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Systematic assessment of mechanistic data for FDA-certified food colors and neurodevelopmental processes

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ABSTRACT

Seven US FDA-batch certified synthetic food colors are approved for use as food additives in the United States. Perceived neurodevelopmental concerns for these colors persist. This study assessed the plausibility of such an association through the evaluation of mechanistic evidence collected from *in vitro* assays or other alternative models. Mechanisms and molecular targets underlying neurodevelopmental processes associated with attention deficit and hyperactivity disorder (ADHD) and other neurodevelopmental-related symptoms (e.g., cognitive function, learning and memory disorder, etc.) were identified. Publicly available data from the ToxCast/Tox21 high-throughput screening (HTS) program and peer-reviewed literature that measure activity of the colors for such molecular targets were analyzed and reviewed. Erythrosine (Red No. 3) was active in several assays mapped to neurodevelopmental processes — specifically, HTS assays that measure signals in neurotransmitter pathways. The remaining six colors do not appear to alter signaling pathways related to neurodevelopmental processes on the molecular or cellular level. This assessment provides an approach for systematically identifying and mapping mechanistic data to putative neurodevelopmental processes as a means to prioritize substances for possible further investigation. The assessment also provides insights into the lack of activity of synthetic food colors for key events in neurodevelopmental signaling pathways.

1. Introduction

Synthetic colors are added to foods in order to meet consumer preferences and expectations, such as to standardize the color of the food due to natural variations in hue from base ingredients, and/or to make up for loss of color due to processing (i.e., cooking) or storage and transportation conditions (i.e., exposure to light), among others. The presence of any food color additive must be labeled on the ingredient statement on packaged food, thereby providing consumers with clear and transparent information (US FDA, 2018b). Nine synthetic color additives have been approved for use in foods by the U.S. Food and Drug Administration (FDA) and are subject to batch certification under the Federal Food, Drug, and Cosmetic (FD&C) Act according to the U.S. Code of Federal Regulations (CFR) Title 21 (Food and Drugs) Chapter I, Subchapter A, Part 74, Subpart A (Foods) (CFR, 2020; US FDA, 2015). This means that the colors are permitted for use only after a sample from every batch produced has been analyzed by FDA chemists and determined to meet composition and purity requirements (e.g., limits for specific contaminants, minimum percentage of the dye in the formulation) as stated in the regulation (21 CFR §74.101–74.706). As Orange B and Citrus Red No. 2 are approved only as external colorants to food (i.e., for casings or surfaces of hot dogs and sausages, and the skins of oranges not intended or used for processing, respectively), the focus of this study is on the remaining seven synthetic food colors that are permitted at Good Manufacturing Practices (GMP) levels in foods generally (see Table 1). FDA batch certification requires that every batch of food color be tested for quality and purity by FDA. Each FD&C color specification also prescribes maximum limits for relevant impurities (21 CFR Part 74). Thus, any evaluation of FDA-approved colors should utilize FDA-certified colors (rather than non-FDA-certified colors) to ensure that the quality and purity of the test material meets FDA standards for food use and findings are not confounded by impurities.

Global regulatory bodies including the US FDA, European Food Safety Authority (EFSA) and the Joint (FAO/WHO) Expert Committee on Food Additives (JECFA), among others, have assessed these seven color additives and found them to be safe for their intended use in foods for all consumers, including children (EFSA, 2008, 2009a, b, c, 2010,

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Table 1
Relevant FDA-approved synthetic colors for use in foods and beverages.

Color	Common Name	CASRN	21 CFR Subpart	FDA ADI (year of most recent evaluation)	Basis for FDA ADI (effect and species)	JECFA ADI[a] (year of most recent evaluation)	Basis for JECFA ADI (effect and species)
FD&C Blue No. 1	Brilliant Blue	3844-45-9	§74.101	12 mg/kg bw/d (1982)	No effects at highest concentration tested in a rat dietary carcinogenicity study	6 mg/kg bw/d (2017)	Decreased body weight and survival in female rats
FD&C Blue No. 2	Indigo carmine	860-22-0	§74.102	2.5 mg/kg bw/d (1982)	No-effects level in a rat dietary carcinogenicity study	5 mg/kg bw/d (2018)	Inhibition of growth (body weight) in male rats
FD&C Green No. 3	Fast Green	2353-45-9	§74.203	2.5 mg/kg bw/d (1982)	No-effects level in a rat dietary carcinogenicity study	25 mg/kg bw/d (2016)	No effects at highest concentration tested in a long-term rat dietary study
FD&C Red No. 3	Erythrosine	16423-68-0	§74.303	2.5 mg/kg bw/d (1969)	No-effects level in a rat dietary carcinogenicity study	0.1 mg/kg bw/d (2018)	Slight increase in thyroid-stimulating hormone (TSH) responsiveness to thyrotropin-releasing hormone (TRH) in humans
FD&C Red No. 40	Allura red	25956-17-6	§74.340	7 mg/kg bw/d (1971)	No-effects level in a rat dietary carcinogenicity study	7 mg/kg bw/d (2016)	Decreased body weight in rats
FD&C Yellow No. 5	Tartrazine	1934-21-0	§74.705	5 mg/kg bw/d (1969)	No-effects level in a rat dietary carcinogenicity study	10 mg/kg bw/d (2016)	Decreased body weight in rats
FD&C Yellow No. 6	Sunset yellow	2783-94-0	§74.706	3.75 mg/kg bw/d (1986)	No-effects level in a rat dietary carcinogenicity study	4 mg/kg bw/d (2011)	Decreased body weight of rat pups exposed *in utero*

[a] JECFA ADIs can be found by searching for individual chemicals here: http://apps.who.int/food-additives-contaminants-jecfa-database/search.aspx.

2011, 2014; US FDA, 2011, 2018a; WHO/FAO, 2011, 2017a, b, 2019). They have also concluded that these colors are generally poorly absorbed and therefore would have limited bioavailability. Nonetheless, the California Office of Environmental Health Hazard Assessment (OEHHA) is currently itself reviewing the evidence on these colors (e.g., toxicology, epidemiology, exposure literature) to conduct a risk assessment that will include neurobehavioral endpoints in children (OEHHA, 2018). The relationship between synthetic food color intake and neurobehavioral effects has been tested in both epidemiological studies and in animal models, all of which have been reviewed in recent EFSA and JECFA assessments with concluding, similar to US FDA (2011), a causal relationship between exposure and response has not been demonstrated (Nigg et al., 2012; Schab and Trinh, 2004; US FDA, 2011).

Estimated daily intakes of these FD&C food color additives in the U.S. population and among U.S. youths have been published (Bastaki et al., 2017; Doell et al., 2016; Tran et al., 2020). The estimated daily intakes for the seven food colors as reported in these studies are generally well below the respective acceptable daily intake (ADI) values established by JECFA and FDA (Table 1). The variation in the JECFA ADIs (2011 through 2018 evaluations) compared to the FDA ADIs (1960s through 1980s evaluations) are mainly a reflection of updates to the evidence with new study information and/or identification of alternative endpoints for ADI derivation.

Neurodevelopmental disorders, including attention-deficit and hyperactivity disorder (ADHD), dyslexia, autism, and other cognitive impairments affect millions of children throughout the world and are complex and not completely understood (Grandjean and Landrigan, 2014). Studying the relationship between specific exposures and their impact on brain development, neurobehavioral and neurological disorders, as well as degenerative changes is challenging (Grandjean and Landrigan, 2014). ADHD is typically characterized by ongoing inattention, hyperactivity, and impulsivity (NIMH, 2019), yet remains a disorder with a complex etiology that is often times diagnosed incorrectly due to lack of consistent and standardized criteria. Differences in diagnostic practices across geographic regions, and parents' and teachers' reporting versus physician examinations and formal questionnaires, continue to be highly variable and preclude consistent determinations across different evaluators (Davidovitch et al., 2017; Heilskov Rytter et al., 2015; Thomas et al., 2015). Additionally, genome-wide association studies (GWAS) have identified DNA variants along with rare insertions and deletions that have been associated with increased susceptibility to developing ADHD (Faraone and Larsson, 2019). Although the exact etiology of ADHD, like other neurobehavioral disorders, remains elusive, current research suggests that ADHD likely results from a combination of factors including genetics, environment, and brain injury, among other factors (AACAP, 2018; NIMH, 2019). There is evidence that ADHD is primarily a genetic disorder with high heritability (Larsson et al., 2014; Zayats and Neale, 2019).

