NT	Electron transport
Rotenone[a]		83-79-4	DTXSID6021248	.0001–20	ToxCast	DNT/NT	Electron transport
S-Bioallethrin		28434-00-6	DTXSID2039336	0.03–20	ToxCast	Pyrethroid	VGSC
Simvastatin[a]		79902-63-9	DTXSID0023581	0.003–20	ToxCast	Pharmaceutical	Statin
Spirodiclofen		148477-71-8	DTXSID6034928	0.03–20	ToxCast	Other	Lipid biosynthesis
Tamoxifen		10540-29-1	DTXSID1034187	0.03–20	ToxCast	Other	Estrogen receptor
Tefluthrin		79538-32-2	DTXSID5032577	0.03–20	ToxCast	Pyrethroid	VGSC
tert-Butylhydroquinone	TBHQ	1948-33-0	DTXSID6020220	0.03–20	ToxCast	Other	
tert-Butylphenyl diphenyl phosphate	BPDP	56803-37-3	DTXSID6024701	0.03–20	NTP 91	Flame retardant	
Tetracycline		60-54-8	DTXSID7023645	0.03–20	ToxCast	Negative control	
Tetramethrin		7696-12-0	DTXSID6032649	0.03–20	ToxCast	Pyrethroid	VGSC
Thiacloprid		111988-49-9	DTXSID7034961	0.03–20	ToxCast	Neonicotinoid	nAChR
Thiamethoxam		153719-23-4	DTXSID2034962	0.03–20	ToxCast	Neonicotinoid	nAChR
Tributyltin chloride[a]		1461-22-9	DTXSID3027403	0.0003–20	ToxCast	Metal	
Tributyltin methacrylate[a]		2155-70-6	DTXSID9035204	0.0003–20	ToxCast	Metal	
Triclosan		3380-34-5	DTXSID5032498	0.03–20	ToxCast	Other	
Tris(methylphenyl) phosphate	TMPP	1330-78-5	DTXSID4021391	0.03–20	NTP 91	Flame retardant	
Trifloxystrobin[a]		141517-21-7	DTXSID4032580	0.001–20	ToxCast	Fungicide	Strobilurin
Triphenyl phosphite		101-02-0	DTXSID0026252	0.03–20	ToxCast	Flame retardant	
Tris(2-chloroethyl) phosphate	TCEP	115-96-8	DTXSID5021411	0.03–20	NTP 91	Flame retardant	
Valinomycin[a]		2001-95-8	DTXSID9041150	0.0001–0.1	NTP 91	Other	

[a]Compound completely inhibited activity over initial concentration range, so additional, lower concentrations were tested.
[b]NT/DNT-compound was noted as potentially neurotoxic (NT) or developmentally neurotoxic (DNT) in categories used for the NTP 91 list.

dissociated, then plated in 25 µl droplets containing 150 000 cells into each well of 48 well MEA plates (M-768-KAP-48; Axion Biosystems, Atlanta, GA). Cells were allowed to attach to the substrate for 2 h after which 500 µl of media was added that contained the appropriate concentration of test material or solvent. For the vast majority of chemicals, test concentrations ranged from 0.03 µM to 20 µM in half-log increments. However, some chemicals were not supplied at 20 mM, as indicated in Table 1.

Electrophysiological assessment of network activity. Assessment of electrical activity using MEAs was conducted as described in Brown *et al.* (2016) and Frank *et al.* (2017). Briefly, spontaneous electrical activity of cortical cells was recorded using the Axion Biosystems Maestro 768-channel amplifier, Middle-man data acquisition interface, and Axion Integrated Studio (AxIS) software v1.9 or later (Axion Biosystems, Atlanta, GA). Spontaneous activity was measured using a gain of 1200× and a sampling frequency of 12.5 kHz. The signal was passed through a Butterworth band-pass filter (0.1–5000 Hz) In AxIS and on-line spike detection (threshold = 8× rms noise) was done with the AxIS adaptive spike detector. All recordings were conducted at 37°C. Activity was recorded for a period of 15 min on day *in vitro* (DIV) 5, 7, 9, and 12 to monitor the development of network activity. After recording on DIV 12, cell viability was assessed using lactate dehydrogenase (LDH) and alamar blue (AB) assays (Promega, Madison, WI) as described in Brown *et al.* (2016) and Frank *et al.* (2017). Each biological replicate of a chemical consisted of triplicate technical replicates (the entire concentration range at 1 well/concentration tested across 3 separate plates from the same culture [$n = 3$/concentration]). Only 1 biological replicate was evaluated for each chemical, except for the 10 chemicals noted above for which 2 biological replicates were compared. In general, concentrations decreased in half-log increments starting at 10µM and below. For 14 chemicals (indicated by a superscript letter 'a' in Table 1), no network activity was observed even at the lowest concentration tested. In these cases, the corresponding cytotoxicity assays indicated cytotoxicity even at the lowest concentration tested. These chemicals were retested at a lower concentration range (where at least some concentrations did not reduce activity or viability); the highest concentrations overlapped with the lowest 2 concentrations originally tested, and data from the two concentration ranges were combined into a single biological replicate.

Data analysis. Analysis of the development of spontaneous electrical activity was conducted as described in Frank *et al.* (2017). Briefly, 17 different parameters describing spiking, bursting, and coordinated network activity were determined by analysis of each recording on each DIV. Trapezoidal area under the curve (AUC) measurements were used to collapse data across time and concentration and generate concentration-response curves for each parameter.

Using the R programming language (R Core Team, 2016) for statistical computing, concentration-response relationships were determined for each network parameter by a change in mean AUC (as percent of median untreated well AUC) over increasing concentration. Initially, data were examined for both increased and decreased responses. However, increased responses were observed in <3% of possible outcomes. Therefore, the analysis focused only on decreased responses. All data and R scripts are available in the published dataset associated with this manuscript (https://doi.org/10.23719/1503191; Accessed March 19, 2019). If at least one of the highest 2 concentrations tested produced a change in mean AUC beyond 3 times the median absolute deviation (MAD) of all untreated control well values, a Hill function was fit to the data with a fixed upper limit of 100% and lower limit of 0%. An EC_{50} concentration was obtained from each curve, representing a 50% reduction from untreated controls. In a few cases where the Hill curve poorly modeled the concentration-response relationship or optimization failed, an EC_{50} was estimated by interpolating between the concentration that caused a greater than 50% perturbation and the next lower concentration. In all cases, if the EC_{50} estimate was outside the tested concentration range, it was discarded due to lack of confidence in extrapolation. Any change that resulted in an EC_{50} value determination for any parameter was considered a positive outcome, or "hit" in a screening context.

Cytotoxicity (Alamar blue and LDH assay) EC_{50} values were determined by fitting a Hill function to DIV12 concentration-response plots with parameterization identical to that used for AUC calculations. Selectivity of a chemical was defined as the difference in concentration between the minimum EC_{50} for network effect(s) and the minimum cytotoxicity EC_{50} value. For selectivity and potency rankings, all chemicals with no AUC EC_{50} values on network parameters were excluded and compounds with undetermined cytotoxicity values were set to the maximum concentration tested (20 µM). Then chemicals were ordered by mean of AUC EC_{50} (potency) and mean selectivity across network parameters. The rule for defining the cutoff for considering an effect to be selective was based on the methods described in Stiegler *et al.* (2011) and Krug *et al.* (2013) for determining the potential selectivity of neurite outgrowth inhibitors. Similar to those publications, the ratio of the EC_{50} (in micromolar concentration units) for viability to the EC_{50} (in micromolar concentration units) for the most sensitive endpoint for network development was evaluated for a number of tested chemicals known to be generally cytotoxic in cortical cultures (bistributylin oxide, colchicine, methotrexate, sodium arsenate, triethyltin, trimethylin). The mean of these ratios was 1.9 (ie, network parameters were on average affected by cytotoxic chemicals at slightly lower concentrations than viability). The upper 95% confidence interval of the ratio was 2.8. Thus, a ratio of 3 was used as a threshold for chemicals to be considered as having selective effects; chemicals that altered parameters of network development. For chemicals where an EC_{50} value for viability could not be determined in either assay, a default of 20 µM (or the highest concentration tested) was used, following the approach used by Frank *et al.* (2017). It should be noted that this results in erring on the side of chemicals not being considered selective.

