Appendix A. In Vitro High-Throughput Screening Assay Systems

A.1. Introduction

OEHHA evaluated high-throughput screening (HTS) *in vitro* data to examine whether such data provided information relevant to mechanisms of action of the seven US FDA-batch certified synthetic food dyes (see Chapter 1, Table 1.1 for dyes evaluated). New approach methodologies (NAMs) based on *in vitro* data integrated with mechanistic data targeting neurological processes may aid in profiling the potential modes of action and effects of these chemicals (Rusyn and Greene 2018). One of the most robust HTS databases is US EPA's publicly available Toxicity Forecaster (ToxCast™) database (Judson et al. 2016; Sipes et al. 2013). As of 2018, ToxCast encompasses more than 9,000 tested chemicals, and more than 1,000 HTS assays. OEHHA developed an approach to profile the food dyes and their metabolites using the ToxCast results for specific molecular targets underlying neurological processes. The results were used to rank the food dyes by their bioactivity and potency for potential target markers using the Toxicological Prioritization Index software.

A.2. Challenges in interpreting HTS data

HTS data can lead to improved chemical screening, reduced data gaps, and provides a basis for prioritization for further research and risk assessment. However, when interpreting HTS data from databases such as ToxCast, it is important to note the challenges and limitations of such data. ToxCast assay results are generally evaluated based on bioactivity and potency (AC_{50S}), efficacy (minimal flags), and cytotoxicity limits; the latter two are components that should be considered when evaluating uncertainty in the results. Data flags are warnings for potential false positive and false negative findings based on methods. In ToxCast, flag assignment is automated and thus prone to some error. Currently, the cytotoxicity limit is defined as the lower bound of the prediction of the median cytotoxicity and therefore is predicted to be lower than many assay hits. Determining the appropriate cytotoxicity threshold is key to differentiating false positives based on bioactivity. Although understanding data quality flags and cytotoxicity thresholds are pivotal to interpreting ToxCast data, filtering out AC_{50}s because there are data flags or the AC_{50} is above cytotoxicity limits is not recommended because such an approach would lead to a marked decrease in the number of candidate assays. Further, as data and methods are optimized, the output of assay AC_{50}s may change, and thus the current flags and cytotoxicity limits will change. Instead, an integrated understanding of the flags and cytotoxicity limits for each assay can assist in identifying potential interference, and can be useful for considering an assay for greater scrutiny and interpreting the significance of the bioactivity and the uncertainty in the result, rather than as a cutoff for relevancy of assay responses (Judson et al. 2016).

A.3. Methods: evaluating food dyes based on *In Vitro* data

Initially, OEHHA screened the food dyes in publicly available aggregate databases including the Comparative Toxicogenomics Database (CTD, Comparative Toxicogenomics Database (CTD); (Davis et al. 2019)), Chemical Hazards Data Commons (CHDC, Chemical Hazards Data Commons (CHDC)), and the Chemistry Dashboard (Williams et al. 2017) to evaluate whether there were any known associations between the food dye chemicals and neurological process targets linked to toxicity *in vivo*. In particular, OEHHA evaluated the food dyes in the Chemistry Dashboard in seven developmental neurotoxicity (DNT) lists to see if there were any hazards established for the food dyes. Presence on these lists would indicate that the dyes demonstrated some effects on neurodevelopment in humans or triggered DNT *in vivo* in animal toxicology studies based on the list sources. However, these aggregate databases yielded limited information on the chemicals in relation to neurodevelopmental processes. For more detailed information on these databases, refer to the last section of this Appendix.

Based on limitations of these initial screening methods, OEHHA developed an approach to map potential associations between the synthetic food dyes and neurological activity based on existing *in vitro* data. OEHHA evaluated the seven dyes as well as the metabolites of the azo dyes (Red No. 40, Yellow No. 5, Yellow No.6), which are known to be primarily metabolized in the gut (Table A.1). This approach is based off of the efforts of Iyer at al. (2019) to integrate different data streams in an effort to characterize chemicals of potential concern that may affect cancer pathways. Using a similar approach, we incorporated a strategy for 1) linking the potential molecular targets examined in assays to neurological processes, and 2) using chemicals with known DNT endpoints to look for potential neurological markers. Toxicological Prioritization Index (ToxPi) visualization software was used to rank the relevant chemical activity observed.

Table A.1: Known metabolites of FDA-certified synthetic azo dyes.

CASRN	Chemical	Known Metabolites
25956-17-6	Red No. 40	cresidine-4-sulfonic acid (2-methoxy-5-methylaniline-4-sulfonic acid) **(6471-78-9);** ANSA (1-amino-2-naphthol-7-sulfonic acid) (**116-63-2)**
1934-21-0	Yellow No. 5	sulfanilic acid (1-amino-4-benzenesulfonic acid) (**121-57-3**); 1-amino-2-naphthol-6-sulphonic acid (**5639-34-9**); (4-ABS) 4-aminobenzenesulfonic acid
2783-94-0	Yellow No. 6	sulfanilic acid (1-amino-4-benzenesulfonic acid) (**121-57-3**); 1-amino-2-naphthol-6-sulphonic acid (**5639-34-9**); (4-ABS) 4-aminobenzenesulfonic acid; aminopyrazolone

A total of 283 ToxCast assays were identified for evaluation at the time of our data collection (May 30, 2019 and April 20, 2020). Chemical quality information from the Chemical Dashboard is provided in Table A.2 below.

Table A.2: Chemical Quality Control information for the seven food dyes from the Chemistry Dashboard (retrieved January 2, 2021).

Dye	Name	CASRN	DSSTox Substance ID	Tox21 ID	QC Grade
Blue No. 1	Brilliant Blue	3844-45-9	DTXSID2020189	Tox21_300516	T0; (Z) MW Confirmed, no purity info T4; (Z) MW Confirmed, no purity info
Blue No. 2	Indigo Carmine	860-22-0	DTXSID1020190	Tox21_113456	T0; (ND) Not Determined, Analytical analysis is in progress T4; (A) MW Confirmed, Purity > 90%
Blue No. 2	Indigo Carmine	860-22-0	DTXSID1020190	Tox21_302732	T0; (C) MW Confirmed, Purity 50-75% T4; (Fns) CAUTION, No Sample Detected; Biological Activity Unreliable
Green No. 3	Fast Green	2353-45-9	DTXSID3020673	Tox21_302086	T0; (A) MW Confirmed, Purity > 90% T4; (A) MW Confirmed, Purity > 90%
Red No. 3	Erythrosine	16423-68-0	DTXSID7021233	Tox21_202932	T0; (ND) Not Determined, Analytical analysis is in progress T4; (Cc) CAUTION, Low Concentration, Concentration 5-30% of expected value
Red No. 3	Erythrosine	16423-68-0	DTXSID7021233	Tox21_302085	T0; (ND) Not Determined, Analytical analysis is in progress T4; (F) CAUTION, Incorrect MW Biological Activity Unreliable
Red No. 40	Allura Red	25956-17-6	DTXSID4024436	Tox21_300393	T0; (A) MW Confirmed, Purity > 90% T4; (A) MW Confirmed, Purity > 90%
Yellow No. 5	Tartrazine	1934-21-0	DTXSID1021455	Tox21_113411	T0; (F) CAUTION, Incorrect MW Biological Activity Unreliable T4; (I) ISOMERS, two or more isomers detected
Yellow No. 5	Tartrazine	1934-21-0	DTXSID1021455	Tox21_201539	T0; (A) MW Confirmed, Purity > 90% T4; (F) CAUTION, Incorrect MW Biological Activity Unreliable
Yellow No. 5	Tartrazine	1934-21-0	DTXSID1021455	Tox21_300554	T0; (A) MW Confirmed, Purity > 90% T4; (Ac) CAUTION, Low Concentration Concentration 5-30% of expected value
Yellow No. 6	Sunset Yellow	2783-94-0	DTXSID6021456	Tox21_201897	T0; (A) MW Confirmed, Purity > 90% T4; (A) MW Confirmed, Purity > 90%
Yellow No. 6	Sunset Yellow	2783-94-0	DTXSID6021456	Tox21_300407	T0; (A) MW Confirmed, Purity > 90% T4; (A) MW Confirmed, Purity > 90%