Although ADHD-like rodent models have been developed (Breese et al., 2005; Ouchi et al., 2013; Pires et al., 2009), these are limited to proxy endpoints that may correspond to only certain aspects of the complex symptoms ascribed to ADHD. While these models may facilitate hypothesis testing relative to underlying mechanistic aspects of ADHD (Russell, 2011; Sontag et al., 2010), these models are not necessarily appropriate for identifying and characterizing hazard for regulatory purposes because they lack sufficient predictability of the complex human condition (Russell, 2011). These models could, however, enable evaluation of "ADHD-like symptoms" rather than serve as *bona fide* models of ADHD *per se* (Sontag et al., 2010). Nonetheless, rodent studies designed to detect neurobehavioral or neurodevelopmental effects have been conducted for several food color additives, including the U.S. certified food colors FD&C Red No. 40, FD&C Red No. 3, FD&C Yellow No. 5, FD&C Yellow No. 6, FD&C Blue No. 1, and FD&C Blue No. 2 (Ceyhan et al., 2013; Dalal and Poddar, 2009, 2010; Doguc

et al., 2013, 2015; Gao et al., 2011; Mohamed et al., 2015; Tanaka, 1994, 2001; 2006; Tanaka et al., 2008, 2012; Vorhees et al., 1983). The results of these studies varied, and both EFSA and JECFA concluded that these studies lacked robustness and were deemed unfit for application in risk assessments of the colors, noting the following limitations: studies tested mixtures, effects were not dose-related or consistent, outcomes were not considered adverse (e.g., improved cognition, or accelerated achievement of developmental milestones) ,along with study design limitations (e.g., small numbers of animals per dose group). Importantly, none of the data collected in these studies are considered relevant for ADI determination/re-evaluation. Additionally, the 2011 FDA Food Advisory Committee concluded that a causal relationship between exposure to food colors and neurobehavioral disorders had not been established for the general population (US FDA, 2011). As noted by the FDA Food Advisory Committee, while symptoms may be exacerbated among susceptible children (those who have been diagnosed with ADHD or other "problem behaviors"), such effects on behavior are attributable to unique specific intolerance to colors rather than as a result of any neurotoxic properties of the food colors. Thus, sufficient evidence upon which to base a risk assessment relative to neurobehavioral endpoints was therefore absent.

The current test guidelines in animals were reviewed in a series of publications in a special issue of *Toxicology and Applied Pharmacology* (Bal-Price and Fritsche, 2018). The guidelines reviewed included neurotoxicity testing based on the Organisation for Economic Co-operation and Development (OECD) test guideline 426 (OECD, 2007) or the U.S. EPA's Health Effects Test Guidelines, OPPTS 870.630 Developmental Neurotoxicity (DNT) Study, and were deemed insufficient to adequately screen and characterize compounds that may affect the developing brain. Therefore, a shift in testing paradigm is needed to better screen chemicals for potential neurodevelopmental effects in order to inform regulatory decisions (Bal-Price and Fritsche, 2018; Fritsche et al., 2018).

Most recently, alternative testing methods to evaluate chemical effects on critical neurodevelopmental processes that represent different stages of human brain development are being developed and include an *in vitro* testing battery for the assessment of neural/glial cell cultures derived from human pluripotent stem cells (Fritsche et al., 2017). However, these assays are not yet ready for hazard or risk assessment purposes. Because of the limited knowledge regarding mechanistic processes that control neurodevelopmental outcomes, *in vitro* data can be used only for screening and prioritizing for further testing. The National Toxicology Program is currently attempting to develop improved screening methods that build upon *in vitro* and new approach methods (NAMs), which represent alternatives to animal models, that may better predict alterations in mechanisms associated with human neurobehavioral or neurologic disorders such as ADHD (Behl et al., 2019).

Importantly, the metrics of any relevant test method or assay may be anchored to key events (KEs) identified in existing neurodevelopmental or behavioral adverse outcome pathways (AOPs), thereby increasing scientific confidence in the mechanistic understanding of the implicated plausible toxicity pathways (Bal-Price and Meek, 2017). These AOPs include identification of molecular initiating events (MIEs) and KEs that together may lead to the adverse outcome of interest. It is important to note that only one signal for any specific event does not in and of itself translate to an adverse outcome. High-throughput screening (HTS) data can be used to prioritize future testing and research, fill data gaps, and/or support existing findings. HTS assays have been run for thousands of chemicals, and data are publicly available through the Toxicity Forecaster (ToxCast™) and the Toxicity Testing in the 21st Century (Tox21) screening programs, which represent a large collaboration of multiple U.S. agencies (Dix et al., 2007; Kavlock and Dix, 2010). The output from the HTS assays lends insight into a wide range of possible molecular or cellular events potentially associated with a diverse array of toxicological outcomes, including those associated with both neurobehavioral and/or neurological outcomes. Evaluation and integration of HTS assay data also represent a type of NAM for toxicological study. While HTS data are best positioned to screen and prioritize substances for further in-depth testing, they may also increase understanding of plausible mechanisms underpinning an AOP, as well as help identify possible data gaps (Punt et al., 2020). As suggested by others, HTS assays, along with other NAMs, could ultimately be integrated *via* the IATA (Integrated Approaches to Testing and Assessment) platform that is designed to provide a fit-for-purpose approach in data generation for regulatory purposes (Bal-Price and Fritsche, 2018).

Based on the complexity of neurobehavioral/neurological disorders (e.g., ADHD) and the concern for identifying exposures that might exacerbate these disorders in children, the objective of this assessment is to identify the current knowledge of proposed mechanisms and/or targets of neurodevelopmental processes. This approach was designed to identify MIEs and KEs in published AOPs for neurodevelopmental adverse outcomes (e.g., cognitive function, learning and memory disorder, etc.), and then to assess the activity of seven synthetic food colors of interest in assays that measure components of these MIEs and or KEs (i.e., HTS assays and *in vitro* or other NAM data reported in the literature). The latter could inform prioritization and further testing.

2. Materials and methods

2.1. Evidence-base for mechanistic data associated with neurodevelopmental processes

As illustrated in Fig. 1 (Step 1), the evidence-base for neurodevelopmental-relevant AOPs, NAMs, and genes were identified by an iterative literature search of primary literature and secondary sources (i.e., review articles) in PubMed and Google Scholar and by online database searches described below. Search terms were customized to adverse outcomes (AOs) (including "neurodevelopmental toxicity," "developmental neurotoxicity," "attention deficit hyperactivity disorder," and "ADHD models," among others) and methods (including "alternative test methods," "*in vitro*," and "zebrafish," among others). Additionally, hand-searching was conducted for references embedded within regulatory documents for synthetic food colors to ensure that key primary literature, reviews, other relevant documents, and consensus statements were identified and possibly included. Information on AOPs (described in selected publications and the AOPWiki, https://aopwiki.org/, accessed August 2019) was critical in the identification of MIEs and KEs possibly relevant to neurodevelopmental processes and outcomes.

The Comparative Toxicogenomics Database (CTD, http://ctdbase.org/, accessed September 2019) was queried to identify genes that have been suggested to have an association with one or more neurobehavioral/neurological disorders (Davis et al., 2019; Mattingly et al., 2003). The CTD is a publicly available database of curated interactions between genes, chemicals, exposures, biological pathways, phenotypes, and diseases. The relevant neurobehavioral disorders listed in the CTD are shown in Table 2.

The Molecular Signature Database (MSigDB) was also queried for gene sets and pathways possibly related to neurobehavioral/neurological signaling or pathologies. Individual genes within relevant gene sets were recorded for use in subsequent assay mapping (http://software.broadinstitute.org/gsea/msigdb; accessed September 2019). Four relevant pathways/gene sets from the MSigDB were identified, three of which were related to gamma-aminobutyric acid (GABA): "Neurite Development" (Gene Ontology Consortium), "GABA Receptor Activation," and "GABA Synthesis Release Reuptake and Degradation" (Reactome database), and "GABA Pathway" (BIOCARTA databases); see Table 2.