Determination of "tipping points". Following determination of EC_{50} values, critical concentrations, or toxicological "tipping points" (Frank *et al.*, 2018; Shah *et al.*, 2016) were determined for chemicals that were active. To determine tipping points, the procedures in Frank *et al.* (2018) were followed. Briefly, using scripts written in R, data were normalized to the plate and *z*-scored, then total scalar perturbations, velocity, and the derivative of velocity relative to concentration were determined. Note that, as per Frank *et al.* (2018), not all network function parameters were used in the determination of the tipping points. Tipping points were based on the following network function parameters: mean firing rate (MFR), number of active electrodes (No. AE), burst rate (BR), number of actively bursting electrodes (No. ABE), percent of spikes in bursts (%SiB), number of network spikes (No. NS), percent of spikes in network spikes (%SiNS),

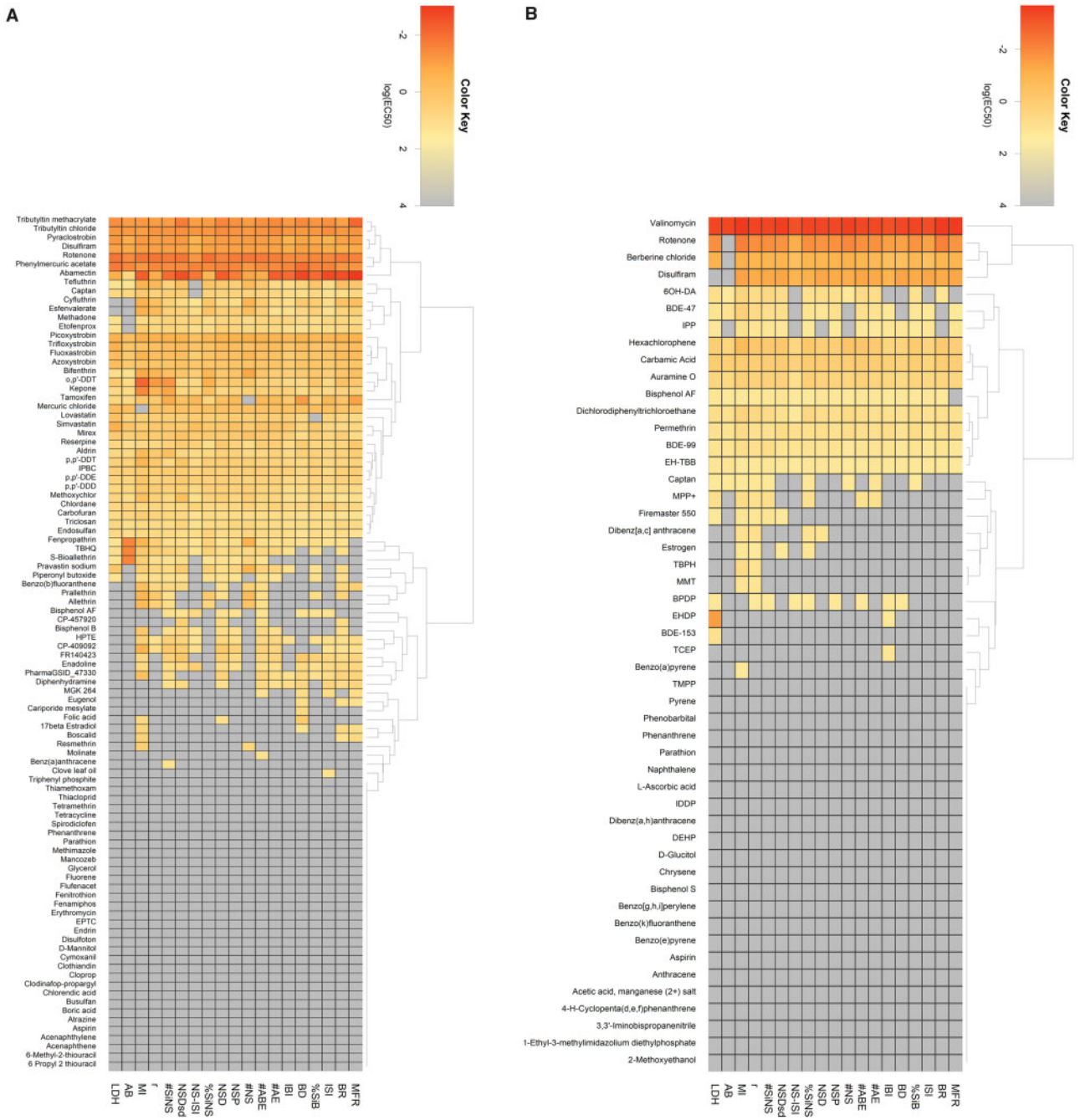

Figure 1. Heatmaps summarizing activity of tested chemicals. Heatmap across the chemicals tested from ToxCast (A) and NTP (B) for concentrations that produced 50% change (EC_{50}) from untreated control median AUC summaries of 17 network activity parameters over developmental time. Concentrations resulting in 50% reduction in 2 cell viability assays, Alamar blue (AB) conversion and lactate dehydrogenase (LDH) release are shown for comparison. Color indicates log-transformed EC_{50} value with gray indicating no EC_{50} established.

Pearson's correlation (r) between activity on electrodes, and normalized mutual information (MI).

Comparison of potency of effects on network development to potency of effects in other ToxCast assays. The potency of the active chemicals in the MEA-NFA was compared to the potency of these chemicals in other ToxCast assays (including Tox21 assay data (Thomas *et al.*, 2018) analyzed using the ToxCast Data Pipeline (Filer *et al.*, 2017)) by comparing the EC_{50} value of the most-sensitive NFA parameter, the tipping point (when 1 was determined), and EC_{50} for cytotoxicity to the potency and "cytotoxicity burst" concentration (Judson *et al.*, 2016) for that chemical in all other ToxCast assays. For those chemicals that were replicated in the present experiments, the most potent value from the 2 replicates was used. The ToxCast data were obtained from invitrodb_v2 (U.S. EPA. 2015). Of the 136 unique chemicals screened using NFA, only 132 chemicals had ToxCast data in invitrodb_v2 (MPP^+ [CASRN 36913-39-0], EH-TBB [CASRN

