DATE: November 9, 2020

PURPOSE: Scientific document review (in 10 pages)

SUBJECT: Health Effects Assessment: Potential Neurobehavioral Effects of Synthetic Food Dyes in Children Public Review Draft, August 2020. Hereinafter referred to as the "Report".

REVIEWED BY: This Report has been reviewed by Peter Spencer, PhD, FANA, FRCPath, Professor of Neurology and Occupational Health Sciences. Dr. Spencer is the former founding director of the Institute of Neurotoxicology at Albert Einstein College of Medicine, and of the Center for Research on Occupational and Environmental Toxicology and the Global Health Center of Oregon Health & Science University. He is a university-based, neuroscience-trained neurotoxicologist with decades of experience studying human and/or animal responses to exposure to chemicals/metabolites present in or added to food, and to exposure to various drugs, workplace and environmental chemicals with neurotoxic potential.

OEHHA TASK. In response to the Legislature's request, the California Environmental Protection Agency's Office of Environmental Health Hazard Assessment (OEHHA) conducted a multifaceted evaluation of the Food, Drug and Cosmetics Act (FD&C) "batch-certified" synthetic food dyes, focusing on seven of the nine food dyes approved by the US Food and Drug Administration for general use in food in the US. Batch-certification refers to a chemical analysis of each batch of dye sold to ensure that specific contaminants are below a legal limit. Color additives subject to batch certification are synthetic, derived from petroleum, and are listed on a product's ingredient label. The seven dyes are considered to contribute the greatest exposure to synthetic food dyes for the general US public. Specifically, the OEHHA was tasked with evaluating the literature designed to assess whether ingestion of food dyes affects the nervous system and behavior of children.

OEHHA REPORT. The study authors conducted a systematic literature search for and analysis of clinical trials examining the neurological effects of food dyes in children and preemptively assigned high confidence for conclusions drawn from the results of these studies. They also identified numerous experimental laboratory studies of mature and developing animals (rodents) designed to assess the effect of treatment with one or more synthetic food dyes. These studies included oral dye exposure during prenatal, infant, and juvenile development, with the examination of neurobehavioral effects in the offspring manifest during development and in adult animals. The effects of dyes were also evaluated with in-vitro high-throughput assay systems. OEHHA contracted with the University of California, Davis to estimate dye exposure from food and over-the-counter medications and vitamins intended for children. OEHHA also contracted with the University of California, Berkeley to combine these food dye levels with 2015–2016 NHANES data and to compute exposure estimates for a finer set of age groupings. Risk characterization compared these exposure estimates with US FDA Acceptable Daily Intakes (ADIs) derived during 1969–1987, and ADIs derived up until 2010 by the Joint FAO/WHO Expert Committee on Food Additives. Exposure risk was also characterized by poverty level, race and ethnicity, and education of the mother.

REVIEWER'S ANALYSIS. Data and conclusions derived therefrom were assessed for scientific validity. This was based on judgement of adherence to established principles of research practice as described in the many reports addressed in the Report. On occasion, individual studies were reviewed by examination of the original publications. Focus was placed on experimental design, study methodology, generated data, and conclusions drawn therefrom. Weaknesses in any of these areas were identified and used to assess the validity of the conclusions stated in the Report.

REVIEWER'S SUMMARIZED FINDINGS. OEHHA in concert with investigators at the University of California Davis and Berkeley carried out a comprehensive study of exposure/effect risk associated with oral intake by children of one or more of seven dyes used as coloring agents in food, medicines and vitamins. The OEHHA study focused on short and long-term risks to the human nervous system and behavior. Assessment was based largely on reports in the professional literature of relevant human, animal and in-vitro studies, the results of which were assessed in relation to contemporary human exposure estimates for Americans with different ethnic, racial, socioeconomic and educational profiles. The resulting August 2020 OEHHA Report for Public Comment represents a comprehensive approach that raises important questions about the safety of current