The genes that were identified as associated with neuro-developmental and neuro-behavioral processes in the CTD and MSigDB were

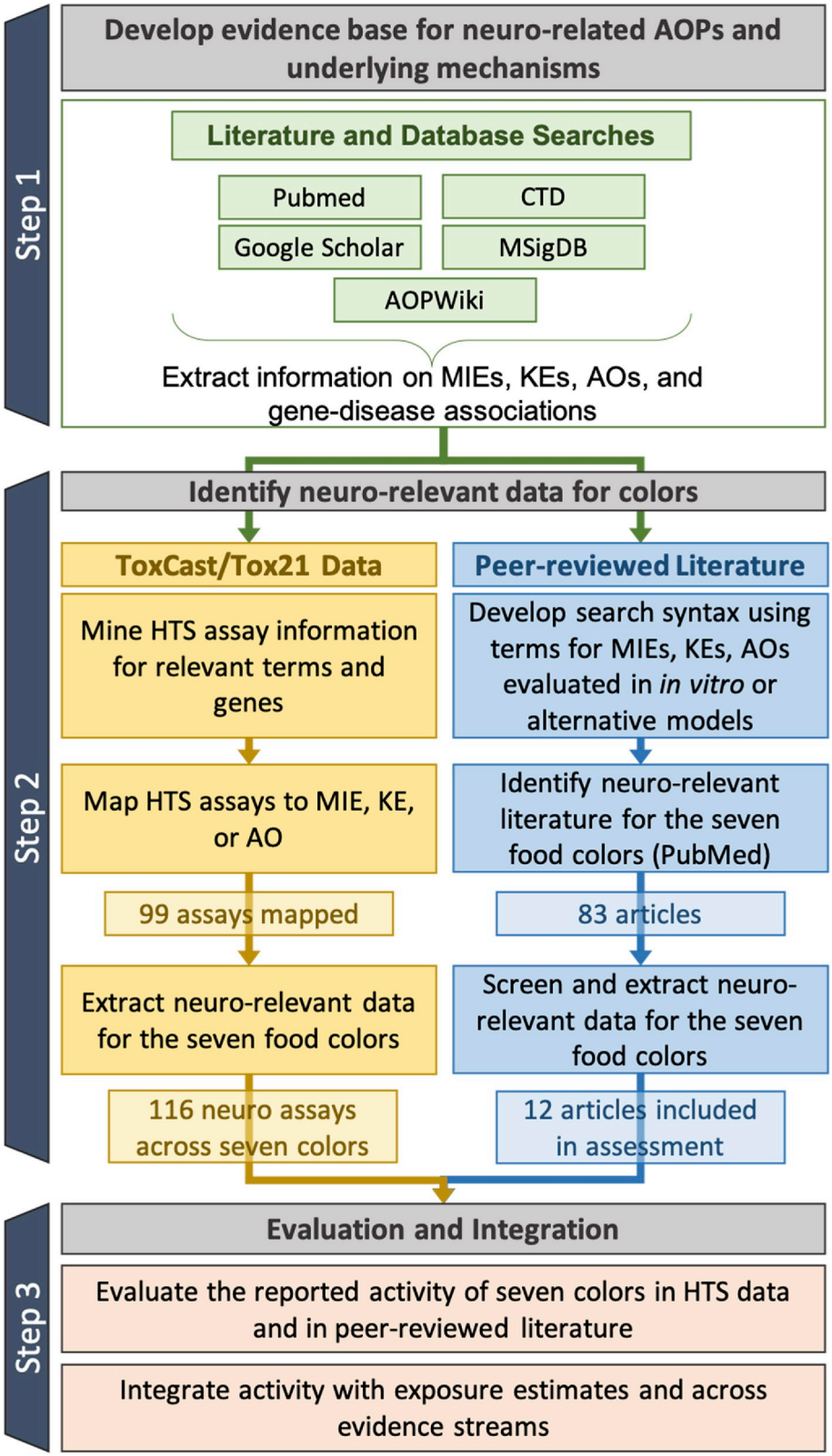

Fig. 1. Flow chart employed in the identification of relevant mechanistic information and signals related to neurodevelopmental processes and neurobehavioral effects, and to evaluate activity for the seven colors as related to such signals reported *in vitro* or in alternative models. AOP: adverse outcome pathway. CTD: Comparative Toxicogenomics Database. MSigDB: Molecular Signature Database. MIE: molecular initiating event. KE: key event. AO: adverse outcome. HTS: high-throughput screening.

Table 2
Neurobehavior-relevant disorders and signaling pathways/genesets listed in the CTD and the MSigDB databases with the number of associated genes.

Neurobehavior-Related Disorders and Pathways	Number of Associated Genes[a]
CTD-listed neurobehavioral disorders	
Attention Deficit and Disruptive Behavior Disorders	2
Attention Deficit Disorder with Hyperactivity	22
Language Delay and Attention Deficit-Hyperactivity Disorder/Cognitive Impairment with or without Cardiac Arrhythmia	1
Conduct Disorder	0
Child Behavior Disorders	0
Learning Disorders	31
Neurobehavioral Manifestations	7
Relevant neuro-related gene set or pathway as listed in the MSigDB	
Neurite Development	53
GABA Receptor Activation	56
GABA Synthesis Release Reuptake and Degradation	19
GABA Pathway	10

[a] Only "curated association" genes within the CTD culled from the literature based on published gene-disease relationships were included (i.e., genes with an "inferred association" were not included).

then used to search the ToxCast/Tox21 HTS assay database, as well as the scientific literature, to identify assays that would map to these gene targets.

2.2. Mapping HTS assays to neurobehavioral/neurological relevant signals

HTS assay data from the ToxCast/Tox21 screening programs as available via the U.S. Environmental Protection Agency (EPA) ToxCast Summary Files database (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data; invitrodbv_v3.2; released August 2019, accessed September 2019 for this study) were downloaded. The ToxCast program includes assay data from a variety of screening facilities, including the National Toxicology Program's Tox21 program. Assay data were reviewed and mapped to neurodevelopmental processes (Fig. 1, Step 2) according to MIEs and KEs identified in relevant AOPs and disease-gene associations. Specifically, assay information was mined for neuro-relevant intended targets (e.g., specific genes) and model types (i.e., species/tissues). Only "curated association" genes within the CTD culled from the literature based on published gene-disease relationships were included in the mapping exercise. Genes from relevant pathways or gene sets from MSigDB were also included in the mapping along with assays for genes related to neurotransmitters. With some of these neurotransmitter genes, there is no evidence of an association with an outcome according to the sources queried [e.g., dopamine receptor D1 (DRD1), histamine receptor H1 (HRH1), among others (see Supplemental Table S1)], and were thus solely included in the category of "Neurotransmission." The inclusion of all neurotransmitters is based on the understanding that alterations to neurotransmitters (type, level and activity) can influence neurodevelopmental processes (Mailman, 1987). Finally, the measure of brain morphology in the zebrafish (*Danio rerio*) embryo assay was also included and is the sole assay mapped to the category "Brain morphology".

All HTS assays relevant to neurodevelopmental processes or disease associations (i.e., risk genes) were assigned to an outcome and mechanistic category. For example, ADHD, a complex disorder, includes targets that encompass at least three categories: neurotransmitters, changes in glutamate release, and genes associated with populations diagnosed with ADHD, the latter based on epidemiology or GWAS studies (Davis et al., 2019; Demontis et al., 2019). The resources used as evidence for the proposed mapping(s) of each HTS assay to specific neurodevelopmental processes (i.e., AOPs, publications, databases, etc), are identified in Supplemental Table S1.

2.3. Food color HTS assay activity relevant to neurodevelopmental processes

The HTS data for the seven food colors (available through the ToxCast/Tox21 database) were extracted from the downloaded summary files. HTS assay data are modeled using an EPA-developed analysis pipeline (ToxCast pipeline, tcpl) that yields an AC_{50} value (i.e., activity concentration at 50% maximum response) per assay-chemical pair (Filer et al., 2017). Cytotoxicity is addressed for many assays run by the Tox21 program, in which cell viability is measured and modeled within the assays that test for the primary biological signals. Overall cytotoxic interference of each tested chemical, as opposed to cytotoxicity measured for a single specific assay, is also modeled and has been described as the cytotoxic "burst" concentration range, characterized by Z-scores (Judson et al., 2016). The Z-score represents the difference between the AC_{50} value and the concentration that elicits cytotoxicity. An assay-specific Z-score for each assay-chemical pair is reported. A large Z-score indicates that activity was observed far below the cytotoxic threshold, with a cut-off of ≥ 3 recommended (Judson et al., 2016). Flags for data quality issues are also reported for each individual chemical-assay pair (e.g., "noisy data" or "hit-call potentially confounded by overfitting," among others). Assay activity is reported as a "hit-call" in the summary files. "Hit-call" is a term used in the ToxCast data summary files and only suggests observed activity for a given assay without further integration of cytotoxicity data or data quality indicators. In the present study, the following criteria were applied to determine assay activity:

- For HTS assays for a biological target that also have cell viability data specifically for that assay, the cell viability information can be applied to determine activity:
  - o Hit-call = 1 and there was no evidence of loss of cell viability. In other words, the hit-call would be 0 for the measurement for loss of cell viability in the assay.
  - o Hit-call = 1 and the AC_{50} value is below the AC_{50} for the loss of viability measure.
- For HTS assays in which cell viability was not measured, the integration of the cytotoxic burst information (as described above) can be applied to determine activity:
  - o Hit-call = 1 and the Z-score is $\geq$ 3.
- For all assays that are considered active according to the hit-call and cell viability criteria, the assay is ultimately not considered active if more than one flag for data quality issues is noted in the summary files.