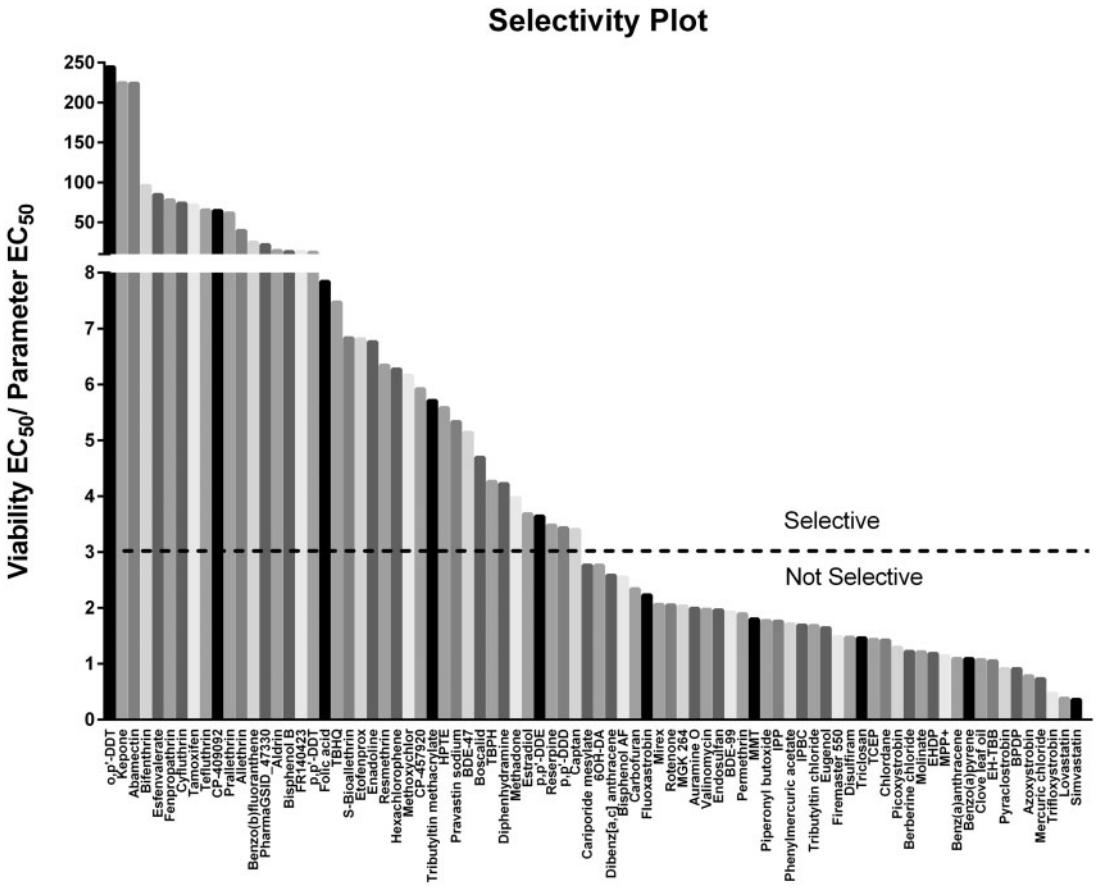

Figure 2. Plot of selectivity. Plot of chemicals from both lists that were active, illustrating the selectivity of the effect. For this plot, the EC_{50} value for effects on viability was divided by the EC_{50} value of the most sensitive network parameter. A ratio of 3 or greater is considered a selective effect. For biological replicates, only the more selective of the 2 replicates is plotted. In addition, the more potent EC_{50} value of the 2 different viability assays was used. If there was no EC_{50} value for viability, the highest concentration tested was used.

183658-27-7], Firemaster 550 [CASRN 860302-33-6], and valinomycin [CASRN 2001-95-8] lacked ToxCast Data). Of the 132 chemicals with data in both the NFA and ToxCast, only 78 chemicals had a minimum EC_{50} value (all 4 chemicals that were not in invitrodb_v2 altered network function). The remaining 54 chemicals for which the NFA was negative, but ToxCast data were available, were used to indicate the range of AC_{50} potency values possible for those chemicals *in vitro*. The ToxCast data and R code used to manipulate these data and create visualizations are available in the Supplementary materials and published dataset, respectively.

Generation of administered equivalent doses and comparison to in vivo doses. For a subset of chemicals where *in vivo* studies reporting adverse neurodevelopmental outcomes in rodents were available, reverse dosimetry was employed to predict the administered equivalent dose (AED) in mg/kg/day units that would be necessary to obtain a steady-state plasma concentration equivalent to the bioactive *in vitro* concentrations (Wetmore *et al.*, 2012, 2014). Using the minimum NFA EC_{50} values and/or tipping points, AEDs were estimated using the high-throughput toxicokinetic (HTTK) information and models available in the httk R package (v1.8; Pearce *et al.*, 2017), which functionalizes an approach similar to the one previously used by Wetmore *et al.* (2012, 2014), as represented by the following equation 1.

$$\text{Administered equivalent dose (AED)} = \text{MinEC}_{50} \text{ or Tipping Point} * \frac{1\frac{\text{mg}}{\text{kg}}}{\frac{\text{day}}{C_{ss}}} \quad (1)$$

Where AED is the administered equivalent dose, $MinEC_{50}$ or Tipping Point are the minimum EC_{50} or Tipping Points determined in the NFA assay, and C_{ss} is the steady-state concentration of compound in the plasma as approximated using a population simulation in the httk R package. The httk R package (Pearce *et al.*, 2017) includes both chemical-specific *in vitro* data and models for predicting *in-vivo* toxicokinetics (ie, absorption, distribution, metabolism, and excretion). The HTTK information, including first-order metabolic clearance and plasma protein binding potential, were used in calculation of the steady-state concentration in plasma, along with estimates of the rate of clearance via the kidney, blood flow across the liver, and intrinsic clearance, assuming 100% bioavailability (Pearce *et al.*, 2017, Wetmore *et al.* 2012). More specifically, the AEDs were calculated programmatically using the "calc_mc_oral_equivalent" function with the following options: the 95th quantile (which.quantile = c(0.95)); restrictive clearance (restrictive.clearance = T); selection of species (species = "Human"); direct resampling of the population data (method = "dr"); a correction for the amount of unbound chemical in whole blood versus plasma (well.stirred.correction = T); the default 3 compartment model (model = "3compartments"); and the output unit as milligrams (output.units = "mg").

Figure 3. Potency versus selectivity. Ranking of active chemicals with network effects by least to greatest potency (mean of network parameter EC_{50} values) and selectivity (mean of distance from equality of each network effect EC50 vs minimum cell viability EC_{50}). The mean value of all 17 measures of network activity is shown to better reflect overall chemical potency and selectivity. For the active biological replicates, only the most potent and selective replicate is plotted. Chemicals circled in solid red are both potent and selective, those circled in large black dashes are potent but less selective, and those circled in small blue dashes are less potent with a range of selectivity.

Results

As previously described (Brown *et al.*, 2016; Cotterill *et al.*, 2016; Frank *et al.*, 2017), the ontogeny of neural network activity in primary cortical cultures began with intermittent, individual spiking activity that occurred on few electrodes on DIV 5 and proceeded to develop into more complex bursting and coordinated network bursts (occurring across all electrodes with activity) between DIV 7 and DIV 12. Generally, exposure to compounds from DIV 0 to DIV12 resulted in decreases in parameters describing network function. Heatmaps summarizing the outcomes of testing of the ToxCast and NTP chemicals are shown in Figures 1A and 1B, respectively, and EC_{50} values for each parameter are provided in the supplementary data. Of the 136 unique chemicals that were tested, 54 did not alter the ontogeny of any network parameter enough to determine an EC_{50} value. This number (54) includes 2, 2', 4, 4', 5, 5'-hexabromodiphenylether (BDE-153), which did not alter any network ontogeny parameter sufficiently enough to determine an EC_{50} value, even though it was cytotoxic. The remaining 82 chemicals had effects on at least 1 parameter of network development, with 40 showing selective effects (more than 3× differences between EC_{50} on network parameter and EC_{50} for viability). A summary of the relationships between potency on the most sensitive network parameter and alterations in viability is shown in Figure 2, whereas comparisons of potency and selectivity are shown in Figure 3.