practices that expose children to the dyes under examination. The report lacks an assessment of the chemical structure of the seven dyes or their theoretically predicted and known metabolites. A more rigorous definition of chemical neurotoxicity is needed. The shortcomings in methodological design and data interpretation of some key human and animal studies should be pointed out, as should limitation in generalizability to the U.S. population. Consideration of neurobehavioral data in the context of data from other studies, notably those addressing the genotoxic properties of the food dyes/metabolites, is needed. Nevertheless, in general, this reviewer agrees with the broad conclusion that ingestion of food dyes may reversibly modify behavior in the short-term, which has special relevance to susceptible children in the context of Attention Deficit Hyperactivity Disorder. There is also scientific merit that certain of the seven synthetic dyes, notably the three azo dyes (Yellow 5, Yellow 6 and Red No. 40) and/or their metabolites, if genotoxic, may have potential to induce persistent nerve cell DNA damage and thus pose a risk for effects on brain function that appear later in life. Four synthetic dyes (Red No. 3, Red No. 40, Blue No. 1, and Green No. 3) appear to affect thyroid tissue, the function of which is required for childhood development, growth and neurobehavioral function.

REVIEWER'S SUMMARY ASSESSMENT OF REPORT CONCLUSIONS

The Reviewer supports the general conclusion" "*The scientific literature provides evidence in humans and animals, as well as mechanistic information, that synthetic food dyes may cause or exacerbate neurobehavioral problems in some children. Data from multiple evidence streams, including epidemiology, animal neurotoxicology, in vitro and high throughput assays providing mechanistic insight, taken together, provide support that some FD&C batch-certified synthetic food dyes impact neurobehavior in children. More evidence is currently available for Red No. 3, Red No. 40, and Yellow No. 5 than the other FD&C batch certified dyes*."

However, the conclusions for human studies rely to a significant extent on results obtained from U.K. and Australian studies of mostly Caucasian children, such that their relevance to the diverse population of U.S. children is unknown. Neurobehavioral changes were only identified in a proportion of children (so called "reactors"). Yellow No. 5 was specifically shown to trigger behavioral changes in so-called reactors. T**he conclusions for animal studies** rely on the aggregated results of diverse studies, several with weaknesses of methodological design, that together report changes in behavioral measures, neurotransmitter-related parameters, and other molecular systems required for normal brain development function. Evidence for induction of brain damage is not supported.

REVIEWER'S COMMENT ON "BIG PICTURE" QUESTIONS

(a) Absent an independent worldwide search of the literature, which is beyond the reviewer's assigned tasks, the Report captures the spectrum of neurobehavioral studies in children challenged with specific food dyes. Missing was presentation of the chemical structures of the dyes and the significance thereof, as discussed below.

(b) Absent an independent worldwide search of the literature, which is beyond the reviewer's assigned tasks, the Report captures available animal neurotoxicology studies relevant to the question of the neurobehavioral effects of the FD&C batch-certified synthetic food dyes. Several significant scientific issues relating to the methodological design of studies were not addressed, as detailed below. Two Iranian studies claiming the presence of neuroanatomical changes in the brain were overinterpreted as described below.

(c) Absent an independent worldwide search of the literature, which is beyond the reviewer's assigned tasks, the Report does not appear to have missed any studies that would inform a safe exposure level for neurobehavioral effects in children for any of the FD&C batch-certified synthetic food dyes. However, in regard to the three azo dyes, it would be valuable to examine their genotoxic potential because of emerging links between food exposure to natural toxins that form genotoxic compounds and induction neurodegenerative disease that may appear long after exposure has ceased.

1.BASIC PRINCIPLES

1.1. Chemical Neurotoxicity

From early development through adult life, there are many types of possible effects on the nervous system that result from overexposure to exogenous chemicals. While the descriptor "neurotoxicity" indicates adverse effects on the nervous system during development or throughout life, the term has shortcomings. The first

problem is that the descriptors “neurotoxin’ and “neurotoxicant” refer respectively to chemicals of natural or synthetic origin that have neurotoxic *potentia*l, but that potential is critically dependent on the subject (human, animal), the dosage and duration of exposure. Even substances required for normal physiological function, such as vitamin B6, have neurotoxic potential when the dosage is sufficiently large and prolonged. Conversely, exposure to minute amounts of high-potency chemicals (such as nerve agents) can be handled physiologically will no detectable adverse health effect. Other factors related to neurotoxic potential include species, sex, age, nutritional status, metabolic and metabolome status and, for humans, ethnicity and racial grouping. Ethanol, for example, has greater acute neurotoxic potential in persons with aldehyde dehydrogenase 2 deficiency, a genetic feature that is more common in Asian people than in other racial groups.