2.4. Investigating the presence of seven food colors in curated lists of developmental neurotoxicants and neurotoxicants

In addition to searching for associations between the seven food colors and neuro-related effects through mechanistic assays, existing chemical lists were likewise searched for the inclusion of any of the seven food colors. A total of 202 such lists have been compiled and are included within EPA's Computational Toxicology (CompTox) online dashboard (https://comptox.epa.gov/dashboard/chemical_lists). These lists include, among others, chemicals shown to have a causal relationship with a number of adverse outcomes along with lists of substances included in various testing programs. Six of the lists are classifications of potential or confirmed neurotoxicants or developmental neurotoxicants (https://comptox.epa.gov/dashboard/chemical_lists/?search=neuro, accessed September 2019; more information in Supplemental Materials).

2.5. Mechanistic evidence for seven food colors and neurodevelopmental processes in peer-reviewed literature

A PubMed search was conducted to identify articles that reviewed

data relevant to one or more of the seven colors and measures of neurodevelopmental process-relevant signals in cell-based, cell-free, and alternative *in vivo* models (zebrafish and *C. elegans*). Search syntax was constructed using key terms for neurobehavioral/neurological outcomes and related MIEs and KEs identified through the process described heretofore (Fig. 1, Step 1) (including "neurobehavio(u)r," "attention deficit hyperactivity disorder," "ADHD" "GABA receptor," and "thyroid hormone," among others), and were concatenated with syntax for the seven food colors, as well as terms specific to *in vitro* and alternative model experiments (including "*in vitro*," and "zebrafish," among others). Exclusionary syntax was used to reduce the number of articles in which a dye was used for cell staining (e.g., fast green staining). The full search syntax is included in the Supplemental Materials. Titles and abstracts returned via the PubMed search were reviewed and deemed relevant for full text review if the exposure to an FD &C color was defined and the response(s) measured were associated with MIEs or KEs for neurodevelopmental processes in *in vitro* or in an alternative model, as described above. Studies were excluded from further analysis if they did not meet these criteria. For all studies that were included or marked as uncertain for inclusion or exclusion, the full text was reviewed by two reviewers (JKB and GAC). For all studies ultimately included, information relevant to the objective of the present study was evaluated and is reported/discussed herein. Author-reported findings and conclusions were considered without a formal evaluation of study reliability or validity.

3. Results

3.1. Identification of plausible mechanisms of neurobehavioral/neurological and developmental neurotoxicity outcomes

Relevant articles in the literature that provided overviews on the current state of developmental neurotoxicity testing methods (both current and alternative), relevant neurodevelopmental processes, and AOP-based approaches were identified including: scientific reviews (Bal-Price et al., 2015a; Bal-Price and Meek, 2017), reports from recent workshops (Aschner et al., 2017; Fritsche et al., 2017, 2018), and regulatory body reviews (Behl et al., 2019; Fritsche et al., 2015). Eight AOPs were identified in the literature, five of which were found in the AOPWiki (see Table 3). Many of the AOPs share key events; for example, reduction of thyroxine (T4) is a KE in three AOPs (AOPs #2, 4, and 5), while altered/reduced neural network function is a KE in four AOPs (AOPs #1, 4, 6, and 7) (see Table 3). Similarly, binding of N-methyl-D-aspartate receptors (NMDARs) is a MIE in more than one AOP. AOPs 1 and 3 share an MIE, most KEs, and AO (impaired learning and memory), while the final KE and the organism-level effects are different (decreased synaptogenesis and decreased neuronal network function, respectively, for AOP 1 versus neuroinflammation and neurodegeneration, respectively, for AOP 3). Most of the AOs for the AOPs are related to learning and memory impairment.

Specific literature on ADHD, along with other neurodevelopmental outcomes, helped identify additional technical terms to inform subsequent searches within the ToxCastTox21 assay data (Aschner et al., 2017; Bal-Price and Fritsche, 2018; Dark et al., 2018; Fontana et al., 2019). For example, Fontana et al. (2019), described the zebrafish as a model organism in which ADHD may possibly be studied based on homologous genes implicated in clinical practice.

Separately, none of the seven colors was included in any of the six chemical lists included in the CompTox dashboard that are related to developmental neurotoxic or neurotoxic adverse outcomes.

3.2. HTS assays mapped to neurodevelopmental processes

Of the 1473 HTS assays included in version 3.2 of the ToxCast database summary files, 99 assays were mapped to neurodevelopmental processes and/or neurobehavioral outcomes (Supplemental Table S1). Additional assays that provide contextual information to these 99 assays, such as assays for specificity or cell viability related to a specific targeted assay, were not included in the 99 total neuro-relevant assays, although they were used to determine assay bioactivity. The majority of these 99 neuro-relevant HTS assay targets (e.g., the measurement of a neurotransmitter-encoding gene possibly relevant to ADHD) from ToxCast/Tox21 was mapped based on CTD-culled information (Supplemental Table S1). The HTS assays were assigned to one or more of the following categories of neurodevelopmental pathways or outcomes based on the evidence as described above: ADHD, learning disorders, behavioral manifestation, brain morphology, and neurotransmission. Assays that measure genes that are related to more than one of these categories would be assigned to all applicable categories. For example, an assay for a gene encoding a neurotransmitter for which evidence exists suggesting an association with ADHD would be mapped to both "ADHD" and "neurotransmission," whereas an assay encoding a

Table 3
Published neurological/neurobehavioral AOPs.

AOP #	AOP Title	AO	Reference(s) and AOPWiki[a] URL (if applicable)
1	Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities[b]	Impaired learning and memory	(Bal-Price and Meek, 2017) https://aopwiki.org/aops/13
2	Up-regulation of thyroid hormone catabolism via activation of hepatic nuclear receptors, subsequent adverse neurodevelopmental outcomes in mammals	Neuronal dysfunction	(Bal-Price and Meek, 2017) https://aopwiki.org/aops/8
3	Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development leads to neurodegeneration with impairment in learning and memory in aging[b]	Impaired learning and memory	(Bal-Price and Meek, 2017) https://aopwiki.org/aops/12
4	Inhibition of Na+/I− symporter (NIS) decreases TH synthesis leading to learning and memory deficits in children	Impaired learning and memory	(Bal-Price and Meek, 2017) https://aopwiki.org/aops/54
5	Inhibition of thyroperoxidase and subsequent adverse neurodevelopmental outcomes in mammals	Decreased cognitive function	(Bal-Price and Meek, 2017) https://aopwiki.org/aops/42
6	Impairment of learning and memory induced by binding of electrophilic chemicals to the SH(thiol)-group of proteins and non-protein molecules in neuronal and glial cells during development	Impaired learning and memory	Bal-Price et al. (2015b) Bal-Price and Meek (2017)
7	The interaction of non-dioxin-like PCBs with ryanodine receptors (RyRs) causes their sensitization affecting neuronal connectivity that results in behavioral deficits	Behavioral deficits: learning, memory, psychomotor, and attention	Bal-Price et al. (2015b) Bal-Price and Meek (2017)
8	Exposure to mixtures of metals and neurodevelopmental outcomes: A multidisciplinary review using an AOP framework	Learning and cognitive deficits	von Stackelberg et al. (2015) Bal-Price and Meek (2017)

[a] AOPWiki URL: https://aopwiki.org/aops/, access date: October 2019.
[b] AOP #1 includes decreased synaptogenesis as the final KE leading to decreased neuronal network function, while AOP #3 has neuroinflammation as the final KE leading to neurodegeneration.

neurotransmitter that has not been associated with any particular outcome would only be mapped to the category "neurotransmission". Thus, because some of the 99 neuro-relevant assays were mapped to more than one category, ultimately there was a total of 125 assay mappings. HTS assays were further sub-categorized according to specific MIEs/KEs or neuro-relevant processes (e.g., calcium influx, thyroid, etc.; Supplemental Table S1). Alterations in human thyroid hormone level, for example, represents an MIE or KE in multiple AOPs for decreased cognitive function and impaired learning and memory (AOPs #2, 4 and 5, respectively, see Table 3). Changes in the blood levels of individual or several related neurotransmitters may represent a mechanism associated with ADHD and learning impairment (Mailman and Lewis, 1987; Nilsen and Tulve, 2019). The category "neurotransmission" represents the mechanistic category with the highest number of assays. These include assays for genes encoding for neurotransmitters such as dopamine, serotonin, GABA, glutamate, histamine, noradrenaline, and adenosine, as well as enzymes that break down specific neurotransmitters, such as acetylcholinesterase (AChE), catechol-O-methyltransferase (Comt), and monoamine oxidases (Maoa and Maob) (Supplemental Table S1).