Folic acid, which altered 4 parameters (Mean Burst Duration, Network Spike Duration, standard deviation of Network Spike Duration and Mutual Information) was the only negative control chemical that altered network parameters beyond the 3 × MAD threshold (Supplementary Table 1). Acetylsalicylic acid was tested twice as biological replicates and was negative in both cases. Of the 10 chemicals tested as biological replicates, hitcalls were qualitatively concordant in all cases; 3 chemicals were negative (acetylsalicylic acid, parathion, and phenanthrene) and 7 were active on at least 1 parameter. Of the 7 positive chemicals, results for biological replicates were also quantitatively similar, with the most potent effect in all cases being less than 10-fold different, and less than 3-fold for 5/7 chemicals. However, only for DDT was the same parameter (Mutual Information) the most sensitive for both biological replicates. Seven chemicals tested here were also tested in Frank *et al.* (2017). Hitcalls with permethrin, phenobarbital, tris-2-chloroisopropyl phosphate (TCEP,), 2, 2', 4, 4'-tetrabromodiphenyl ether (BDE-47), and hexachlorophene, were all consistent with the previously published outcomes, whereas hitcalls for glucitol (sorbitol) and di(2-ethylhexyl) phthalate (DEHP) differed between the present study and Frank *et al.* (2017). Different salts of manganese were also tested between the present study and Frank *et al.* (2017), with concordant (negative) hitcalls. Additional outcomes are discussed below by chemical category for the combined lists of ToxCast and NTP 91 chemicals.

Polyaromatic Hydrocarbons

Polyaromatic hydrocarbons were the largest class of chemicals tested, with 17 total chemicals, only 4 of which (benz(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, and dibenz[a, c] anthracene) were active. Potencies for these chemicals were generally in the 1μM–20μM range. None of the PAHs exhibited cytotoxicity for the concentration ranges tested, but given the criteria for selectivity, of the 4 active chemicals, only benzo(b)-fluoranthene was selective. None of the PAHs had tipping points.

Flame Retardants

Fourteen chemicals classified as flame retardants were tested in the present study. Nine of the 14 chemicals were active in the assay with only BDE-153, IDDP, TMPP, triphenyl phosphite, and chlorendic acid without effect on any network parameter. BDE-153 was cytotoxic. In general, EC_{50} values for the 9 active chemicals were in the 1μM–20μM range, and with the exceptions of BDE-47 and TBPH, were not selective when compared to effects on viability. Tipping points could be generated for BDE-47, EH-TBB, and BDE-99 in all 3 cases were more potent than the minimum network parameter EC_{50} value.

Pharmaceuticals

Including the negative control chemicals aspirin, folic acid, erythromycin, and tetracycline, there were 21 unique chemicals in this category. Fifteen of these chemicals (including folic acid) altered at least 1 parameter of network development. EC_{50} values ranged from 0.095μM for disulfiram to 18μM for clove leaf oil. Of those chemicals that were active, 9/15 were selective when compared to effects on viability. Tipping points were determined for 11 chemicals and in all cases were more potent than the network parameter EC_{50} value. This includes 1 replicate of 6-methyl-2-thiouracil, for which no EC_{50} values for network parameters could be determined. Targets included in this group of chemicals included opioid receptors, voltage-gated sodium channels and gamma amino butyric acid (GABA) receptors.

Pyrethroids

There were 12 pyrethroid insecticides and 2 commonly used synergists (piperonyl butoxide and MGK-264) tested; 11/12 pyrethroids (only tetramethrin was without effects) and both synergists were active on at least one parameter. Minimum parameter EC_{50} values ranged from 0.14μM to 10μM and were selective for 10/11 of the active pyrethroids (permethrin was not), but neither of the synergists (MGK264 and piperonyl butoxide). Interestingly, mutual information appeared to be an endpoint especially sensitive to pyrethroids, as this was the most sensitive endpoint for 8 of the 10 active pyrethroids. Tipping points were determined for 7/11 active pyrethroids and 1 of the synergists (piperonyl butoxide) and were in all cases more potent than the network parameter EC_{50} value.

Organochlorines

Twelve chemicals were considered organochlorines, and 11/12 chemicals altered the development of at least 1 network parameter, with endrin being the only chemical that was without effect. Eight of the 11 active chemicals were selective. *Para-para*-DDT was tested in both the NTP and ToxCast cohorts, and was active in both cases, but interestingly was only selective in the "ToxCast" cohort. Mirex, endosulfan, and chlordane were the other chemicals that were not selective in their actions. Minimum parameter EC_{50} values ranged from 0.03μM to 4.33μM, and tipping points could be determined for all active chemicals. Only the tipping points for kepone and one biological replicate of DDT exceeded the minimum parameter EC_{50} value.

Organophosphates and Neonicotinoids

Five organophosphate and 3 neonicotinoid chemicals were tested in the assay, and all 8 chemicals were negative.

Metals

Six different metals and organometals were tested, including 2 different salts of tributyl tin. Only manganese (II) acetate tetrahydrate was without effects on any parameter, whereas both salts of tributyl tin produced similar effects. Minimum parameter EC_{50} values ranged from 0.008μM to 11.1μM. However, only tributyltin methacrylate was selective in its effects on network parameters. A tipping point could only be determined for both salts of tributyl tin.

Fungicides and Herbicides

Eleven fungicides and 4 herbicides were tested in the current study. For the fungicides, 9/11 chemicals altered at least 1 parameter of network development, with 3-Iodo-2-propynyl-N-butylcarbamate and captan being tested in both the NTP and ToxCast cohorts as biological replicates with concordant results. All 5 strobilurin fungicides were active, as were both carbamate fungicides. Only 1 of the 4 herbicides was active. Minimum network parameter EC_{50} values ranged from 0.07μM to 16.57μM. However, molinate, the active herbicide, altered only 1 network parameter with EC_{50} value >15μM, suggesting that it has low potency. Of all the active chemicals in these categories, only the fungicides captan (from ToxCast) and boscalid had selective effects. Tipping points could be determined for 10/15 of these chemicals and were in all cases more potent than the minimum network parameter EC_{50} values.

Plasticizers and Estrogen Receptor Agonists

The estrogen receptor agonists estrogen and tamoxifen disrupted network formation in the present set of experiments, with estrogen being tested in both the ToxCast and NTP cohorts and being positive in both instances. In addition, bisphenol AF, bisphenol B and bisphenol S, all potential replacements for bisphenol A, a putative estrogen receptor agonist, were tested and only bisphenol S was without effects. Bisphenol AF was tested and positive in both cohorts. Of the active chemicals, all had low cytotoxicity, but with the exception of tamoxifen (minimum network parameter $EC_{50} = 0.081\mu M$), potencies for effects on network parameters were generally in the 1μM–20μM range. Only bisphenol B and tamoxifen had selective effects. Tipping points could be determined for 4 of these chemicals and the tipping point concentration was lower than the minimum network parameter EC_{50} value in 3 of 4 cases.

Potential Targets

Of the pharmaceutical or pesticidal chemicals tested here, many are known to act on specific receptors or ion channels as potential targets mediating their mode of action and possibly mediating their toxicity. The $GABA_A$ receptor is an established pharmaceutical and insecticidal target and known to be important to development of the nervous system (Schmidt and Mirnics, 2015). Of the 10 chemicals that may act on this receptor, 9 of them disrupted at least 1 parameter of network development, and 6/9 were selective in their actions on network parameters (chlordane, mirex, and endosulfan were not). Voltage-gated sodium channels are also well established pharmaceutical and insecticidal targets and 20/21 unique chemicals with actions on this channel disrupted network development, with 18 of those chemicals acting in a selective manner. All 3 opioid receptor-active chemicals (enadoline, FR140423, and methadone) were active and selective, whereas the 5 organophosphate insecticides acting on acetylcholinesterase and the 3 neonicotinoids that act on nicotinic acetylcholine receptors were inactive.