The method for selecting the assays involved several criteria.First, ToxCast assays from the NovaScreen (NVS), Attagene (ATG), and Tox21 platforms were selected to assess target binding as an indicator of protein activity, translated as an association between receptor binding and potential effect. We initially explored just these three platforms to demonstrate a proof of concept while maintaining manageability. There were 108 NVS

assays, 50 ATG assays, and 24 Tox21 assays selected based on whether the assays: 1) had a neurological-related gene target; 2) were conducted in brain tissue (regardless of species); or 3) targeted the specific receptors of aryl hydrocarbon, androgen, estrogen, or the thyroid hormone. The neuro-relevant HTS assays selected also include the 15 assays identified by (Spinu et al. 2019) as applicable to adverse outcome pathways (AOPs) known to induce neurotoxicity. Assays in the first criterion were identified by expert judgment. Scientific literature identified through PubMed (PubMed) was used to support putative neurological target associations. Furthermore, the CTD provided curated associations (published gene-disease relationships) based on peer-reviewed literature to support links between a molecular target and outcomes/diseases in the category of "neurological or developmental disorders". This category includes conditions such as motor skills disorders, developmental disabilities, neural tube defects, neurotoxicity syndromes, and prenatal exposure delayed effects. OEHHA used CTD and its curated associations to confirm genes potentially connected to neurodevelopmental mechanisms and/or neurological disorders to determine relevant targets of interest. For the second criterion, assays were included if their assay description listed "brain" as tissue. These were selected regardless of species.

The rationale to include the third criterion was based on literature reporting interactions between food dyes and these receptors (Axon et al. 2012; Dees et al. 1997; Jennings et al. 1990; Mathieu-Denoncourt et al. 2014; McCarthy, 2008; McEwen et al., 2012; Wu et al. 2019; Choudary et al., 2020). There is substantial neuroendocrinology literature demonstrating several pathways by which estrogen regulates many neuronal processes, including behavior (McEwan et al., 2012). Estradiol actively modulates many processes, both genomic (e.g., modulating gene expression) and non-genomic (e.g., interactions with cell membrane receptors resulting in direct, rapid effects on synaptic function involving cells with varied neurotransmitter systems) in the brain. Furthermore, ARs and ERs often act and regulate in concern, and both nuclear and non-nuclear androgen receptors are present in cells in many parts of the brain. Although not specifically with food dyes, the aryl hydrocarbon receptor has been shown to mediate zebrafish neurogenesis and gliogenesis (Wu et al., 2019) and has been linked to the mediation of neurological activities in other studies (Wójtowicz et al., 2017; Choudhary et al., 2020). Such interactions may have downstream effects on targets underlying neurological processes, and therefore, these assays were pertinent to explore as well. Assays were not included if they did not clearly meet any of these three categories. Cell viability assays from these platforms were identified but were not included in the evaluation of target markers related to neurological processes. There were 183 total assays from these three criteria.

Table A.3: Organophosphates with known DNT/neurotoxicity activity.

Chemical	CASRN
Acephate	30560-19-1
Carbaryl	63-25-2
Carbofuran	1563-66-2
Chlorpyrifos	2921-88-2
Chlorpyrifos-oxon	5598-15-2
Dichlorvos	62-73-7
Dicrotophos	141-66-2
Methamidophos	10265-92-6
Methyl Parathion	298-00-0

To further expand the assay coverage, another subset of assays were identified based on potential neurological process markers from ToxCast data for known DNT candidates, such as pesticides. The identification of chemicals with DNT potential was based on studies from the California Department of Pesticide Regulation's (DPR) database. Candidate chemicals were determined by evaluating DPR's Risk Characterization Documents (RCDs). For more information on these studies, refer to the chemical-specific RCDs (DPR 2005-2018). Identified pesticides were then screened to see which were tested in ToxCast. Organophosphates (OPs) were of particular interest, given their presence in one of the eight AOPs highlighted by Bal-Price (2017) as being relevant to DNT. There were nine OP pesticides in the DPR database that are tested in ToxCast (Table A.3). All of the ToxCast assays were then screened across these pesticides. Assays were selected as potential markers if they were a hit for at least 3 pesticides (activity in a third of the total pesticides evaluated); as a result, 63 ToxCast assays were identified for this subset.

Lastly, oxidative stress and inflammation are proposed mechanisms linking the food dyes with potential downstream effects leading to toxicity. We took the subsets of assays in Iyer et al. (2019) categorized under "induction of oxidative stress" and "induction of chronic inflammation" and screened the chemicals through the 50 assays from these subsets as well. Refer to Figure A.1 for the flow chart of methodology. Further details on the development of the full assay set can be found in Appendix C.