Because oxidative stress is reported to be a KE in an AOP for learning disorders associated with exposure to a mixture of metals (AOP #8, Table 3; (Bal-Price and Meek, 2017; von Stackelberg et al., 2015), we also searched for HTS assays related to oxidative stress. This AOP (#8) is based on information specific to metal exposure, which is linked to oxidative stress in astrocytes along with disruption in calcium signaling and thyroid hormone levels (reviewed in von Stackelberg et al., 2015). The HTS assays in ToxCast/Tox21 that are relevant to oxidative stress were primarily tested in hepatic cells, with no such comparable assays tested in neuronal cell models. As such, the HTS assays are not relevant for inclusion in this assessment as presented herein. Nonetheless, none of the colors were active in the HTS oxidative stress assays in which they were tested , according to the criteria described above (data not shown).

The HTS assay mappings described in this assessment were cross-referenced with those of a recent study that developed an adverse outcome pathway network for human neurotoxicity (Spinu et al., 2019). In the study described by Spinu et al. (2019), 15 ToxCast HTS assays were identified that evaluate signals related to KEs in neurotoxicity-relevant AOPs. The authors only included ToxCast HTS assays that use a brain tissue model. The neuro-relevant HTS assays mapped in the assessment presented herein included all 15 assays identified by Spinu et al. (2019). However, because we did not limit the mapping to brain tissue models in the present study, and because we mapped HTS assays more broadly across neuro-relevant mechanisms, there were a number of other assays included in our assessment.

3.3. HTS evidence-base for seven food colors relevant to neurodevelopmental processes

Across the seven food colors, a total of 116 neurodevelopmental process-relevant assay measures/color pairs were tested (Table 4, Fig. 1). Note that the maximum number of assays would be 693 if all seven colors were tested for all 99 mapped assays. The number of neuro-relevant assays in which each color was tested ranged from as low as eight (FD&C Blue No. 2) to 26 (FD&C Red No. 40). Only FD&C Blue No. 1 (brilliant blue), FD&C Green No. 3 (fast green FCF), FD&C Red No. 3 (erythrosine), and FD&C Yellow No. 5 (tartrazine) were found to have activity in a limited number of assays (Table 4). Of these, only FD&C Red No. 3 had more than two active HTS neuro-relevant assays. Using FD&C Red No. 3 as an example, Fig. 2 shows the process for identifying the overlap between all HTS assays in which the color was tested, and those that were mapped to neurodevelopmental processes or outcomes.

All seven FD&C colors were also tested in twelve autofluorescence assays within the Tox21 program. Autofluorescence assays are used for artifact detection and provide background controls. Changes to fluorescence intensity signals in these assays indicate that the test article has a physical feature that alters or influences the background fluorescence. All seven of the FD&C colors were inactive in all twelve of these autofluorescence assays.

The limited assays – mostly rodent-based systems – for which there was reported activity for a given food color included signals for thyroid antagonism (FD&C Blue No. 1 and FD&C Green No. 3), neurotransmitter receptor expression for dopamine and serotonin (FD&C Red No. 3 and FD&C Yellow No. 5, respectively), and transport and breakdown of the neurotransmitters serotonin, dopamine, and noradrenalin and serotonin (FD&C Red No. 3) (Fig. 3). These are contrasted with inactivity observed for many other assays tested in similar systems and querying similar signaling pathways (Fig. 3).

In the assays tested by the National Toxicology Program (Tox21 assays), analytical quality confirmation and stability were evaluated for FD&C color test articles. In several cases, test articles did not meet chemical quality criteria due to sub-optimal purity or low concentration (Table 5, Supplemental Table S2), an important consideration in the interpretation of activity elicited by the test article (or lack thereof). In the ToxCast assays other than those tested in the Tox21 program, which are conducted by a variety of laboratories, chemical quality of the test article remains unknown. More detail is provided in the sections below.

FD&C Blue No. 1 was active in a single assay: the reporter gene

Table 4
Number of active HTS assays in the ToxCast/Tox21 database per total number of assays in which each FD&C color was tested, considering only the assays mapped to neurodevelopmental processes.

Color[a]	Common Name	Number of Active HTS Assays/Total Number of HTS Assays[b]	
		Activity without integration of cytotoxicity data[c]	Activity including integration of cytotoxicity data[d]
FD&C Blue No. 1	Brilliant blue	4/11	1/11
C.I. Acid Blue 74	Indigo carmine	2/8	0/8
FD&C Green No. 3	Fast green FCF	5/11	2/11
FD&C Red No. 3	Erythrosine	8/15	4/15
Allura Red C.I.16035	Allura red	10/27	0/27
FD&C Yellow No. 5	Tartrazine	7/21	1/21
FD&C Yellow No. 6	Sunset yellow	7/23	0/23

[a] As listed in the CompTox Dashboard, December 2019 https://comptox.epa.gov/dashboard.
[b] The term "assays" used here reflects the individual "assay component endpoints" as named in the ToxCast/Tox21 summary files and database. Assays that are included for contextual information, i.e. cell viability assays, are not included in the counts in the respective columns.
[c] These assays are indicated as active in the CompTox dashboard based solely on a hit-call of 1, with no other contextual consideration, such as the cytotoxic concentration burst range.
[d] These assays are indicated as active according to criteria that include consideration of potential cytotoxic interference as described in the Materials and methods section.

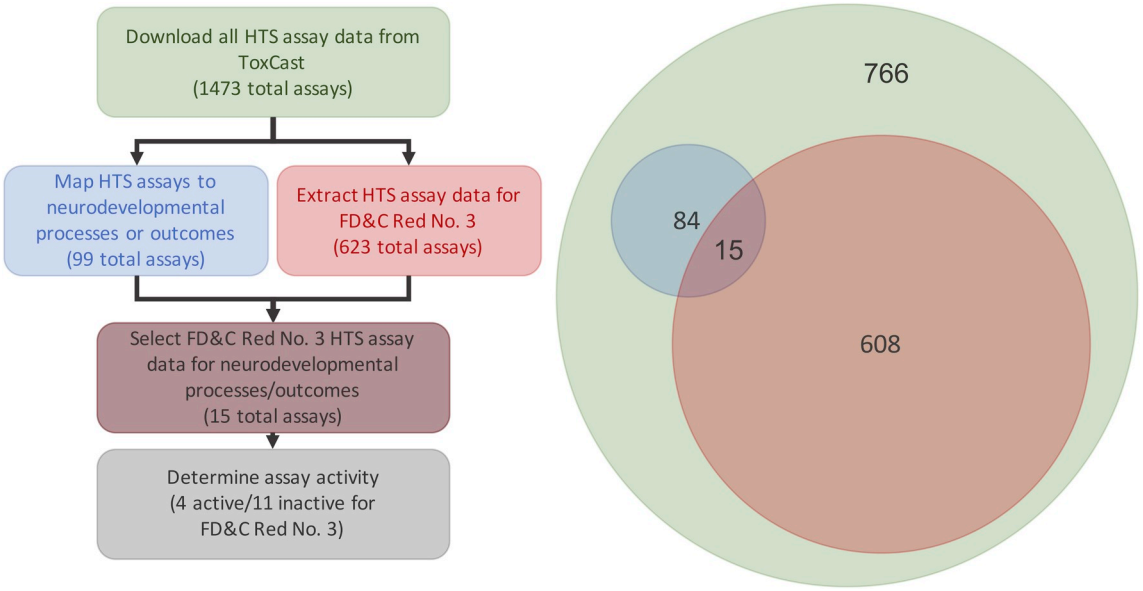

Fig. 2. Process for the identification of HTS assays with activity related to MIEs and KEs identified in neurodevelopmental AOPs, or genes potentially related to neurodevelopmental processes or outcomes, using FD&C Red No. 3 as an example. The Venn diagram shows the number of HTS assays tested for FD&C Red No. 3 among all HTS assays in the ToxCast/Tox21 database, and the overlap of the FD&C Red No. 3 assays with neurodevelopmental-relevant assays.

assay "TOX21_TR_LUC_GH3_Antagonist" that detects loss of signal via bioluminescence after binding the promoter region of thyroid hormone receptor-alpha and -beta (*THRA* and *THRB*) genes in the rat pituitary gland GH3 cell-line. The molecular weight of the sample used for this assay was confirmed by chemical quality control (QC) analytical testing, but no purity information was available. For this reason, while the identity of the test sample was confirmed, it is unknown whether the purity is sufficient to provide confidence that activity (or inactivity) is attributable to FD&C Blue No. 1. However, FD&C Blue No. 1 was inactive in seven other thyroid-related assays, including assays for antagonist activity against thyroid stimulating hormone receptor (TSHR) and the thyrotropin releasing hormone receptor (TRHR) (see Supplemental Table S2 for complete set of assay results). In addition to the reported inactivity of these seven thyroid assays, other reported inactivity included absence of a loss of expression in the serotonin receptor *HTR7*, absence of gain of expression in the selectin P gene *SELP* (which has been associated with "neurological manifestations," CTD), and a lack of change to zebrafish brain morphology in an embryo model. Collectively, all but one of the HTS assays representing plausible KEs in neurodevelopmental outcomes tested with FD&C Blue No. 1 were inactive. These inactive assays included thyroid signaling, changes to neurotransmitter activity, and alterations in brain morphology in a zebrafish embryo model.