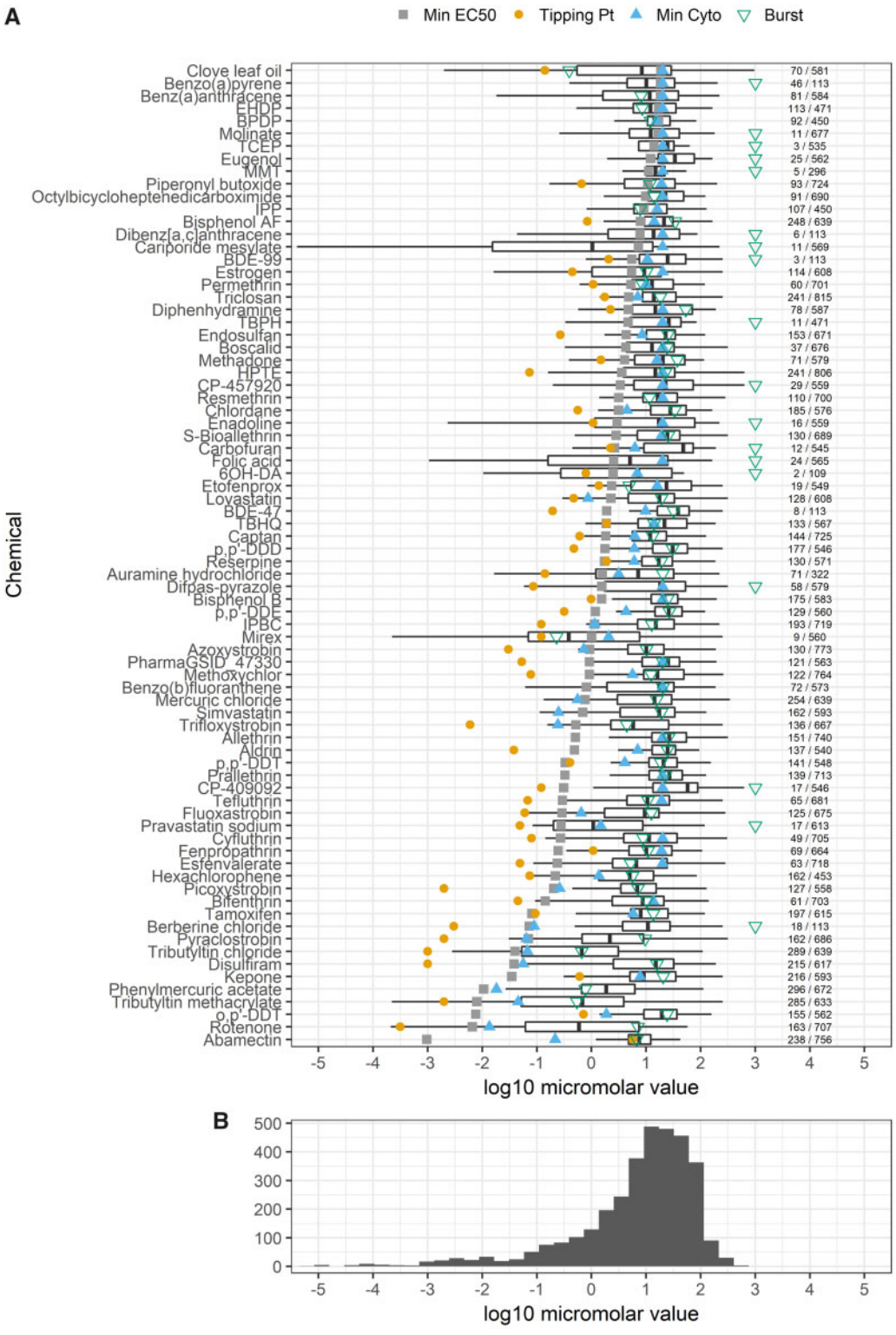

Figure 4. Comparison of effects in the NFA to results from other ToxCast assays. A, Comparison of the minimum EC_{50} (grey box, "Min EC50"), tipping point (orange circle, "Tipping Pt," where established) and minimum viability EC_{50} from the NFA (blue triangle, "Min Cyto") to the median and interquartile range of ToxCast AC_{50} values from all positive assay endpoints from multi-concentration screening for a given chemical, as well as the "cytotoxicity burst" (inverted green triangles, "Burst") is illustrated. The numbers on the right-hand side of the figure indicate the number of multi-concentration assays the chemical was active in (numerator) compared to the total number of multi-concentration assays that chemical has been tested in (denominator). B, Histogram depicting the AC_{50} values from other ToxCast assays for the chemicals that were inactive in the NFA. Note that there are many instances of AC_{50} values well below the 20μM concentration that was tested in the NFA, indicating that inactive chemicals in the NFA do have activity in other assays, and are not just biologically inert.

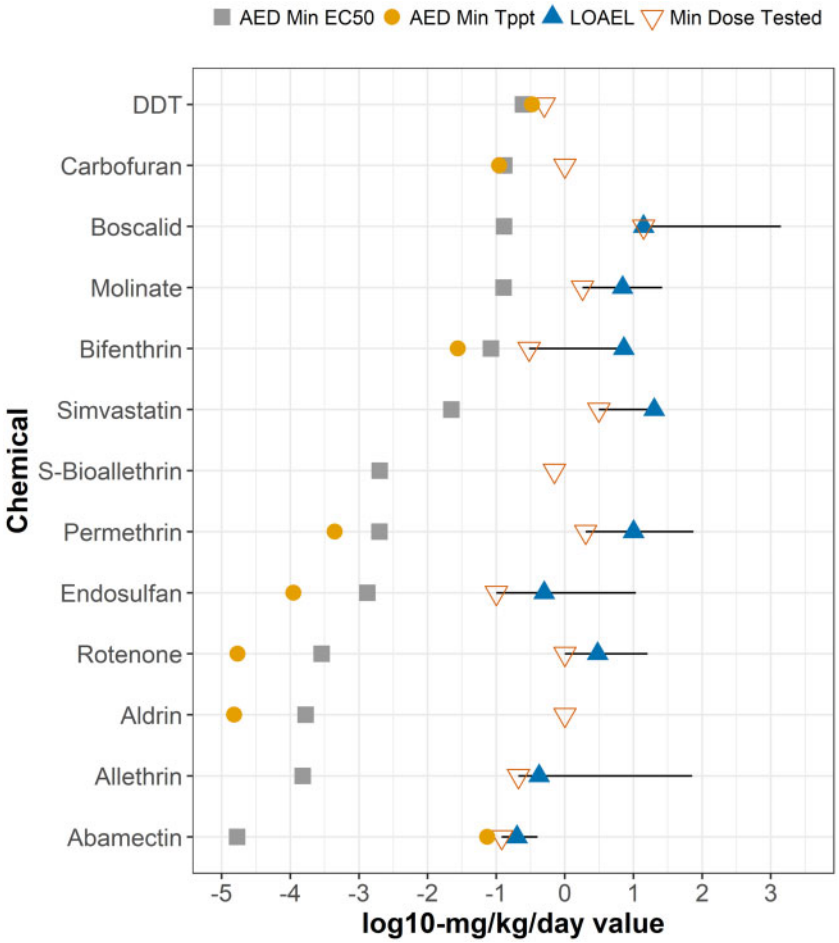

Figure 5. Comparison of administered equivalent dose *in vitro* with *in vivo* dose levels associated with neurodevelopmental effects. *In vivo* data represented as a range of doses tested (black horizontal line), the minimum dose tested (inverted red triangle, "Min Dose Tested") and the minimum observed LOAEL (blue triangle, "LOAEL") across studies, summarize the data in Table 2 on a log10-mg/kg/day scale. Administered equivalent doses (AEDs), also in log10-mg/kg/day, are illustrated for the minimum EC_{50} (gray box, "AED Min EC50") and the minimum tipping point (orange circle, "AED Min Tppt") from the NFA.