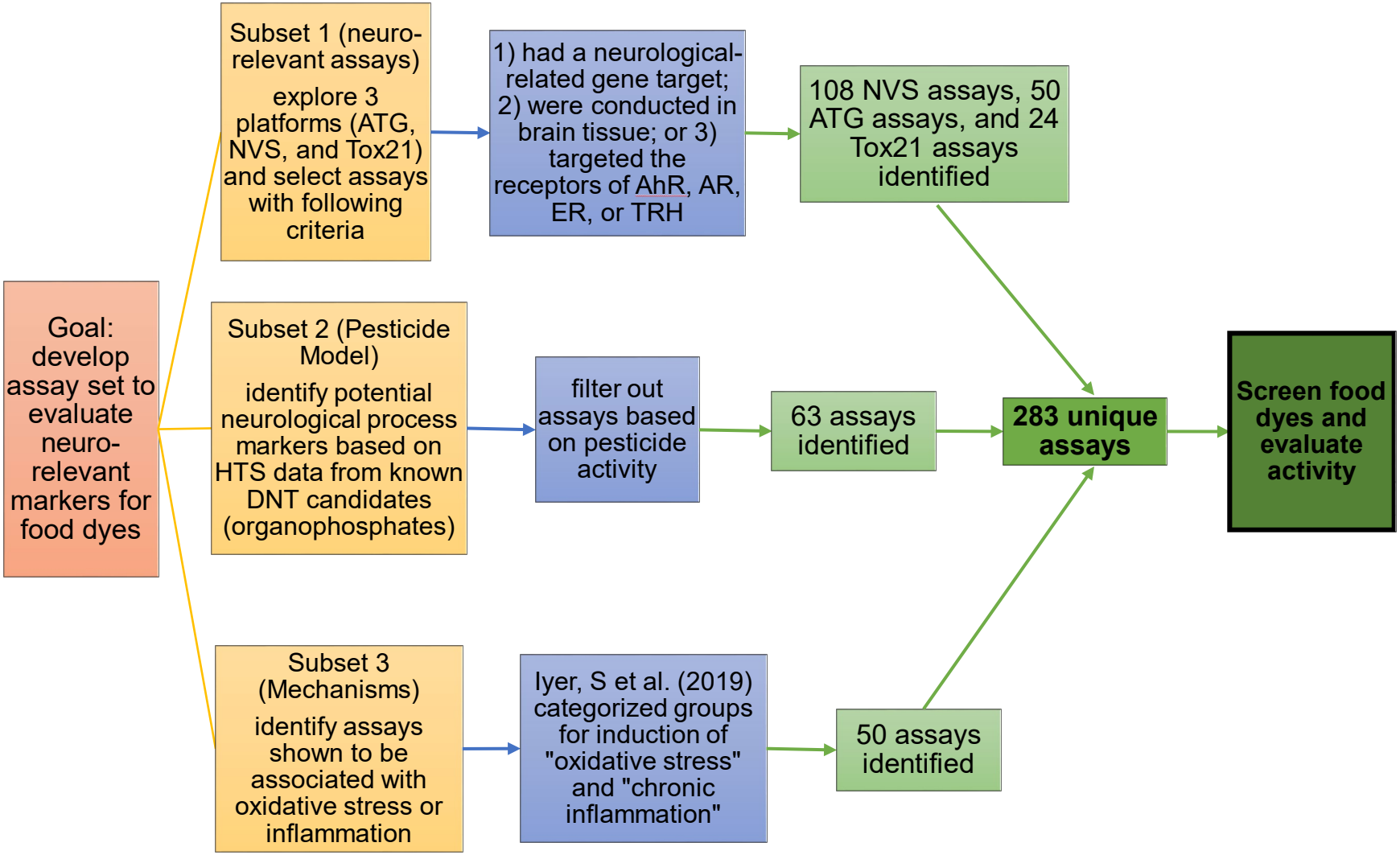


Figure A.1: Method flow chart for developing the 283 assay list used to screen food dyes.

A.3.1 ToxPi data

Using the Toxicological Prioritization Index (ToxPi) software (version 2.3; Marvel et al., 2019), we assessed the NVS assay subset further. Only data from this subset was selected for input into the ToxPi software in order to preserve the categorical comparisons and maintain manageability in the resulting outputs. The simplicity of this selection allowed us to make direct comparisons between the chemicals in enzymatic and receptor signaling assay activities. The ToxPi software calculates a unitless index score that represents a relative ranking of biological activity across multiple assays. This output can be used to rank order the food dyes to inform relative potency and activity. A ToxPi image is composed of "pie" slices that represent individual components being compared, or aggregations of multiple-related components. For our approach, each ToxPi represented a food dye, and slices represented assays that fell into one of six types of the NVS Intended Target Family Subtype (as categorized by the Chemistry Dashboard) (Figure A.2).

Input data for the software are the AC_{50}s of active chemical-assay pairs. Inactive assays are assigned an AC_{50} of 10^6 in order to use the ToxPi scaling, $-\log_{10}(AC_{50}) + 6$. Each ToxPi slice length is proportional to the normalized potency of the assay values ($-\log_{10}(AC_{50}) + 6$) included in that slice (UNC 2009). For the ToxPi analysis, weighting was applied based on the number of mapped "assay component endpoints" making up the slice. There were:

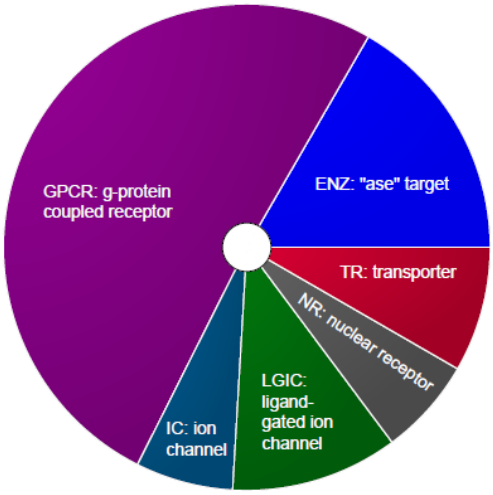
- 18 assay component endpoints for NVS_ENZ; enzymatic assays with intended targets often ending in "ase". Targets include acetylcholinesterase (AChE), adenylyl cyclase, thyroid peroxidase, and monoamine oxidase.
- 55 assay component endpoints for NVS_GPCR (G-protein coupled receptor). Targets include angiotensin, dopamine, adenosine, serotonin, opiate, adenosine, adrenoceptor, cholinergic, GABA, glutamate, and tachykinin.
- 7 assay component endpoints for NVS_IC (ion channel assays); assays conducted in brain tissue. Targets include channels for calcium, potassium, and sodium.
- 12 assay component endpoints for NVS_LGIC (ligand-gated ion channel assays). Targets include receptors for GABA, nicotinic cholinergic, glutamate, and glycine.
- 7 assay component endpoints for NVS_NR and NVS_OR (combination of two types of assay component endpoints). Targets include receptors for thyroid hormone, androgen, estrogen, and aryl hydrocarbon.
- 9 assay component endpoints for NVS_TR (transporter assays). Targets include transporters for neurotransmitters, nucleosides, and vesicular monoamine.

These values sum up to 108 assays in the NVS subset. To correspond with the variable numbers of assay component endpoints within each ToxPi slice, weights of 18, 55, 7, 12, 7, and 9, were applied for each of these slices, respectively. The slices were normalized so that their percent contributions summed up to 100% (Figure A.2). Using

this approach, the food dyes were ranked by activity. For more information on which assays were included for each slice, refer to Appendix C.

Figure A.2: ToxPi slice breakdown.

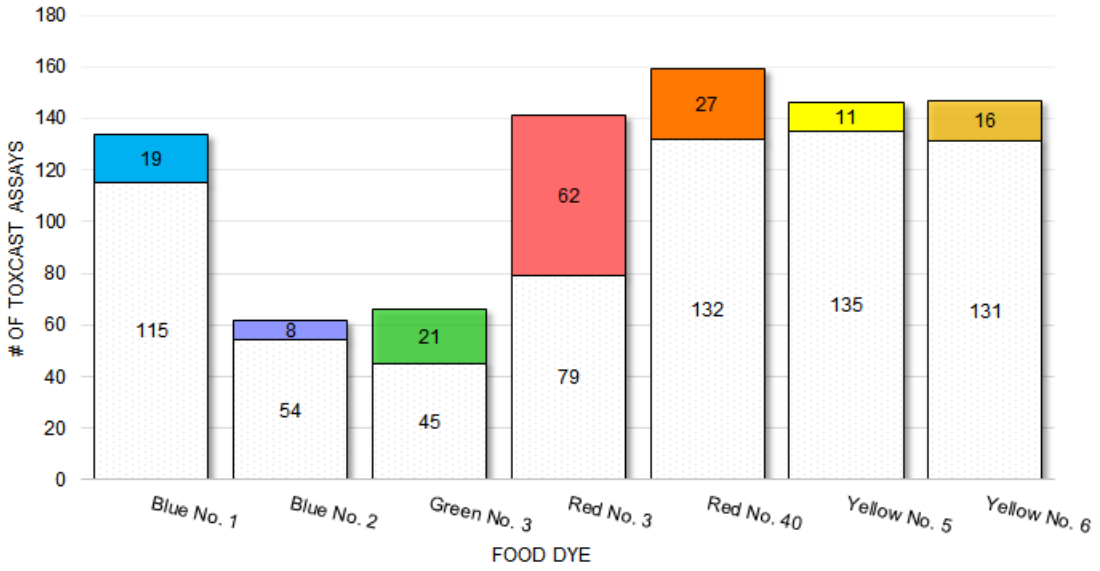
Slice	Weight	Targets Included
GPCR	55 (50.5%)	G-protein coupled receptors: opiate, dopamine, cholinergic, serotonin
ENZ	18 (16.7%)	acetylcholinesterase, monoamine oxidase, peroxidase
TR	9 (8.3%)	neurotransmitter transporters
NR (OR)	7 (6.5%)	nuclear receptors: androgen, estrogen, glucocorticoid, thyroid hormone
LGIC	12 (11.1%)	Ligand-gated ion channel receptors: glutamate and GABA
IC	7 (6.5%)	ion channel assays tested in brain tissue