The only neuro-relevant assays in which FD&C Green No. 3 was tested were related to thyroid. FD&C Green No. 3 was active in two assays – both are related to thyroid antagonism, one of which is the reporter gene TOX21_TR_LUC_GH3_Antagonist assay. The other, NVS_NR_hTRa_Antagonist, uses a binding reporter chemiluminescence signal to understand changes in the binding relative to the gene *THRA*. Loss of signal (when compared to dimethyl sulfoxide (DMSO) negative

Fig. 3. HTS assays mapped to neurodevelopmental processes and/or MIEs and KEs identified in neurodevelopmental AOPs. Tiles represent each color-assay pair as denoted by ToxCast assay endpoint ID (aeid) on the x-axis and color on the y-axis. Each tile is color-coded to represent the testing status and activity for each color-assay pair. The 99 neuro-relevant mapped assays are grouped by their respective mechanistic category, as denoted across the x-axis at the top of the graphic. None of the colors were tested for assays related to GABA signaling or AChE, and there was very little coverage of assays for glutamate release, all of which are within the "Neurotransmission" category. All results and additional assay information can be found in a tabular format in Supplemental Table S2.

Table 5
Test article quality information for each food color.[a]

Color[b]	CASRN	DTXSID[c]	SampleID[d]	Identity	Purity
FD&C Blue No. 1	3844-45-9	DTXSID2020189	Tox21_300516	Molecular weight confirmed by LCMS	No purity information
			TP0000395E06	No information	No information
			TP0001207L10	No information	No information
				No information	No information
			TP0000972F06	No information	No information
			TP0000477A08	No information	No information
C.I. Acid Blue 74	860-22-0	DTXSID1020190	Tox21_113456	No information on molecular weight	Impure by NMR
			Tox21_302732	Molecular weight confirmed by LCMS	Purity 50–75%
			TP0000714E07	No information	No information
			TP0001401D09	No information	No information
FD&C Green No. 3	2353-45-9	DTXSID3020673	Tox21_302086	Molecular weight confirmed by NMR	Purity > 90% by NMR
			TP0001515H09	No information	No information
			TP0001468L05	No information	No information
			TP0001397A05	No information	No information
			TP0000721E09	No information	No information
			TP0001397A05	No information	No information
FD&C Red No. 3	16423-68-0	DTXSID7021233	Tox21_202932	Incorrect molecular weight by NMR	Purity > 90% by LCMS, “acceptable” by NMR
			Tox21_302085	No information on molecular weight	Minor impurities by NMR
			TP0001390D02	No information	No information
			TP0001468F01	No information	No information
			TP0001343E08	No information	No information
			TP0001180B05	No information	No information
			TP0001468F01	No information	No information
			TP0001397A07	No information	No information
Allura Red C.I.16035	25956-17-6	DTXSID4024436	Tox21_300393	Molecular weight confirmed by NMR	Purity > 90% by NMR
			TP0000395G02	No information	No information
			TP0001206O14	No information	No information
			TP0000967B08	No information	No information
			TP0000422G02	No information	No information
			TP0000474A04	No information	No information
FD&C Yellow No. 5	1934-21-0	DTXSID1021455	Tox21_113411	Stereoisomers detected by NMR	“Acceptable” by NMR
			Tox21_201539	Molecular weight confirmed by LCMS	Purity > 90% by LCMS
			Tox21_300554	Molecular weight confirmed by NMR	Purity > 90% by NMR
			TP0000394B12	No information	No information
			TP0000968B11	No information	No information
			TP0000471A11	No information	No information
			TP0000410B12	No information	No information
			TP0000394B12	No information	No information
FD&C Yellow No. 6	2783-94-0	DTXSID6021456	Tox21_201897	Molecular weight confirmed by NMR	Purity > 90% by NMR
			Tox21_300407	Molecular weight confirmed by NMR	Purity > 90% by NMR
			TP0000397E03	No information	No information
			TP0000970G04	No information	No information
			TP0000493A10	No information	No information
			TP0000131D04	No information	No information

NMR: nuclear magnetic resonance, LCMS: liquid chromatography-mass spectrometry, GCMS: gas chromatography-mass spectrometry.

[a] As provided by the EPA CompTox program via personal communication. Concentration information was either not reported, or the expected concentration (a necessary metric to understand absolute concentration) according to the methodology used was not clear.

[b] As listed in the CompTox Dashboard.

[c] DSSTox substance identifier. DSS: Distributed Structure-Searchable Toxicity.

[d] As listed in the invitrodb_v3.2 summary files (released August 2019, accessed September 2019 for this study). Multiple samples exist for each FD&C color, each of which was analyzed. The assays in which each sample was run can be found in Supplemental Table S2.

control) indicates antagonist activity of the test article. For the other nine thyroid-related assays in which FD&C Green No. 3 was tested, it was inactive including assays for TSHR and TRHR (see Supplemental Table S2 for a complete set of results). No chemical QC analytical data were available for the sample used in the NVS_NR_hTRa_Antagonist assay, while the sample used in the Tox21 assay passed chemical QC analysis with a confirmed molecular weight (identity) and purity > 90%. Overall, the weight of *in vitro* evidence for FD&C Green No. 3 suggests a lack of interaction with the ThR, which is only one MIE or KE among a collection of MIE/KEs in the AOPs noted in Table 3. Aside from the thyroid-related assays, FD&C Green No. 3 was not tested in any other HTS assays that were mapped to neuro-relevant processes.

FD&C Red No. 3 was evaluated for activity in a total of nine thyroid-relevant assays, five neurotransmitter-relevant assays, and one assay for the selectin P (*SELP*) gene (associated with “neurological manifestations,” CTD). While no activity was reported in any of the thyroid-relevant assays, activity in four neurotransmitter-relevant (i.e., degradation or transport) assays shows a *loss* of signal for four genes: rat monoamine oxidase A (*Maoa*), human solute carrier family 6 (neurotransmitter transporter) member 2 (*SLC6A2*), solute carrier family 6 (neurotransmitter transporter) member 4 (*Slc6a4*), and human dopamine receptor D1 (*DRD1*). Chemical QC analyses of the FD&C Red No. 3 samples are available for six assays, all of which evaluate activity in thyroid signaling. These samples were reported as having acceptable purity, but one or more issues with the sample quality were reported: either the molecular weight was incorrect by chemical analysis or there was a low concentration of FD&C Red No. 3 in the sample (Table 5, Supplemental Table S2). The concentration in the test substance does not represent the final concentration tested in the assays, such that the significance of a low concentration in the test sample on the final concentration in the well of the assay is not clear. Thus, the reliability of the results for these six FD&C Red No. 3 thyroid signaling assays is questionable. Chemical QC analytical and purity data were not available for all other FD&C Red No. 3 assays, including those

neurotransmitter assays that were identified as active. Although the weight of *in vitro* evidence evaluated in this assessment suggests that Red No. 3 lacks activity in thyroid assays, the test article used in these assays did not pass QC analysis and, as such, these results may be unreliable. However, FD&C Red No. 3 may play a role in altering genes responsible for normal function of neurotransmitters, which is a key event in some neurobehavioral disorders.

FD&C Yellow No. 5 was inactive in 20 of the 21 neuro-relevant assays in which it was tested. Inactivity was reported for: eight thyroid-relevant measures; the zebrafish embryo brain morphology assay; and assays of various types that measure gain or loss of binding or activity of target genes that encode either dopamine receptors 1, 2, and 4, neuronal nitric oxide synthase (*Nos1*), a calcium channel subunit (*Cacna1a*), an adreno receptor (*ADRA2C*), selectin P (*SELP*), or serotonin receptors 1A, 4, 5A, and 7. Activity was reported for loss of signal for the 5-hydroxytryptamine (serotonin) receptor 1A (*Htr1a*) in rat cortical membranes using a G protein-coupled receptor (gpcr) cell-free binding assay. The serotonin receptor binding appears to be species-specific, because other serotonin receptor binding assays for human and guinea pig serotonin receptor genes were inactive for FD&C Yellow No. 5. No other alterations to neurological/neurobehavioral-related signals mapped to the available HTS assays were observed. Also, no chemical QC analytical data were available for the single assay with activity (the *Htr1a* assay). For the six thyroid assays in which chemical QC analytical data were available, there was no activity. Issues were reported for the identity and stability of the chemical according to analytical testing (Table 5). Specifically, the samples passed structural identity tests but appeared to degrade over time, based on the detection of isomers or impurities. The loss of test article over time resulted in a low concentration of FD&C Yellow No. 5 in stored samples. Since the timepoint at which the bioactivity assays were run relative to the storage of the test article(s) is not available, how these stability results influence the assay results is unclear. Overall, FD&C Yellow No. 5 was inactive in thyroid or other HTS assays mapped to neuro-relevant processes.