Analysis of "Toxicological Tipping Points"

Toxicological tipping points determine the critical concentration at which a biological system transitions from homeostasis to loss of fidelity (Shah *et al.*, 2016). In the case of network development, this is at the point wherein network development no longer proceeds in a manner that reaches a similar state of function after the 12-day development period evaluated in the present study (Frank *et al.*, 2018). As with the previous study, an analysis of the data was conducted to determine the critical concentrations (or tipping points) at which active chemicals perturbed the development of network function. Overall, tipping points were determined for 58 of the 83 active chemicals. Tipping points could not be determined for any of the 4 active PAHs, or for 6/9 active flame retardants. By contrast, tipping points were determined for 10/15 active pharmaceuticals, 7/11 active pyrethroids, 11/11 active organochlorines, and 8/10 active fungicides and herbicides. In all but 7 cases (abamectin, fenpropathrin, kepone, *p, p'*-DDT, *o, p'*-DDT, tamoxifen, and tert-butylhydroquinone) the tipping point occurred at a lower concentration than the EC_{50} value of the most sensitive network parameter.

Figure 3 compares the active chemicals in terms of their potency and selectivity rank. It is clear from this figure that a group of chemicals including but not limited to several pyrethroids (tefluthrin, bifenthrin, cyfluthrin, esfenvalerate) metals (tributyl tins, mercuric chloride, phenylmercuric acetate) and GABAergics (abamectin, kepone, aldrin, o, p-DDT) act with both high potency and selectivity. However, not all chemicals in these classes are highly potent and selective, as the pyrethroids fenpropathrin and S-bioallethrin are potent but not highly selective, as are organochlorines such as chlordane and mirex. As a group, the strobilurin fungicides and statin drugs tended to exhibit lower potency and specificity, as they were largely in the lower half, or lower left quadrant of this figure.

To compare the outcomes of the MEA-NFA to those of other ToxCast assays, the EC_{50} of the most sensitive network parameter, as well as the tipping point and minimum cytotoxicity value were plotted against AC_{50} values from the ToxCast assays in which that chemical was active. As the boxplots in Figure 4A illustrate, in many cases, the tipping point and/or minimum EC_{50} values obtained from the MEA-NFA are more potent than the lowest quartile of AC_{50} values from other ToxCast assays. Toward the top of this figure, the values from the MEA-NFA tend to not differ from the values obtained from other ToxCast assays, indicating that effects on network formation are not more sensitive than many other endpoints measured in ToxCast. However, toward the bottom of this figure, the EC_{50} and tipping point values more frequently fall outside of the lower quartile of potencies for ToxCast assays, indicating that network formation is more sensitive to the effects of these

Table 2. Chemicals With *In Vivo* DNT data

Chemical	Species	Dosing Period	Dose	Time	LOAEL	Critical endpoints	References
S-Bioallethrin	Rodent	Postnatal	0.7 mg/kg, oral	PND10–16		Neurochemistry	Pauluhn and Schmuck (2003)
Permethrin	Rodent	Postnatal	34 mg/kg, oral	PND6–15		Behavior, neurochemistry	Nasuti *et al.* (2007)
	Rodent	Prenatal	2–75 mg/kg, oral	GD10.5	10 & 50 mg/kg	Vascular morphology, behavior	Imanishi *et al.* (2013)
Bifenthrin	Rodent (rat)	Prenatal	0.3–9.0 mg/kg/day	GD6–21	7.2 mg/kg/day	Behavior	Nemec (unpublished data)
	Rodent (rat)	Perinatal	3.6 mg/kg/day	GD1-PND 21		Behavior/Biochemistry	Syed *et al.* (2016)
Bioallethrin	Rodent (mice)	Postnatal	0.7 mg/kg	PND10 & 16		Biochemistry	Eriksson and Fredriksson (1991)
	Rodent (mice)	Postnatal	0.72 & 72 m/kg	PND10–16		Biochemistry	Eriksson and Nordberg (1990)
	Rodent (mice)	Postnatal	0.21–4.2 mg/kg	PND10–16	0.42 mg/kg/day	Behavior	Ahlbom *et al.* (1994)
DDT	Rodent (mice)	Postnatal	0.5 mg/kg	PND10		Behavior/biochemistry	Eriksson *et al.* (1993)
Fenvalerate	Rodent (rat)	Prenatal	10 mg/kg	GD5–21		Behavior	Husain *et al.* (1992)
	Rodent (rat)	Postnatal	10 mg/kg	PND1–15		Biochemistry	Malaviya *et al.* (1993)
Diphenhydramine	Rodent (rat)	Prenatal	20 mg/kg, ip	GD16–21		Behavior/biochemistry	Moraes *et al.* (2004)
	Rodent (rat)	Prenatal	20 mg/kg, ip	GD0–21		Behavior	Chiavegatto *et al.* (1997)
Aldrin	Rodent (rat)	Prenatal	1 mg/kg sc	GD1–21		Behavior	Castro *et al.* (1992)
Boscalid	Rodent (rat)	Perinatal	14–1400 mg/kg/day	GD6-PND21	14 mg/kg/day	Behavior	Kaufmann, Schilling, Mellert, and van Ravenzwaay (unpublished data)
Rotenone	Rodent (rat)	Postnatal	1–16 mg/kg	PND5	3 mg/kg	Behavior	Ishido *et al.* (2017)
			1 mg/kg/day	PND5–8		Behavior	Ishido *et al.* (2017)
Endosulfan	Rodent (rat)	Perinatal	3.74–10.8 mg/kg/day	GD6-PND21	3.74 mg/kg/day	Body wt, food consumption	Gilmore, Sheets, and Hoss (unpublished data)
	Rodent (mice)	Perinatal	1 mg/kg	Preconception-PND21		Biochemistry	Wilson *et al.* (2014)
	Rodent (mice)	Postnatal	0.1 or 0.5 mg/kg	PND10	0.5 mg/kg	Behavior/biochemistry	Lee *et al.* (2015)
Carbofuran	Rodent (rat)	Prenatal	1 mg/kg/day	GD7–21		Biochemistry/behavior	Mishra *et al.* (2012)
Molinate	Rodent (rat)	Perinatal	1.8–26.1 mg/kg/day	GD7-PND11	6.9 mg/kg/day	Behavior/morphometrics	Horner (unpublished data)
Simvastatin	Rodent (rat)	Prenatal	3.125–12.5 mg/kg b.i.d.	GD6–17			
	Rodent (rat)	Postnatal	5–20 mg/kg p.i.d.		20 mg/kd b.i.d.	Behavior	Wise *et al.* (1990)
Abamectin	Rodent (Rat)	Perinatal	0.12–0.40 mg/kg/day	GD7-PND21	0.2 mg/kg/day	Body wt reductions	Moxon (unpublished data)

Table 3. Confusion Matrix for MEA-NFA and DNT Reference Chemicals[a]

	Actual Positive	Actual Negative	
Predicted positive	49	14	63
Predicted negative	2	11	13
	51	25	76

True positive rate (sensitivity) = True positives (49)/Known positives (63) = 0.78.
True negative rate (specificity) = True negatives (11)/Known negatives (14) = 0.84.
Precision = True positives (49)/(True positives [49] + False positives [2]) = 0.96.
Accuracy = (True positives [49] + True negatives [11])/(Known positives [63] + Known negatives [14]) = 0.78.
[a]DNT reference chemicals are based on Mundy *et al.* (2015). Negatives are as identified in the present paper and in Frank *et al.* (2017). Data for this table are drawn from the present study as well as Frank *et al.* (2017).