Grouping of the NVS assay subset by NVS Intended Target Family Subtype. Respective weights were applied to each slice so that they could be normalized to one another and their percent contributions would sum up to 100%.

A.4. Results

Figure A.3: Food dye activity in ToxCast assay subset.



A total of 283 assays were evaluated; not every dye was tested in all 283 assays. Colored bars indicate number of active assays for each chemical; dotted bars below indicate number of inactive assays. For example, there were19 assays active for Blue No. 1 out of 134 assays tested.

This section briefly summarizes the HTS results for food dyes in the ToxCast assays associated with neuro-relevant target markers. Overall food dye activities in the assays are shown in Figure A.3; results are for chemical-assay pairs deemed active by US EPA. The mapping of food dye activity to potential targets in neurodevelopmental processes are summarized in Table A.4. For expanded details of ToxCast assay selection and results, refer to Appendices C and D. There were a total of 27 viability assays in the subset, but these were not included in our overall evaluation. Flags and potential cytotoxicity limits should be taken into consideration when evaluating results, although as noted above, should not be used to dismiss the relevance of a particular result.

Red No. 40 was tested in in the most number of assays in this set, but Red No. 3 had the most activity. Red No. 3 was active for all neuro-relevant molecular targets it was tested in; however, this dye was not tested in several pertinent neuro-relevant molecular targets. Like Red No. 3, Green No. 3 was also active in assays for all neuro-relevant molecular targets it was tested in; however, the dye was only tested in a select few (Table A.4). Although the two yellow dyes were tested in as many assays as the red dyes, they had much less activity, comparatively. The relatively low assay activity by Blue No. 2 can be attributed to the fact that this dye was tested in the least amount of assays; activity was not observed for Blue No. 2 with GPCRs, ion-channel receptors, or enzymes such as hydrolases, esterases, peroxidases, and oxidases.

Both red and yellow dyes had a range of activity in the assays mapped to GPCRs and were active in assays targeting a range of dopaminergic and opioid receptor subtypes. The trimethylamine dyes (blue dyes and Green No. 3) were not tested in many GPCR assays, and therefore observations of their activity are inconclusive. Only Blue No. 1 was tested in assays mapped to serotonergic receptors and had a hit for subtype 5HT7 (also a hit for Red No. 40 and both yellow dyes). The GPCR ion channels, glutamate and GABA, were not tested extensively in the food dye set, and only slightly among the pesticides. Pesticides were not tested in assays targeting the glutamatergic receptors, and although some were tested in assays mapped to the GABA receptors, only chlorpyrifos had a hit for one assay.

Assays mapped to the nuclear receptors for androgen, estrogen, and thyroid hormone were tested across all the food dyes. Their extended coverage compared to the other molecular targets is due to a higher number of these assays from platforms ATG and Tox21. All the food dyes were active for the androgen receptor assays that they were tested in. The dyes, except for Blue No. 2 and the yellow dyes, were active for the receptor-based antagonist assays for the estrogen receptor, potentially indicative of antagonism for this receptor. Except for the yellow dyes, all other dyes were active for antagonist assays for the thyroid hormone receptor. Red No. 3, Red No. 40, Blue No. 1, and Green No. 3 were also active for an assay mapped to thyroid peroxidase (TPO). This assay measures TPO activity as a loss of signal; TPO inhibition may lead to a decrease in thyroid hormone synthesis, which ultimately could lead to altered neurodevelopmental processes (AOP-Wiki, AOP 4). These same four dyes were also all active (and the only dyes tested) for an assay targeting the glucocorticoid (GC) receptor NR3C1. We noted that there were some overlap between the pesticides evaluated and the food dyes for the active assays targeting the receptors androgen, estrogen, thyroid hormone, and glucocorticoid.

All the dyes were tested and active for assays mapped to the aryl hydrocarbon receptor. Yellow No. 5 was the only dye associated with downregulation of the gene, while all other dyes were associated with upregulation. Red No. 3 was the only dye with activity for monoamine oxidase (it was also the only dye tested for monoamine oxidase). The food dyes were not tested in assays mapped to the targets AChE and adenylyl cyclase. Like the food dyes, the pesticides were also not tested in the assays targeting adenylyl cyclase. However, several pesticides were active for assays targeting AChE (carbaryl, carbofuran, chlorpyrifos, chlorpyrifos-oxon, and dichlorvos).

A.4.1 Oxidative stress and inflammation pathways

All the assays mapped to the induction of oxidative stress and inflammation (Iyer et al. 2019) were from the Bioseek platform (BSK). The molecular targets for these assays covered a variety of cytokines, including chemokines, interleukins, and growth factors. Of the seven food dyes, only Red No. 3 had activity in these assays, all of which were associated with the downregulation of the signal.

A.4.2 Metabolites

The activity for azo dye metabolites (Red No. 40, Yellow No. 5, Yellow No. 6) were explored in this current assessment. Of the six metabolites, four (cresidine-4-sulfonic acid, 1-amino-2-naphthol-7-sulfonic acid, sulfanilic acid, and 1-amino-2-naphthol-6-sulphonic acid) were found on the Chemistry Dashboard, but none were tested in ToxCast.

Table A.4: Summary of food dye activities in *in vitro* assays.

The "Molecular target" column addresses both protein and related receptors. "Pathways" represents suspected modes of action by the food dyes potentially linked to DNT. A "✓" represents a hit in at least one of the assays mapped to that target for that food dye (regardless of how many total assays were active or inactive for that target). Active hits do not differentiate between receptor subtypes or species. A "-" represents a chemical that was tested but inactive in all assay(s) mapped to the molecular target. "NT" means not tested and denotes that the food dye was not tested in assays related to the receptor. Active viability assays were not regarded as hits for the molecular targets. Comments under "Notes" indicate activity for different target subtypes. AOPs mentioned in this section do not imply a direct association to neurological outcomes, but instead are general AOPs linked to the molecular target. Supporting data for this table can be found in appendices C and D.