Remaining are C.I. Acid Blue 74, Allura Red C.I.16035, and FD&C Yellow No. 6, for which all neurobehavioral/neurological-relevant assays tested (i.e., 8, 27, and 23 relevant assays, respectively) were reported to be inactive (see Table 4). The samples of FD&C Yellow No. 6 and Allura Red C.I.16035 for which chemical QC data were available passed identity and purity analytical tests. However, the C.I. Acid Blue 74 samples for which analytical chemical QC data were available (seven thyroid signaling assays) were impure and unstable in storage (although the time of testing is not known) (Table 5).

3.4. Relevant literature on colors and neurologic/neurobehavioral linkages measured in vitro or in alternative models

A total of 83 articles were identified in PubMed using curated search syntax based on terms identified from neurodevelopmental mechanistic evidence, with 12 articles across the seven colors identified as containing data from *in vitro* or alternative models (Table 6, Fig. 1). Of these, nine studies investigated Red No. 3 (Augustine and Levitan, 1983; Bole and Ueda, 2005; Brosemer, 1985; Logan and Swanson, 1979; Mailman, 1987; Mailman et al., 1980; Mailman and Lewis, 1987; Shimizu et al., 2013; Wade et al., 1984). For FD&C Blue No. 1, a single study was identified (Lau et al., 2006), and two studies were identified for FD&C Yellow No. 6 (Qu et al., 2017; Swarnalatha et al., 2017). A full list of articles returned via the PubMed search, and whether or not they were excluded or included can be found in Supplemental Table S3. The most common reason for excluding studies was because *in vitro* or other alternative models were not used in the study. Other main themes among the excluded studies were the use of the color as a dye for the indication of an endpoint that is not relevant to the present study, or studies conducted in plants.

Two studies were identified that reported the potential for FD&C Red No. 3 to interfere in neurotransmitter uptake and neuronal function; one in isolated cutaneous pectoris nerve-muscle preparation from frogs (*Rana pipiens*) (from 10 μM to 1 mM) (Augustine and Levitan, 1983) and another in rat brain homogenates (1 μM) (Logan and Swanson, 1979). Another study evaluated Red No. 3 in rat brain synaptosomal preparations based the hypothesis that the neurotoxicant properties observed in the 1979 study (Logan and Swanson, 1979) may be due to methodological artifacts related to the amount of tissue used in the incubation systems (Mailman et al., 1980). The authors of this follow-up study found that the synaptosomal protein concentration present in the incubation medium significantly influenced the inhibitory effect of FD&C Red No. 3 on synaptosomal dopamine uptake in rat brain preparations, with an inverse relationship between the percentage of dopamine uptake observed and the synaptosomal tissue concentration, while erythrosine and dopamine concentrations in the test medium were held constant. The authors surmise that the dopamine inhibition may be the result of non-specific interactions with neural membranes (Mailman et al., 1980). Additionally, three *in vitro* studies with erythrosine reported effects on neurotransmitter function related to GABA and glutamate in rat brain tissue preparations (Bole and Ueda, 2005; Brosemer, 1985; Wade et al., 1984), with one study reporting FD&C Red No. 3 inhibition of iodotyrosine deiodinase, an enzyme involved in thyroid hormone homeostasis (Shimizu et al., 2013). Based on the studies by Mailman et al. (1980), the use of *in vitro* model systems in interpreting any findings without model validation is questionable. In fact, there were a number of reviews by Mailman and colleagues in which the results in neurotransmitter changes evaluated in various *in vitro* models were conducted at concentrations that would not be achieved in the brain under conditions of human dietary exposure (Mailman and Lewis, 1987). It was noted in these studies that low ingested doses of FD&C Red No. 3 (erythrosine) are extremely unlikely to produce effects on the central nervous system (Mailman, 1987; Mailman et al., 1980).

Only one neuro-relevant mechanistic study was identified in the

Table 6
Relevant articles on food colors tested in *in vitro* or other alternative models evaluating potential MIEs or KEs for neurobehavioral/neurological outcomes.

Color	Common name	Number of articles returned in PubMed search[a]	Number of relevant articles[b]	References for relevant articles
FD&C Blue No. 1	Brilliant Blue	12	1	Lau et al. (2006)
FD&C Blue No. 2	Indigo Carmine	8	0	–
FD&C Green No. 3	Fast Green	22	0	–
FD&C Red No. 3	Erythrosine	28	9	Augustine and Levitan (1983); Bole and Ueda (2005); Brosemer (1985); Logan and Swanson (1979); Mailman (1987); Mailman et al. (1980); Mailman and Lewis (1987); Shimizu et al. (2013); Wade et al. (1984)
FD&C Red No. 40	Allura Red	1	0	–
FD&C Yellow No. 5	Tartrazine	10	0	–
FD&C Yellow No. 6	Sunset Yellow	6	2	Swarnalatha et al. (2017); Qu et al. (2017)

[a] Search conducted on October 1, 2019.
[b] Articles were considered relevant based on full text review that provided mechanistic, exposure and neuro-related assay information.

literature for FD&C Blue No. 1, which was confounded by secondary cytotoxicity considerations in the *in vitro* cell system. In this study, Lau et al. (2006) evaluated the potential neurotoxic effects of FD&C Blue No. 1 (0.05–500 nM) alone and in combination with glutamate in a neurite outgrowth model of mouse NB2a neuroblastoma cells that were induced to differentiate and grow neurites in the presence of additives. Neurite outgrowth is a measure used to assess normal brain development and maturation, and is used to measure neuronal differentiation in culture [(Bal-Price and Fritsche, 2018; Lau et al., 2006)]. In this *in vitro* model, FD&C Blue No. 1 reduced neurite outgrowth with an IC_{50} concentration (i.e., the concentration at which there was a 50% reduction) of 51.4 nM (SEM = 21.2 nM). Cytotoxicity was evaluated by Trypan Blue dye exclusion, in which there was ~35% cell death when exposed to 0.05 nM of FD&C Blue No. 1. This suggests that, in this model, reduction may be due to cytotoxicity of the cells *in vitro* at a level much lower than the IC_{50} for neurite outgrowth reductions in an *in vitro* non-dynamic system.

Embryogenesis was evaluated in zebrafish embryos exposed to FD&C Yellow No. 6 (Swarnalatha et al., 2017). Growth defects were observed during day 1, the brain and optical primordium development was initiated but was not complete by day 2, embryo development slowed at days 4–6, and mortality increased in exposed embryos in relation to controls. Study limitations include unclear exposure conditions – it is unclear if the embryos were exposed to 0.1 and 0.5 mg or mg/L, with different units reported in the abstract compared to the rest of the manuscript – and media and other conditions were not reported. An *in vitro* study reported increased intracellular calcium levels in HepG2 cells following 24 h of exposure to Sunset yellow (Qu et al., 2017). While the authors propose the increase in calcium levels are involved in general toxicity, alterations to calcium signaling may be associated with neurotoxicity and neurodegenerative disorders (von Stackelberg et al., 2015), and increased intracellular calcium may lead to excitotoxicity, which has been related to neuronal cell death and acute neurologic disease (Arundine and Tymianski, 2003). However, the increase in intracellular calcium in the HepG2 cells treated with Sunset yellow was accompanied by a significant loss of mitochondrial membrane potential and an increase in cell membrane permeability. This suggests that the calcium fluctuation related to Sunset yellow is likely a result of diffusion across a gradient, as intracellular calcium levels are typically much lower than extracellular calcium in a normal state. In contrast, perturbations in calcium levels are considered a key event in AOPs related to impairment of learning and memory, when intracellular calcium levels *decrease* (i.e., AOP #1, Table 3). It is notable that FD&C Yellow No. 6 was inactive in an assay for changes to the calcium channel, voltage-dependent, N type, alpha 1B subunit (*Cacna1b*) gene in tissue-based cell-free format using rat cortical membranes, according to the HTS data (Supplemental Table S2).

Overall, a limited number of relevant mechanistic studies were identified in the peer-reviewed literature for these seven food colors. FD&C Red No. 3 appears to have the most information published in the dated literature (> 20 years ago), although the study authors have questioned the relevance of findings due to unvalidated models and unclear extrapolation of test article concentrations and tissue levels to human exposures.