1.1.1. Developing Nervous System

The nervous system of the developing fetus is susceptible to chemical-induced changes as a function of the *stage of development* and secondarily the dosage. For example, substances that selectively interfere with molecular mechanisms required for cellular migration will express their neurotoxic potential *only* during periods of cellular migration. Importantly, chemical-induced changes in the developing brain have the potential for induction of *permanen*t adverse effects on neural structure and function.

1.1.2. Mature Nervous System

1.1.2.1. Pharmacological Effects. Single doses of neuroactive substances, such as the psychoactive substance caffeine, produce temporary and reversible changes on the nervous system function of adults. Both positive (brain stimulation, muscle strength) and negative behavior changes (sleep changes, anxiety) may result during the period of the chemical’s pharmacological activity. Mild physical dependence and withdrawal symptoms may accompany abstinence of caffeine intake. Substances with neuroactive potential, while not neurotoxic at low doses, are therefore relevant to an assessment of the health effects of food dyes. High doses of neuroactive agents or of agents that interfere with factors (such as glucose levels) critically required for normal neurological function can induce severe acute toxicity, including death. Additionally, chemicals that perturb the physiological balance of excitatory and inhibitory transmitters, or which act directly on their receptors, can induce acute and potentially chronic effects on neurological function. For example, the glutamate-receptor agonists beta-*N*-oxalylamino-L-alanine and domoic acid, which are present in certain plants and animals used by humans for food, can in certain doses induce neuroexcitatory effects that interfere with human motor and memory function, respectively.

1.1.2.2. Structural Damage. Single doses of some substances with neurotoxic potential may have delayed effects on the infant, juvenile and adult nervous system, with or without immediate effects. A classic example is the organophosphate compound tri-ortho-cresylphosphate which, with sufficient exposure, may have little or no immediate neurotoxic effect but, over the course of weeks, may induce distal degeneration of elongate nerve fibers in the spinal cord and peripheral nerves. Another example is carbon monoxide, a gas with acute toxic potential (narcosis, coma) but which may also precipitate a post-exposure and largely reversible movement disorder (e.g. Parkinsonism, dystonia, tremor) or even a progressive and fatal encephalopathy that appears weeks after the initial insult. Another example is the sugarcane mycotoxin 3-nitropropionic acid, which in large doses can induce coma and, upon reawakening, leave the subject with lifelong dystonia. These types of delayed-onset neurotoxic changes arise from structural damage to the nervous system.

1.1.2.3. Insidious Disease Onset. Repeated exposure to a large number of chemicals with neurotoxic potential may result in the insidious development of changes in nervous system structure and function; such changes are usually self-limiting after exposure ceases, and nervous system damage is repaired, albeit slowly by the re-growth of damaged structures. Examples include repeated oral exposure of rodents to the food flavor ingredient Musk Ambrette, which induces a peripheral neuropathy associated with distal nerve fiber (axonal) degeneration.

1.1.2.4. Long-latency Effects. There is growing evidence that single or multiple exposures to some chemicals may trigger molecular changes in the nervous system that do not surface clinically until years or decades later. This subject has particular relevance to progressive neurodegenerative diseases. One possible explanation is that exposure to culpable substances lowers the normal anatomical reserve of nerve cells but to a degree insufficient to surface in the form of clinical disease. However, with the addition of selected neuronal attrition

with the advance of age, certain damaged nerve cell populations eventually decline to a level that clinical disease surfaces. More recently, there is evidence that exposure to chemicals that induce certain types of DNA damage (i.e. genotoxins) may activate a silent pathological process that appears years or decades later in the form of a progressive neurodegenerative disease. The genotoxic property of food dyes is a subject investigated in relation to carcinogenic potential/risk but not for potential long-latency adverse effects on the nervous system. Nevertheless, identification of chemicals with genotoxic potential, whether or not they have been associated with experimental mutagenicity or carcinogenicity, has become relevant to safety assessment in relation to the nervous system.

2. ASSESSMENT OF NEUROTOXIC POTENTIAL

2.1. Study Design

Studies involving human subjects, animals or *in vitro* test systems should be based on a cogent hypothesis and effective design. In addition to the desirable controls discussed below, the study should have sufficient statistical power to provide a definitive answer to the hypothesis under study. A power analysis is used to estimate the minimal sample size based on a declared significance level, effect size, and statistical power. Studies that lack a sound study design may yield results with conclusions that are open to question.