Molecular Target	Blue No. 1	Blue No. 2	Green No. 3	Red No. 3	Red No. 40	Yellow No. 5	Yellow No. 6	Notes
GPCRs								
Adenosine: agonism linked to neurotoxicity; receptors predominantly expressed in the brain	NT	NT	NT	✓	NT	NT	✓	Red No. 3 active for assay targeting A1, while Yellow No. 6 active for assay targeting A2a
Adrenoceptor: inhibits adenylate cyclase. Involved in release of NTs from nerves and adrenergic neurons in CNS	NT	NT	NT	NT	✓	-	✓	Red No.40 active for assay targeting α2c; Yellow No. 6 active for assay targeting α2a.
Dopaminergic: predominantly expressed in brain and CNS. Receptors regulate neuronal growth and development, and modulate behavioral responses	NT	NT	NT	✓	✓	✓	✓	Red No. 3, Red No. 40, Yellow No. 5, and Yellow No. 6 all active for assay targeting D1. Red No. 40 and Yellow No. 6 active for assays targeting D2 and D4; Yellow No. 5 active for assay targeting D4
Gamma-aminobutyric Acid: receptor for inhibitory NT in mammalian brain	NT	NT	NT	NT	NT	NT	NT	No dyes tested in subset assays mapped to this target. Target linked to AOP 10.[1]
Glutaminergic: dysregulation of receptor and associated NMDA receptors linked to abnormal neuronal development, abnormal synaptic plasticity, and neurodegeneration	NT	NT	NT	NT	-	NT	-	Red No. 40 and Yellow No. 6 were tested in an assay targeting Grik1 but both were inactive. Glutamate receptor binding is a key event in the two AOPs relevant to DNT and NT. Target linked to AOP 48. [1]
Muscarinic (cholinergic): binding of AChE leads to responses such as adenylate cyclase inhibition and potassium channel mediation	NT	NT	NT	NT	✓	-	✓	Red No. 40 active for assays targeting m2, m3, and m5; Yellow No. 6 active for assay targeting m3
Nicotinic (cholinergic): ion channels serving as muscle and neuronal receptors in CNS	NT	NT	NT	NT	✓	NT	NT	Red No. 40 active for assay targeting α2
Opioid: expressed in the brain. Agonist-mediated activation leads to the modulation of many biological functions	NT	NT	NT	✓	✓	✓	✓	Red No. 3 active for assay targeting μ1; Red No. 4 active for assay targeting δ1; Yellow No. 5 and 6 active for assays targeting κ1
Serotonergic: found in the central and peripheral nervous system; mediate both excitatory and inhibitory neurotransmission	✓	NT	NT	NT	✓	✓	✓	Blue No. 1, Red No. 40, Yellow No. 5, Yellow No. 6 active for assays targeting 5HT7. Red No. 40 active for assays targeting 5HT1, 5HT3, and 5HT4; Yellow No. 5 active for assay targeting 5HT4; Yellow No. 6 active for assays targeting 5HT1 and 5HT5A

[1] AOP-Wiki available at AOP-Wiki (accessed on March 21, 2020).

Molecular Target	Blue No. 1	Blue No. 2	Green No. 3	Red No. 3	Red No. 40	Yellow No. 5	Yellow No. 6	Notes
Nuclear Receptors								
Androgen: receptor activated by binding of ligands and then is translocated into the nucleus	✓	✓	✓	✓	✓	✓	✓	All dyes, except Blue No. 2, active for Tox21_AR_LUC_MDAKB2_Antagonist, suggesting antagonistic role. Blue No. 2 active for NVS_NR_cAR
Estrogen: steroid hormone receptor activated by binding of ligands and then is translocated into the nucleus	✓	-	✓	✓	✓	-	-	Most dyes active for antagonism assays. Yellow dyes active for viability assays, but not receptor activity assays
Glucocorticoid: transcription factor binds to response elements in promoters of responsive genes, regulates other transcription factors	✓	NT	✓	✓	✓	NT	NT	Activity of food dyes based on assay NVS_NR_hGR.
Thyroid Hormone: receptor for tyrosine-based hormones that are primarily responsible for regulation of metabolism.	✓	✓	✓	✓	✓	-	-	Activity based on TH and TSH. Green No. 3 active for NVS_NR_hTRa_Antagonist - looks at ability of chemical to bind and displace T3 from receptor α. Neither yellow dye was active for receptor assays. Associated with AOPs 8, 152, and 300.[2]
Oxidases, esterases, transcription factors, and transporter proteins								
***Acetylcholinesterase*:** In CNS, binding by acetylcholine (AChE) plays a role in the function of peripheral neuromuscular junctions.	NT	NT	NT	NT	NT	NT	NT	No dyes tested in subset assays mapped to this target
Adenylyl Cyclase: catalyzes the formation of cyclic AMP and pyrophosphate from ATP.	NT	NT	NT	NT	NT	NT	NT	No dyes tested in subset assays mapped to this target
Aryl Hydrocarbon: protein involved in the regulation of biological responses to aromatic hydrocarbons	-	✓	✓	✓	-	✓	-	Activity for target based on ATG_AhR_Cis (AOP 150).[1] Only Yellow No. 5 associated with downregulation; others associated with upregulation
Monoamine Oxidase: regulates metabolic degradation of catecholamines and serotonin in neural/target tissues.	NT	NT	NT	✓	NT	NT	NT	Red No. 3 was only dye tested. The dye was tested in 2 assays and active in 1
Soluble Carrier Protein 6: member of sodium NT symporter family, responsible for reuptake of norepinephrine into presynaptic nerve terminals.	NT	NT	NT	✓	-	NT	NT	Activity observed in assays: NVS_TR_HNET, NVS_TR_HSERT, NVS_TR_RSERT. Red No. 3 active for assay targeting member 2.
Thyroid Peroxidase: oxidoreductase; inhibition leads to a decrease in thyroid hormone synthesis	✓	NT	✓	✓	✓	NT	NT	Four dyes associated with downregulation; targeting the loss of signal of TPO activity. The assay is associated with AOP 42.[1]
Pathways								
Oxidative Stress: Targets include intercellular adhesion molecules, chemokines, and interleukins.	-	-	✓	✓	-	-	✓	Green No. 3 and Yellow No. 6 were active in one assay each; Red No. 3 was active in three assays.
Inflammation: Targets include tumor necrosis factor and transforming growth factor	-	NT	-	-	-	-	-	No activity observed

[2] AOP-Wiki available at AOP-Wiki (accessed on March 21, 2020).

A.4.3 ToxPi results

The ToxPi analysis was limited to the NVS platform so that direct comparisons of receptor-based assays from similar test methods could be made. We looked at six groups of receptor families. For the analysis, we input AC_{50} data values as a quantitative measure of chemical activity; the values can be customized based on the scaling options available in ToxPi. In Figure A.4, each ToxPi represents a food dye composed of slices from the different receptor families. These ToxPis give an overall ranking of the food dyes' activity relative to one another based on the potency. In comparison to the overall activity in the 283 assays, by selecting out a smaller subset and focusing on NVS receptor binding assays, activity mapped to specific target types highlighted activity for different dyes. In order of activity observed, the most active to least were: Yellow No. 6, Yellow No. 5, Red No. 40, Red No. 3, Blue No. 1, Green No. 3, and Blue No. 2. Although the yellow dyes were not active in as many assays as some others, their biological activities in their active assays were greater than the other dyes. Based on the results, GPCR assays had the most hits and the most number of assays (at least 50% of the assays evaluated in the ToxPis) which may influence how much overall activity was observed. The second most active group was the "ENZ" assays which included lyases, oxidases, and esterases. As expected, the slices representing the ion channels and ligand-gated ion channels had the least amount of activity, given that the food dyes were not tested extensively in these assays. Within the NVS subset, Blue No. 2 also had the least amount of activity.