Table 4. Outcomes for Chemicals With Evidence of DNT From One Laboratory[a]

Chemical	Outcome
Abamectin	Hit, selective
Acetylsalicylic acid (aspirin)	Negative
Aldrin	Hit, selective
Atrazine	Negative
Boscalid	Hit, selective
Bifenthrin	Hit, selective
Busulfan	Negative
Carbofuran	Hit, not selective
Chlordane	Hit, not selective
Clodinafop-propargyl	Negative
Clothianidin	Negative
Cyfluthrin	Hit, selective
Cymoxanil	Negative
DDT	Hit, selective
Diphenhydramine	Hit, selective
Disulfoton	Negative
Endosulfan	Hit, not selective
Endrin	Negative
EPTC	Negative
Etofenprox	Hit, selective
Fenamiphos	Negative
Fenitrothion	Negative
Flufenacet	Negative
Molinate	Hit, not selective
Spirodiclofen	Negative
2-tert-butyl hydroquinone (TBHQ)	Hit, selective
Thiamethoxam	Negative
Triphenyl phosphite	Negative

[a]Based on Supplementary Table 2 from Mundy *et al.* (2015).

chemicals. This is particularly true for the pyrethroids and chemicals acting on the $GABA_A$ receptor, such as organochlorines and abamectin. The histogram in Figure 4B plots the AC_{50} values in other ToxCast assays for those 54 chemicals that were not active in the NFA assay. As this illustrates, many of these chemicals have biological activity with potencies well below the highest concentration tested here (20μM); thus, inactive chemicals in the NFA are not simply biologically inert.

In Figure 5, the AED based on the EC_{50} and or tipping point values from the NFA is compared to administered doses from studies that reported an adverse neurodevelopmental outcome *in vivo*. Although *in vivo* data and parameter estimates for HTTK were available for only 13 chemicals (Table 2), in 9/13 cases, AEDs based on the NFA were equivalent to or lower than minimum dose tested or the lowest observable adverse effect level (LOAEL). These data indicate that the levels at which changes in development of network ontogeny are observed *in vitro* are potentially relevant to doses that result in neurodevelopmental alterations.

DISCUSSION

The present study demonstrates the feasibility of screening large numbers of chemicals in the MEA-based NFA. In addition, the study demonstrates that the assay has high concordance and responds differentially to a wide variety of chemical classes. As outlined by Crofton *et al.* (2011), this assay was first evaluated using assay positive control chemicals (Brown *et al.*, 2016), and then with a larger "training set" (Frank *et al.*, 2017), wherein literature citations support their designation as DNT reference chemicals. Here, the testing of 136 unique chemicals, where the ability to cause DNT *in vivo* is not well established, represents the application of this assay to screening. In addition, the overall data set of chemicals tested in this assay is now over 200, which makes it one of the larger chemical sets tested in any DNT *in vitro* assay.

The present data provide an opportunity for further evaluation of the NFA beyond that in Frank *et al.* (2017). In the present experiments, 8 negative control chemicals were tested, and 7/8 were without any effect on ontogeny of network parameters, including 2 biological replicates of acetylsalicylic acid. The 1 negative control chemical that was active here was folic acid. In a previous study (Frank *et al.*, 2017), acetaminophen was tested as a negative control chemical, and found to be without effect on any network parameter, as were amoxicillin, glyphosate, sodium benzoate, propylene glycol, and saccharin. All of these chemicals are either approved for use during pregnancy, are sweeteners, are negative in many other DNT assays (glyphosate) or are on the FDA "Generally Regarded As Safe (GRAS)" list. In Frank *et al.* (2017), the sweetener sorbitol did alter ontogeny of some parameters of network function, however, it was without effect in the present study. Thus, of these 13 unique chemicals, only 2 (folic acid and sorbitol/glucitol) had activity and might be considered as "false positives." It should be noted that for both of these compounds, only 3 (folic acid) and 2 (sorbitol; See Frank *et al.*, 2017) of the 17 parameters of network function evaluated were altered sufficiently enough to generate EC_{50} values. The criterion for a "hit" defined here and in Frank *et al.* (2017) was the alteration of a single network parameter sufficiently to generate an EC_{50} value. From a screening perspective, this is conservative in that it may result in a greater number of false positives due to the large number of parameters being considered without any statistical correction.

With respect to chemicals that have *in vivo* evidence of DNT (Mundy *et al.*, 2015), only 3 additional chemicals (3, 3'-iminobispropanenitrile, methamizole, and parathion) beyond the 60 tested in Frank *et al.* (2017) were tested here, and all 3 were negative. However, 4 of the 60 chemicals from Frank *et al.* (2017) were repeated, with concordance in 3 of 4 cases (only DEHP was not concordant). In addition, 2 different salts of manganese were tested between the present study and Frank *et al.* (2017), with concordant results. As reported in Frank *et al.* (2017), the assay identified 49/60 DNT reference chemicals. Including the 3 tested here, the assay identified 49/63 positive chemicals.

These results from Frank *et al.* (2017) and the present study can be used to generate the Confusion Matrix in Table 3, which allows evaluation of assay performance. Based on the results from this study and that of Frank *et al.* (2017), the assay has a

sensitivity (correct identification of positives) of 0.78, a specificity (correct rejection of negatives) of 0.84 and a precision (or positive predictive value) of 0.96. It is not surprising that the sensitivity is the lowest of these 3 values. This is because the NFA does not evaluate *all* of the important processes that could be disrupted by developmentally neurotoxic chemicals. For example, if the DNT of a chemical were due primarily to alterations in neuroprogenitor proliferation or myelination, it is unlikely that it would be active in the NFA, as these neurodevelopmental processes do not occur in the culture model used here. It is for this reason that the concept of a battery of *in vitro* assays will be required (Fritsche *et al.*, 2018) for reliable DNT screening. By contrast, the higher specificity of the assay reflects a high rate of true negative responses, and a false positive rate of 16%. Whether this could be lowered without negatively impacting overall performance of the assay is a topic for future evaluations of the data. For example, by applying a more stringent treatment of the data such as correcting for the use of multiple endpoints, or by organizing the different endpoints into "domains" that might reflect different aspects of network function, this might decrease false positive responses such as those observed with folic acid and sorbitol. Finally, the precision indicates that when a chemical tests positive, that in most cases, it truly is positive, and any future treatments of the data should endeavor not to negatively impact this measure.

Several caveats should be kept in mind regarding the outcomes from the Confusion Matrix. First, the total number of known negative chemicals tested is small (13), and the balance between positive and negative chemicals is skewed (63 vs 13). In part, this is because it is difficult to identify truly negative chemicals. In this case, chemicals that are known not to result in DNT following exposure in mammals (including humans) are considered negatives. Identification and testing of additional negative chemicals may change the specificity and precision outcomes. Second, the results presented in Table 2 include chemicals that were active, whether or not they were selective. Elimination of chemicals that were not selective would decrease the true positive count and decrease the sensitivity of the assay. However, it cannot be ruled out that cell death via these chemicals might contribute to their DNT *in vivo*. In addition, the criteria for selectivity used here were conservative, and may have considered a chemical not selective simply because viability data were not available at concentrations above 20μM. Therefore, nonselective chemicals were not excluded from this evaluation. Finally, as stated above, this is only one assay out of a potential battery of assays, and in terms of using this battery for environmental decision-making, the overall performance of the battery as a whole will be more important than that of any individual assay.

The data to date also support that this assay provides highly consistent results. Ten chemicals were tested as biological replicates in the present study. Of these 10 chemicals, qualitatively concordant hitcalls were observed in all cases, and the most sensitive parameters had EC_{50} values that were less than 10-fold (in 5/7 cases, $<3\times$) different. In addition, 7 chemicals that were tested in Frank *et al.* (2017) were retested in the present experiments; 5/7 chemicals had concordant hitcalls, with sorbitol (glucitol) and DEHP the only chemicals that were not concordant between the 2 studies. Finally, there were 6 chemicals replicated between Brown *et al.* (2016) and Frank *et al.* (2017), with concordant results. Thus, of 23 total chemicals that have been replicated in the over 200 chemicals evaluated, only 2 have resulted in discordant hitcalls between the replicates (>91% concordance). There are several factors that may contribute to failure to replicate the hitcalls for DEHP and sorbitol. The sources of the compounds themselves were different, and a higher concentration range (up to 30μM) was tested in Frank *et al.* (2017). In addition, testing of these chemicals was separated by months or years between testing of replicates, as well as different staff members conducting the assays. Despite these differences, similar responses were observed between the 2 studies; effects of either compound just did not reach the criterion for a "hit" as defined here (Supplementary Figure 1). That only 2 discordant results were observed in 23 replicated compounds indicates that the assay provides robust results.