2.1.1. Human Studies

A cardinal principle is adherence to a strong study design and observers who are blind to study interventions. This was not the case in the study by Bateman and colleagues (1987), which assessed the effects of artificial food coloring and benzoate preservatives on the behavior of 3-year-old children. Parents who were not blinded reported changes in behavior while validated psychological tests failed to register changes. A follow-up community-based, double-blinded, placebo-controlled food challenge reported replication of the first study (McCann et al., 2007), but the generalizability of these results in unknown. The two studies utilized children of families who lived in the U.K. Isle of Wight, which had a population of about 125,000 in 1991 of which, 2.7% were classified as “non-white” in 2011. Income levels were similar or somewhat lower than in other parts of the U.K. Whether or not the study groups were economically and ethnically representative of the population of the Isle of Wight, the results of these two studies are strictly only applicable to the subject population and have unknown relevance to other populations worldwide. For the U.S. population, the present Report concludes that: “Overall, non-Hispanic Black participants had significantly higher intake compared to other ethnic groups (Hispanic, non-Hispanic White, and Asian or other categories)” but that “Higher income was inversely, albeit weakly, associated with food dye exposure”.

2.1.2. Animal Studies

Species, strain, age, sex, nutritional status, route, method, dose, duration and purity of the administered article should all be controlled when designing and assessing the results of a study designed to measure the effects of a chemical on the nervous system. Most studies administer food and water *ad libitum*, but the composition of the diet and the presence of any contaminants in the diet, drinking water or administered article are rarely assessed. This has relevance because some food dyes contain contaminants with carcinogenic potential.

Studies are rarely performed with animals subjected to dietary restriction/excess, even though human subjects that eat food containing dyes are under/overfed. A minimum of three doses of the test article is required to assess dose-response but additional doses permit stronger assessment of a dose-dependent effect, a criterion commonly used as evidence that the test article was responsible for the outcome. Commonly omitted from experimental studies to assess the neurotoxic potential of one or more test articles are additional sets of animals that receive doses of a substance that is known (positive control) and is known not (negative control), to induce the neuro/behavioral effect of interest. Inclusion of a positive control compound that induces a response that matches previously published experience provides confidence both for the experimenter and for external observers that the test laboratory is performing to standard and, thus, the results from parallel studies of the test article(s) are credible. Further credibility is provided by the inclusion of a negative control compound that also performs according to previous experience. Since a negative control compound may itself induce changes in nervous system function, it is important to compare results generated by the test article not only with those associated with the test article’s vehicle (e.g. distilled water) but also with the effects of the negative control compound. A majority of studies seeking to assess the neurotoxic potential of test articles (including synthetic dyes) fail to include positive and negative control groups in experimental animal studies.

2.1.3. Comparison of Human and Animal Studies

Most studies utilized various mixtures of synthetic dyes to assess and define their effects on the nervous system and behavior. Such study designs yield results that can be very difficult, if not impossible, to interpret. Disparate chemicals and their metabolites may interact one with another in unpredictable ways, as illustrated by the following example. Peripheral neuropathy developed among adults who deliberately inhaled an organic solvent mixture for euphoric purposes. Since the solvent mixture was comprised mostly of methyl ethyl ketone (MEK), this was determined to be the culpable neurotoxic agent. However, animal studies demonstrated the culpable neurotoxic agent was a minor component of the solvent mixture (*n*-hexane) and that co-exposure to MEK potentiated the neurotoxic potency of *n*-hexane, while co-exposure to toluene did the reverse. This is an exceedingly rare example in which the interaction of concurrent exposure to two chemicals was delineated; in most cases, such interactions are rarely explored. and there are no examples of studies using mixtures of more than 2 chemicals that have yielded interpretable results. This example demonstrates the importance of controlled animal studies of individual substances in the interpretation of the effects of chemical mixtures on human subjects. Such studies are lacking for most of the seven synthetic dyes.

2.1.4. Cell/tissue Culture Studies

Studies of chemicals that test for effects in cell or tissue culture require special care in study design and interpretation. Direct application of a chemical to cells or neural tissues can elicit radically different responses from that seen when the same substance is administered systemically. Exposure *in vitro* is continuous. Whether the chemical applied to the in *vitro* system is metabolized over the course of exposure is rarely determined. The ability of the system to respond to chemical exposure in a manner that can be meaningfully interpreted is often questionable. While concentration-effect designs are commonly employed, positive and negative control compounds are often omitted. Exposure duration is often short and cellular responses may be non-specific. Determining whether there is any relationship between an *in vitro* observation and a behavioral effect in children is highly problematical.