4. Discussion

Understanding the risk factors associated with neurobehavioral/neurological disorders in children, such as ADHD, is challenging, especially given the paucity of test models and methods to study these disorders. The development of testing strategies that evaluate potential disruptions in neurodevelopmental processes upon exposure to substances (e.g., synthetic food colors assessed herein) to predict potential neuro-related hazards are ongoing. In the present assessment, ToxCast/Tox21 HTS assays, along with mechanistic data reported in the peer-reviewed literature collected from *in vitro* assays and other NAMs, were mapped to MIEs and KEs identified in published neurodevelopmental-relevant AOPs (Table 3) or to other signaling pathways or mechanisms related to neurodevelopmental processes and/or outcomes, as identified in the literature and in public databases. Because the HTS assays typically measure changes to single genes, they represent a single component of a MIE or KE in an AOP in which all MIEs/KEs would need to occur in the proposed temporal and directional manner for the adverse outcome to arise. The objective of the assessment presented herein was to understand whether these data provide insights into the plausibility of an association between neurodevelopmental effects and exposure to any one of these seven FDA approved food colors.

Overall, FD&C Red No. 3 was the only food color out of the seven evaluated showing evidence, albeit limited, of neurodevelopmental-related activity in HTS assays from the ToxCast/Tox21 database and in *in vitro* assays published in the literature. Based on the number of AOP-mapped MIE- and/or KE-relevant assays in which FD&C Red No. 3 was tested, most were inactive. The predominant category of HTS assays that showed activity for FD&C Red No. 3 was specific to loss in neurotransmitter activity, although none of these assays had chemical analysis data for substance quality. Also of interest, we evaluated the bioactivity of resveratrol, a natural polyphenol proposed to have antioxidant, antitumor, and neuroprotective effects (Rege et al., 2014), in the 99 neuro-relevant HTS assays. Resveratrol, like FD&C Red No. 3, was active in several neurotransmitter assays (resveratrol HTS assay data are included in Supplemental Table S2). This highlights the current understanding that bioactivity in selected individual HTS assays does not necessarily indicate adverse effects.

Alterations in the neurotransmitter-encoding genes are purported to be associated with ADHD and other behavioral outcomes in children and in rodent models of behaviors related to anxiety and ADHD (Chen et al., 2004; Fontana et al., 2019; Lawson et al., 2003; Nymberg et al., 2013). Monoamine oxidase A (*MAOA*) oxidizes neurotransmitters, specifically serotonin, norepinephrine, and dopamine. *MAOA* regulation is essential to the maintenance of normal mental states (Shih et al., 1999). The loss of expression of *MAOA* may be associated with anxiety-like behavior, as evidenced by observations in a *Maoa/b* knockout mouse model (Chen et al., 2004). *MAOA* gene mutations and polymorphisms have been suggested to be associated with ADHD incidence in humans as well (Lawson et al., 2003; Nymberg et al., 2013). The members of the solute carrier 6 (*SLC6*) gene family encode transporters for neurotransmitters, including serotonin, dopamine, norepinephrine, GABA, and glycine (Kristensen et al., 2011). Alterations to *SLC6* family members have likewise been linked to ADHD and other neurobehavioral or mental disorders (Hahn and Blakely, 2007; Pramod et al., 2013). ADHD linkages to these genes are attributable primarily to genetic polymorphisms or mutations, not to alterations related to chemical exposures. In older literature, a number of *in vitro* assays showed the potential of FD&C Red No. 3 to interfere in neurotransmitter uptake and neuronal function (Augustine and Levitan, 1983; Logan and Swanson, 1979). However, subsequent evaluation identified methodological artifacts *in vitro* related to the amount of tissue used in the incubation systems that may have confounded these findings. Thus, the results of these studies are placed into question, underscoring the need for validation of *in vitro* model systems prior to interpretation of the findings.

Important considerations for the interpretation of any *in vitro* findings include extrapolation of observed activity (AC_{50} concentration) to circulating human blood levels under normal dietary intake scenarios, which would require knowledge of toxicokinetic information. Although *in vitro*-to-*in vivo* extrapolation (IVIVE) modeling could be used to estimate human doses (mg/kg bw/day) from *in vitro* AC_{50} values, these modeling efforts are dependent on, at a minimum, knowledge of the intrinsic clearance and protein binding information for the test substance in question. For example, there is evidence that FD&C Red No. 3 is poorly absorbed, with nearly 100% excreted unchanged in the feces in exposed rats, and only 1% estimated to be absorbed from the GI tract

in humans (studies summarized in EFSA, 2011; WHO/FAO, 2019). More detailed knowledge of both the bioavailability of FD&C Red No. 3 and its potential protein binding in humans is necessary to improve the accuracy of IVIVE modeling for the extrapolation of *in vitro* activity concentration (AC_{50}) to estimated circulating human blood levels. The other six colors are also poorly absorbed (summarized in EFSA, 2009a, b, c, 2010, 2011, 2014; WHO/FAO, 2011, 2017a, b, 2019). However, as there is a lack of consistent signal for activity across assays for any one of these colors, along with questionable analytical quality of the test substance evaluated, applying IVIVE modeling is less important at this point.

For some assays, a clear understanding of actual concentration to which cells were exposed based on the physical/chemical properties of the test substance (i.e., stability and protein binding, among others) was not possible due to either a lack of data or poor analytical quality testing results. Therefore, *in vitro* screening of these colors for activity in assays that measure signals related to neurodevelopmental process-related MIEs or KEs may require further refinement and validation. This assessment serves as a first step in mapping available HTS assays and literature-reported assays to these processes, but is limited by both the extent of assay coverage across KEs in identified AOPs and lack of knowledge on all processes involved in disorders such as ADHD. Also, in some cases, the findings were hindered by unknown or poor analytical quality of the test substance.

It should also be noted that limitations generally exist in the sensitivity, reproducibility, and relevance of existing test methods utilized as a surrogate to evaluate disorders such as ADHD, even in tests conducted *in vivo* according to current guidelines (Bal-Price et al., 2015a). For example, the relevance of brain and optical primordium development measurements – such as those reported in zebrafish embryos exposed to FD&C Yellow No. 6 (Swarnalatha et al., 2017) – for the evaluation of potential changes in neurodevelopmental processes in humans is unclear. Additionally, based on the differences in routes of exposure between zebrafish embryo assays and humans, it is unclear how the experimental concentrations translate to *in utero* human exposure. Uncertainties would similarly be introduced in the extrapolation of rodent data to human outcomes, primarily related to toxicokinetics, timing of exposure relative to brain development stages, unreliable sensitive functional assays, and collective endpoints measured in rodent studies that do not sufficiently capture and/or inform underlying biochemical or behavioral traits associated with human neurobehavioral/neurological disorders (Behl et al., 2019).

At this time, there are no fit-for-purpose or validated testing frameworks to evaluate neurodevelopmental endpoints in alternative assays that better represent human diseases/disorders, as compared to *in vivo* models. However, a recent review (Behl et al., 2019) described a set of cell-based and alternative models that have been proposed to capture signals related to neurodevelopmental processes using receptor-based cellular and cell-free assays for the identification of MIEs; human induced pluripotent stem cells, immortalized human dopaminergic neuronal precursor cells, and peripheral neurons for KEs; and rat primary cortical cells for cortical connectivity, zebrafish, and planaria for neurobehavioral adverse outcomes. Further testing and validation of such methods may provide additional opportunities for the testing of molecular and cellular events related to neurodevelopmental processes. Such models, coupled with additional research to identify factors related to susceptibility to alterations in neurodevelopmental processes and events, may be used to better understand and model unique susceptibilities that may not be relevant to the general population.

In conclusion, the results of our assessment of available *in vitro* mechanistic data collected from assays that measure signals related to MIEs or KEs involved in neurodevelopmental processes indicate that the seven FDA-approved food colors (when batch certified) have limited or no activity for such signals. While available information on FD&C colors and genes or enzymes that may have a role in mechanisms of neurodevelopmental alterations may be limited, FD&C Red No. 3 was the only color (of the seven assessed) that showed activity associated with neurodevelopmental pathways. Additional follow-up assays, especially with test articles that pass analytical QC criteria, would provide clarity and increased confidence in these findings. Overall, the FD&C colors do not appear to alter signaling pathways related to neurodevelopmental processes on the molecular or cellular level.

CRediT authorship contribution statement

G.A. Chappell: Conceptualization, Methodology, Investigation, Visualization, Writing - original draft. **J.K. Britt:** Investigation, Writing - review & editing. **S.J. Borghoff:** Conceptualization, Methodology, Supervision, Writing - original draft.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: This work was funded by the American Beverage Association. No authors received personal reviews. ToxStrategies regularly provides consulting services related to food and beverage safety, including assessments of food additives, to various entities within the private sector.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2020.111310.

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