Finally, with respect to how the NFA compares to other assays in ToxCast, Figure 4 illustrates 2 important points. First, for many chemicals, EC_{50} and tipping point values from the NFA are among the most sensitive responses recorded for those chemicals in all ToxCast assays. As previous studies have demonstrated that the most potent effects in ToxCast assays can provide conservative estimates of points-of-departure for adverse *in vivo* effects (Thomas *et al.*, 2013) or can be used to provide an approach to prioritization for further testing (Wetmore *et al.*, 2013), it may be important to evaluate the potential for effects on neural network formation *in vitro* to ensure that points-of-departure based on *in vitro* data are conservatively protective for very potent and selective putative neurotoxicants/developmental neurotoxicants. Second, the NFA is neither overly sensitive, nor insensitive. Many chemicals active in the NFA are also active in other ToxCast assays at similar concentrations, suggesting that those chemicals that are especially potent in this assay are truly altering biological processes important to network development. Further, for those chemicals not active in this assay, part B of Figure 4 illustrates that these same chemicals demonstrate a range of potencies in other ToxCast assays, suggesting that it is possible to obtain negative responses in the NFA assay for chemicals that demonstrate other *in vitro* bioactivities in the same concentration range.

Based on the comparison of AEDs to *in vivo* minimum or LOAEL doses, changes in network development *in vitro* can provide a conservative estimate of the levels at which adverse neurodevelopmental outcomes might be expected, consistent with previously demonstrated outcomes for other ToxCast assays (Thomas *et al.*, 2013). Although this analysis indicates that concentrations at which changes in network development are observed *in vitro* are relevant to *in vivo* doses, there are important caveats to consider. The values here are administered dose levels, and do not reflect brain concentrations of these chemicals, which may be either lower or higher than the estimated steady-state plasma concentration used to derive an AED. Further, the specific *in vitro* to *in vivo* extrapolation decisions made herein (eg, to use the steady state concentration approximation corresponding to the upper 95th percentile of the distribution for the population and restrictive clearance) may have resulted in lower AED estimates to produce plasma concentrations equivalent to the *in vitro* bioactive concentrations. In the future, improvements to modeling of brain concentrations and *in vitro* to *in vivo* extrapolation techniques overall may improve confidence in AED values corresponding to NFA activity. In addition, metabolism of the chemicals tested *in vitro* is not measured even if it is likely to be minimal As such, it remains an uncertainty in this vitro to *in vivo* extrapolation.

For the majority of the chemicals tested in the present experiments, their potential to cause DNT *in vivo* is not well established (data for most of the positive chemicals in Table 3 were drawn from Frank *et al.*, 2017). As such, they could be considered "unknowns." Several different classes of chemicals

were screened in the present study, and therefore rather than a chemical by chemical evaluation of the results, examination by chemical class may be more informative and efficient. In addition, the results in the present experiment were relatively consistent within classes of chemicals. Among the more active and selective chemical classes were the pyrethroid and organochlorine insecticides, as well as the metals. By contrast, among the least active classes in the present assay were the PAHs, organophosphates and neonicotinoids.

These results could be informative as part of an Integrated Approach to Testing and Assessment (IATA; Bal-Price *et al.*, 2018; Casati, 2018; Worth and Patlewicz, 2016;) to evaluate these individual (or classes of) chemicals for potential DNT. In this respect, there were 28 chemicals tested in the present study (Table 4) that were listed in Supplementary Table 2 of Mundy *et al.* (2015) as having evidence of DNT from a single laboratory. For the 13 chemicals that were positive in the NFA assay, this provides additional support of potential DNT hazard from a "weight of evidence approach." By contrast, for the 15 chemicals that were negative in the NFA, it would be less than conclusive, and additional data or knowledge may be needed. For example, both clothianidin and thiamethoxam were listed in the Supplementary Table 2, but were negative in the NFA assay. However, the lack of activity of the neonicotinoid insecticides in the present assay is consistent with previous results in our laboratory, wherein nicotine and neonicotinoid insecticides are generally without activity in our cortical cultures (Mack *et al.*, 2014; McConnell *et al.*, 2012; Valdivia *et al.*, 2014). Thus, an IATA may give the results of the present study less weight in a weight of evidence approach when considering the neonicotinoids. By contrast, the results with the organophosphate insecticides may be misleading, as chlorpyrifos oxon was a potent inhibitor of NFAs (Frank *et al.*, 2017), and when testing a larger set of 27 organophosphates, approximately 1/3 of them were active (unpublished results). Finally, consider results with flame retardants and PAHs in the current study. Nine of 14 flame retardants were active in the current study, but only 2 were selective, whereas only 4/17 PAHs were active (only 1 selective). By contrast, 9/10 flame retardants and 11/14 PAHs altered parameters of cardiac function, with the majority of both being selective after 30 min of exposure (Sirenko *et al.*, 2017), suggesting that an IATA for these classes might give greater concern to cardiac effects.

Use of an IATA approach with this dataset could also incorporate comparisons of the results from this assay to other *in vitro* assays. A comparison of the potency and tipping point values for the MEA-NFA to AC_{50} values from the ToxCast battery of assays indicates that this assay can provide "value added," possibly by identifying the nervous system as a sensitive target for some chemicals, or by identifying other chemicals that may be "promiscuous" (active in a number of *in vitro* assays). Among the chemical classes where network formation was a more sensitive endpoint than other ToxCast assays were many known neurotoxic/neuroactive chemicals, including the pyrethroids, organochlorines, and heavy metals. Surprisingly, although they were not selective, the strobilurins and statins were also active in the NFA and generally more potent in this assay than many other ToxCast assays. Because many chemicals were not more potent in this assay when compared to their effects in other ToxCast assays, this indicates that the assay is also not simply very sensitive to chemical effects in general. For example, of the 4 active PAHs, benzo(b)fluoranthrene was the only chemical where the minimum EC_{50} value was in the lower quartile of all ToxCast values, suggesting the actions of these chemicals on network formation are not among their more potent *in vitro* effects. Similarly, of the flame retardants, only BDE-47 (2, 2', 4, 4'-tetrabromodiphenyl ether) had tipping point and EC_{50} value that was lower than the lowest quartile of ToxCast assays. In these cases, an IATA might then rank DNT as a lower concern than other endpoints (except in the case of BDE-47). Finally, this analysis sheds some interesting light on the positive outcome with folic acid, which appears to have a broad range of potencies across the various ToxCast assays. The minimum EC_{50} values in the NFA falls very near the median for all of the ToxCast assays, indicating that the outcome here may simply reflect general biological activity of folic acid, and not any sort of specific effect on network development.

These results begin to provide one approach to prioritizing chemicals for additional testing or review of other available data. By comparing the potency and selectivity of the chemicals amongst each other in this assay (eg, Figure 3), one could develop a prioritized list of chemicals. Comparing the outcomes of the NFA to other ToxCast assays where data are available for the same chemical (eg, Figure 4), could be used to refine this list. Finally, one could use estimates of *in vivo* exposure to further select the chemicals that are most likely to be present at levels that overlap with concentrations in this assay that alter development of network activity.

In summary, the present results demonstrate that screening and prioritization of potential DNT using the NFA is feasible, and that this assay provides excellent sensitivity, specificity and concordance. The results can be compared to outcomes from other ToxCast assays, incorporated in to IATAs and used to select chemicals for additional testing and hazard characterization. That other laboratories have demonstrated alterations in network activity following chronic (Dingemans *et al.*, 2016) or developmental exposures (Hogberg *et al.*, 2011) indicates that the approach can be adopted across laboratories, although standardization of approach is needed. As such, this assay can be a valuable addition to a battery of higher throughput approaches to characterizing chemicals for DNT hazard.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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