Figure A.4: ToxPi ranking of food dyes.

Chemicals ranked in order of biological activity in NVS assay subset. Most active to least were: Yellow No. 6, Yellow No. 5, Red No. 40, Red No. 3, Blue No. 1, Green No. 3, and Blue No. 2.

A.5. Discussion

The large suite of *in vitro* assays within the ToxCast program, along with the integration of aggregate databases and AOPs, has the potential to be a useful tool for predictive assessment of potential neurological activity following chemical exposure. Identification of assays mapped to markers associated with neurologic activity is consistent with the NAS (2017) recommendations to evaluate chemicals according to their ability to perturb toxicity pathways. Our current approach is based off of a proof-of-concept exercise (Iyer et al. 2019) utilizing mechanistic data to identify chemicals potentially linked to known hazard traits (Chiu et al. 2018; Iyer et al. 2019). We used those methods to

evaluate the potential activity of food dyes in ToxCast and assessed how the results could help identify targets that play a role in neurodevelopment processes ultimately leading to DNT or neurotoxic or neurobehavioral effects.

ToxCast activity for the food dyes ranged widely making it difficult to make strong correlations between what was observed, and adverse effects or potential mechanisms that have been reported in the literature. The lack of substantial correlations can be due to several factors. For one, the assays used in ToxCast do not represent the entire spectrum of biological processes that might be relevant to human health, including neurobehavioral effects. Therefore, there are gaps in biological coverage of the available assays. Biological coverage gaps persist even after expanding the assay selection to additional markers using the pesticide candidates and the pertinent pathways. Red No. 3 and Green No. 3 had hits for all the neuro-relevant molecular targets that they were tested in. It is unknown whether or not these dyes would show activity in the molecular targets in which they have yet to be tested. Because of this, no conclusion can be drawn with respect to dye activity in a number of the *in vitro* assays targeting several markers (including AChE, adenyl cyclase, and the ion channels GABA and glutamate). In our sub-analysis with ToxPi, we see that the number of active assays is just one component for evaluating the biological activity of a chemical. Another important factor to take into account is the potency of the chemical-assay pair – although a dye may be active in fewer assays, the potency in those active assays may be much higher, or vice-versa.

The current lack of metabolic activation and design limitations of the assays may also contribute to a higher number of inactives than expected. Known mechanisms linking food dye exposure to neurotoxicological effects include induction of oxidative stress and inflammation, which are thought to be primarily mediated through the active metabolites of the azo dyes. Typically, the azo dyes are substantially cleaved in the gut and the metabolites are absorbed. Thus, even *in vivo*, the synthetic azo dyes themselves would be less likely to reach the targets measured in the ToxCast assays. Therefore, a lack of observed activity *in vitro* does not necessarily translate to the absence of activity *in vivo* and may explain the lack of activity of several dyes (i.e., yellow dyes) across many of the molecular targets. Although Red No. 3 has activity in assays mapped to oxidative stress, which supports some literature findings (Floyd 1980) and indicates an area that may need to be explored further, none of the known metabolites have been tested in ToxCast (however, four were identified in the Chemistry Dashboard). Therefore, the role of metabolic activation in the toxic action of the food dyes could not be clearly assessed using ToxCast data.

Even with the limitations of the *in vitro* data, in contrast to a recent study published by Chappell et al. (2020), our approach resulted in more assays being included in the assessment (283 for OEHHA compared to 99 for Chappell et al. (2020)) and more corresponding active “hit-calls” for OEHHA in comparison to Chappell et al. (2020). These differences could be explained by the fact that (i) we cast a much wider net to

include indirect effects that may have potential associations with neurobehavioral outcomes and (ii) we did not integrate cytotoxicity and flags of efficacy as hard cutoffs when determining activity calls, in contrast to the approach Chappell et al. (2020) took. As mentioned previously, although data flags increase uncertainty, it is not recommended to use data flags and cytotoxicity limits as hard cutoffs to discount assay results, but rather they should be utilized as a set of cautions for users when considering the data (Judson et al. 2016). For instance, AC_{50s} observed for chemical-assay pairs above the cytotoxicity threshold are more likely to be associated with an interference that may lead to cell death. However, certain quantitative uncertainties in AC_{50}s (hit-calls are binary currently, but improvements are being done to integrate confidence intervals) still exist as well as our lack of true understanding of the dynamics between observed activity, cell stress, and cytotoxicity. Furthermore, there is not an established standardized way of incorporating cytotoxicity when interpreting ToxCast HTS data. Viability assays were given limited consideration in our evaluation due to a number of factors including 1) the variable number of cytotoxicity assays for each chemical; 2) appropriateness of utilizing Tox21 cytotoxicity assays in application to ToxCast assays outside the Tox21 platform (potentially important differences in the methods across platforms which would affect cell viability and non-concurrent measurements); 3) many cytotoxicity assays have flags of efficacy, which by the same rules, would render them less reliable. Due to their integration of cytotoxicity data in their analysis, the Chappell study had little or no hits for most of the seven food dyes. By comparison, our approach resulted in a significant number of assay hits for potentially relevant molecular targets underlying neurological processes (Figure 2).

Although our approach had certain limitations, much of our results showed concordance with the literature. ToxCast data supports the estrogenic activity observed in literature for Red No. 3 (Dees et al. 1997), but does not support the estrogenic interactions of Yellow No. 5 and No. 6 as reported by Axon, 2012. All the FD&C synthetic food dyes (except for yellow dyes) are active for antagonistic effects with the thyroid hormone receptor based on activity for assay TOX21_TR_LUC_GH3_Antagonist. Some have data flags and therefore the results should be viewed as less reliable. There are no data flags for Red No. 3, but because the assay is part of the Tox21 platform, viability assay results associated with this assay target should be given weight. Because of this, there is a likelihood that the AC50s observed for the Red No. 3 and Red No. 40 activities are influenced by cytotoxicity. However, the extent of cytotoxicity influence is unclear and therefore it should be noted that there may still be some concordance with effects on thyroid hormone homeostasis reported in the literature. In particular, Red No. 3 is active for assays targeting the thyroid hormone supporting literature findings for the inhibitory effects of Red No. 3 on the conversion of T4 to T3 in rats and increased release of TRH from the pituitary (Jennings et al. 1990). Red No. 3, along with Red No. 40, Blue No. 1, and Green No. 3, were also all active (albeit with AC_{50s} above that of the associated viability assay) for an assay mapped to TPO that measures TPO activity as a loss of signal and is linked to the AOP key event TPO inhibition, leading to the decrease in

thyroid hormone (TH) synthesis and subsequently a decrease in circulating concentrations of THs in serum and tissue. Alterations in human thyroid hormone levels have been associated in multiple AOPs for decreased cognitive function and impaired learning and memory (Bal-Price and Meek 2017; J Li et al. 2019). These results combined with literature reporting that thyroid hormone interactions and the reduction of thyroxine (T4) may be linked to developmental neurotoxicity (O'Shaughnessy and Gilbert 2019) may be suggestive of another mode of action of the food dyes. These four dyes were also the only active dyes for an assay targeting the glucocorticoid (GC) receptor NR3C1. GCs and their receptors exert widespread actions in the central nervous system, ranging from the regulation of gene transcription, cellular signaling, and modulation of synaptic structure. Elevated GC levels are linked to neuronal plasticity and neurodegeneration (Vyas et al. 2016).

All azo dyes were active in assays targeting dopaminergic and opioid receptor subtypes. From CTD, there are inferred associations between the opiate receptor kappa 1 (evaluated in our set) and neurotoxicity syndromes, neural tube defects, and neurobehavioral manifestations. Other opiate receptor subtypes also have inferred associations with neurological diseases on CTD. The activity of the yellow dyes with the opiate receptor subtype assays do have flags (three flags each) and therefore should still be considered but potentially viewed as less reliable. Blue No. 1, Red No. 40, and both yellow dyes were also active for serotonergic receptors. It has been noted in the literature that the presence of certain red and yellow dyes may lead to the increased release of neurotransmitters like dopamine and serotonin (Lafferman and Silbergeld et al., 1979) (Gao et al., 2011). ToxCast data also supports cholinergic activity for Red No. 40 and Yellow No. 6 as observed in a study evaluating mixtures of dyes (Ceyhan et al., 2013). The cholinergic activity for Yellow No. 6 was based on activity noted for assay NVS_GPCR_hM3, which had 4 out of 8 data quality flags. Although the flags do not render the chemical-assay pair data as inactive, other supporting information should also be considered in evaluating the cholinergic activity for Yellow No. 6. The yellow dyes were tested in as many ToxCast assays as the red dyes, but had significantly less activity.

Our approach was developed based on the current knowledge of molecular mechanisms underlying DNT or neurotoxicity (NT). Moving forward, further analysis should be done on other molecular targets beyond the current scope. Continuing work can include organizing ToxCast data mapped to future established key characteristics of neurotoxicants and correlating assay information with continuing updates from CTD. Other avenues to explore include grouping chemicals (despite their differences in chemical structure), according to their biological activity, i.e. the capacity to trigger an impairment of certain similar neurodevelopmental process. Integration of the battery of *in vitro* assays with other data streams and AOPs should be explored further for potential markers indicative of neurologic activity. There are currently ten existing AOPs relevant to DNT (Li et al. 2019) and eight AOPs, either fully developed or in development, relevant to NT (Bal-Price and Meek 2017). Specifically, there are two

AOPs relevant to DNT and NT that include the binding of glutamatergic receptors as a triggering key event for downstream adverse neurodevelopmental effects (Fritsche et al. 2015) however, only two dyes were tested for interaction with this receptor and both were inactive. Although it is currently difficult to link the activity (or lack thereof) of the food dyes with the molecular targets in the assay subset to key events in these AOPs, further analysis can be done on assays outside the scope of the current subset to explore other potential markers. Additionally, it may be possible to utilize *in silico* modeling to evaluate the potential of structurally similar chemicals to trigger key events based on the chemico-physical properties.

Here, we highlighted several pertinent associations between the dyes and certain molecular targets of interest. The selection of assays for our approach does not purport to be complete, but spans a good representation of currently suspected molecular targets that underlay neurodevelopmental, neurological or neurobehavioral processes. While the ToxCast results did not provide overwhelming support for *in vivo* neurological alterations for the food dyes, data gaps and lack of biological coverage in ToxCast shine a light on areas to pursue. This exploration of ToxCast was intended to provide initial information on whether the *in vitro* HTS assays could be linked with the ability of the FD&C synthetic food dyes to promote a biological response in the nervous system. These assays are limited in predicting long term or indirect adverse effects in biological systems, in part due to the complexity of the mechanistic processes that underlie detrimental neurotoxic or neurobehavioral outcomes compared to the current limited spectrum of the ToxCast assays. Ongoing refinement of the *in vitro* platforms, including expansion of biological coverage, alongside increasing knowledge of mechanism of action will lead to the generation of stronger predictive outcomes. Evaluation of the food dyes in future iterations may offer more refined results and provide information on roles that these gene markers play in mechanisms of potential neurodevelopmental, neurobehavioral or neurotoxic effects.

A.6 Information from Aggregate Databases on Food Dye Activity

Chemical hazards data commons

The Chemical Hazards Data Commons (CHDC, or Data Commons) is a public database that integrates data sources and analyses to provide known information about the hazards of certain chemicals to facilitate comparisons. As of 2020, Data Commons identifies 22 specific human and environmental health endpoints identified by governmental and professional authorities (based on the Healthy Building Network's Pharos Chemical and Material Library). The database is an aggregate of over 40 authoritative hazard lists and uses the GreenScreen protocol to assess and identify known chemicals of varying concern and hazard. The GreenScreen protocol benchmarks the inherent hazards of chemicals across a broad range of health endpoints.

In this database, developmental toxicity is defined as: Ability to cause harm to the developing child including birth defects, low birth weight and biological or behavioral problems that appear as the child grows. It is considered that classification under the heading of developmental toxicity is primarily intended to provide a hazard warning for pregnant women and men and women of reproductive capacity. Therefore, for pragmatic purposes of classification, developmental toxicity in this context essentially means adverse effects induced during pregnancy, or as a result of parental exposure. These effects can be manifested at any point in the life span of the organism. Note that developmental toxicity can occur from postnatal exposures to chemicals as an organism matures. The major manifestations of developmental toxicity include death of the developing organism, structural abnormality, altered growth and functional deficiency.

For CHDC, Developmental Toxicity includes developmental neurotoxicity. None of the food dyes have hits in developmental toxicity, developmental neurotoxicity, or single/multiple exposure neurotoxicity as defined by CHDC. The following food dyes have hits for endocrine activity (as defined as ability to interfere with hormone communication between cells, which controls metabolism, development, growth, reproduction and behavior):

- Green No. 3
- Yellow No. 6
- Red No. 3

These chemicals have high to moderate hazard for endocrine activity (CHDC).

Results from CHDC

- Blue No. 1 did not have enough support to classify it as having either a neurotoxicity or developmental neurotoxicity hazard. The hazards database listed this chemical as part of “safer chemicals as defined by DfE: Green tagged chemicals in the list meet DfE’s safer chemical criteria and are among the safest chemicals for their particular function."
- Blue No. 2 did not have enough support to classify it as having either a neurotoxicity or developmental neurotoxicity hazard. It also did not have enough support for endocrine toxicity hazard. It is worth noting that it had high hazard level for skin sensitization and eye irritation as classified by the list New Zealand –GHS: 6.4A and 6.5B.
- Green No. 3 did not have enough data support to classify it as having either a neurotoxicity or developmental neurotoxicity hazard. However, it was listed as having a high to moderate hazard in endocrine toxicity. This was supported by its presence in the TEDX - Potential Endocrine Disruptors: Potential Endocrine Disruptor List. Fast Green was listed as having a very high hazard in persistence as listed by EC - CEPA DSL: Persistent.
- Red No. 3 did not have enough support to classify it as having either a neurotoxicity or developmental neurotoxicity hazard. However, it was listed as having a high to moderate hazard in endocrine toxicity. This was supported by its

presence in the TEDX - Potential Endocrine Disruptors: Potential Endocrine Disruptor List.

- Red No. 40 did not have any identified hazards in the CHDC. It was listed as being present on one list: US EPA - DfE SCIL: Green Circle - Verified Low Concern.
- Yellow No. 5 did not have enough support to classify it as having either a neurotoxicity or developmental neurotoxicity hazard. It also did not have enough support for endocrine toxicity hazard. It is worth noting that it had high hazard level for persistence by the list EC-CEPA DSL: Persistent.
- Yellow No. 6 Sunset Yellow did not have enough support to classify it as having either a neurotoxicity or developmental neurotoxicity hazard. However, it was listed as having a high to moderate hazard in endocrine toxicity. This was supported by its presence in the TEDX - Potential Endocrine Disruptors: Potential Endocrine Disruptor List.

Comparative toxicogenomics database

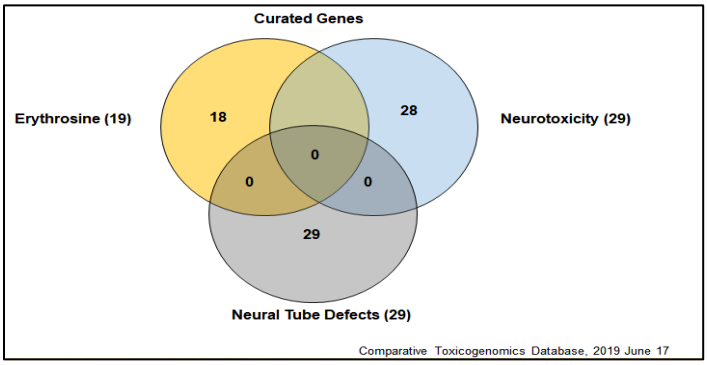
The Comparative Toxicogenomics Database (CTD) is a publicly available database that provides literature-based manually curated information about chemical–gene/protein interactions, chemical–disease and gene–disease relationships. These data are integrated with functional and pathway data to aid in development of hypotheses about the mechanisms underlying environmentally influenced diseases.

The purpose of screening the food dyes through CTD is to search whether any of the dyes have reported curated gene-interactions of relevance to development neurotoxicity or neurotoxicity. To do so, CTD's MEDIC was used to identify curated associations between human neurological and developmental diseases with either 1) the chemical of interest, or 2) the relevant genes associated with the chemical. Curated associations are established through a marker or a mechanism of a disease while inferred associations are established via curated chemical-gene interactions. Therefore, curated interactions bear more weight than inferred interactions.

There were more than five diseases related to neurological or development disorders available in CTD, however only the ones below had gene sets associated with them to perform the analyses of interest for this study:

Motor Skills Disorders
Developmental Disabilities
Neural Tube Defects
Neurotoxicity Syndromes
Prenatal Exposure Delayed Effects

After identifying which genes had curated associations with the food dyes, those genes were cross-searched with the five established neurotoxicity or developmental neurotoxicity-labeled "diseases" listed above to see if there were any overlaps between the dyes and the identified neurological and developmental diseases.



Results from CTD

- Blue No. 1 has a curated disease association with Prenatal Exposure Delayed Effects (Mikkelsen, 1978). The dye has the following gene hits: GRIN2A, CHRNA4, and CHRNB2.
- Blue No. 2 has a curated disease association with Prenatal Exposure Delayed Effects (Ceyhan 2013). The dye has the following gene hits: CYP1A1, AHR, GRIN2A, CHRNA4, CHRNB2, CYP1A, CYP1B1, CYP1C1, CYP1C2, and CYP2A6. CHRNA4 has a curated disease association with Development Disabilities.
- Although the chemical is in CTD, there have been no curated disease or gene associations for Green No. 3 yet.
- Red No. 3 has a curated disease association with Neurotoxicity Syndromes (Yamauchi, 2002; Yankell, 1977). The dye has the following gene hits: CDK2, UGT1A6, UGT2B7, ABCB1, ALB, CCND1, CYP19A1, CYP3A4, FOSL1, GSTP1, HPGDS, IYD, JUN, ORM1, PHEX, TP53, TRH, TSHB, and VEGFA. ABCB1 has a curated disease association with Neurotoxicity Syndromes.
- Red No. 40 has a curated disease association with Prenatal Exposure Delayed Effects (Ceyhan, 2013). The dye has the following gene hits: GRIN2A, CHRNA4, CHRNB2, CYP19A1, and ESR1. CHRNA4 has a curated disease association with Development Disabilities.
- Yellow No. 5 has curated disease associations with Neurotoxicity Syndromes (Tanaka, 2006) and Prenatal Exposure Delayed Effects (Ceyhan, 2013). The dye has the following gene hits: ESR1, GRIN2A, TFF1, CAT, CHRNA4, CHRNB2, and LYZ. CHRNA4 has a curated disease association with Development Disabilities.
- Yellow No. 6 has a curated disease association with Prenatal Exposure Delayed Effects (Ceyhan, 2013). The dye has the following gene associations: ESR1, GRIN2A, IFNG, IL6, TFF1, TNF, CHRNA4, CHRNB2, IL2, and IL4. IFNG has a curated disease association with Neural Tube Defects; CHRN4 has a curated disease association with Development Disabilities.

Comptox Chemistry Dashboard (Comptox Chemistry Dashboard)

The seven food dyes were screened through eight developmental neurotoxicity chemical lists for their presence.

CHEMISTRY DASHBOARD DNT LISTS

CASRN	FD&C NAME	DNTEFFECTS	DNTINVIVO	DNTSCREEN	HUMANNEUROTOX	NEUROTOXINS	LITMINEDNEURO	DNTPOTNEG
3844-45-9	Blue No. 1	-	-	Y	-	-	-	-
860-22-0	Blue No. 2	-	-	-	-	-	-	-
2353-45-9	Green No. 3	-	-	-	-	-	-	-
16423-68-0	Red No. 3	-	-	-	-	-	-	-
25956-17-6	Red No. 4	-	-	Y	-	-	-	-
1934-21-0	Yellow No. 5	-	-	Y	-	-	-	-
2783-94-0	Yellow No. 6	-	-	Y	-	-	-	-

DNTEFFECTS: Chemicals demonstrating effects in Neurodevelopment. Mundy et al 2015 (Chemicals demonstrating effects in Neurodevelopment. Mundy et. al. 2015)
DNTINVIVO: Chemicals triggering developmental neurotoxicity in vivo. Aschner et al 2017 (Chemicals triggering developmental neurotoxicity in vivo. Aschner et. al. 2017)
DNTSCREEN: DNT Screening Library.
HUMANNEUROTOX: Human Neurotoxicants. Grandjean and Landrigan, The Lancet, Volume 368, No. 9553, p2167–2178, 16 December 2006. Human Neurotoxicants. Grandjean and Landrigan, The Lancet, Volume 368, No. 9553, p2167–2178, 16 December 2006
NEUROTOXINS: Neurotoxicants Collection from Public Resources
LITMINEDNEURO: Neurotoxicants from PubMed
DNTPOTNEG: Potential negative controls for DNT Assays. of Aschner et al 2017 (Potential negative controls for DNT Assays. of Aschner et. al. 2017)

A.7. Other related appendices

Appendix B shows the complete list of ToxCast assays (tested, inactive, and active) for the food dyes. At the bottom of each column, the numbers of total tested assays and total active assays by chemical are given.

Appendix C shows the subsets of ToxCast assays developed for evaluation in this study. Criteria for assay selection are addressed in the Methods section. The subset included receptor assays, viability assays, active assays for pesticides with known DNT endpoints, and assays mapped to oxidative stress and inflammation. ToxCast results are for chemical-assay pairs deemed active by US EPA. There are a total of 283 assays.

Appendix D covers the expanded details for the ToxCast assay results. Details include AC_{50}s, flags, associated gene target, and a description of the assay where available.

References – see References Section for Entire Report