2.2. Chemical Structure

The Report provides no information on the chemical structures of the synthetic dyes under review. Chemical structure is of cardinal importance because it may provide information on the presence or absence of a previously established active/inactive moiety for which effects may be forecast. Additionally, the structure of the chemical may provide information on probable metabolites and their potential for biological activity. An example of these principles is provided by the former food additive Musk Tetralin, the metabolite of which reacted with proteins to generate a blue pigment that predicted neurotoxicity in the form of nerve damage. Although the entire body of test animals turned blue after repeated treatment with Musk Tetralin, which indicated widespread reactivity with proteins, only the nervous system underwent pathological changes because of the unique architecture and functional requirements of neurons and their elongate axons. Subsequent studies of compounds related to Musk Tetralin demonstrated that the neurotoxic property was specifically dependent on the spacing of keto groups, such that 1,2-diacetylbenzene was chromogenic and neurotoxic, while 1,3-diacetyl benzene lacked both properties. This provides a clearcut demonstration that chemicals with closely related structures cannot be assumed to have comparable effects on the nervous system. As noted above, the simultaneous administration of multiple synthetic dyes adds a great deal of further complexity since the interactive effects the parent compounds and their metabolites cannot be predicted. Most studies analyzed in the Report tested chemical mixtures; only Yellow No. 5 (tartrazine) was tested as a single chemical.

Although the chemical structures of the food colors under review was omitted from the Report, mention is made of three so-called azo dyes, namely Yellow 5, Yellow 6 and Red No. 40, compounds that known to produce mutagenic metabolites. The first step in enzymatic metabolism of azo dyes appears to depend on the azoreductase activity of intestinal microbiota, the composition of which varies with ethnicity and other factors. Azo compounds/metabolites are of special concern because of their potential for genotoxicity that results in DNA damage and repair responses, oxidative stress, genetic instability, mutations, cell death and inflammation. Such properties have been previously related to cancer risks. Increasingly, there is concern that DNA damage/repair mechanisms may underly the genesis of certain sporadic neurodegenerative diseases that may incubate for years or decades prior to clinical expression. For this reason, it is important to consider the potential neurotoxic effects of azo dyes and their metabolites in relation to their genotoxic properties. While the

present report does not assess the genotoxic properties of azo dyes in relation to either carcinogenic or neurotoxic potential, there is sufficient information in the literature to express serious concern over the use of azo dyes food and medicine consumed by children. In accord with the precautionary principle as it relates to human health, it is recommended that azo dyes should not be used in food.

2.3. Chemical Access to the Nervous System

Chemicals that enter the blood stream have differential access to the nervous system as a function of chemical structure and developmental stage. In humans, the blood-brain regulatory interface, which is often described as a "blood-brain barrier" (BBB), matures within months of birth. Thus, compounds with neurotoxic potential have greater access to the nervous system of the developing fetus and newborn infant. However, even in the adult, certain substances can not only traverse the BBB but also enter brain tissue via the circumventricular organs (area postrema, median eminence of the hypothalamus, pineal gland, and the posterior pituitary), where there is normally no BBB in the infant, juvenile or adult human subject. Additionally, in the peripheral nervous system, a comparable blood-nerve barrier is normally absent in spinal and autonomic ganglia, such that chemicals circulating in the blood stream have immediate and direct access to both central and peripheral neural tissue.

Carotid artery injection of radiolabeled Red No. 3 in anesthetized rats resulted in radiolabel entering brain tissue (cerebral cortex, hippocampus, caudate, thalamus/hypothalamus) but the conclusion that chemical entry was solely via the BBB is likely incorrect. The hypothalamus is associated with specialized brain regions where the BBB-based capillary epithelium is fenestrated, such that free transfer occurs from blood to brain tissue. This possibility was recognized in a related study in which Red No. 3 was injected in the veis of conscious rats, when radioactivity was detected in 14 brain regions.

2.4. Secondary Effects on Nervous System Development

Chemicals that perturb thyroid function can interfere with normal brain development. Thyroid hormones are essential for brain development through specific time windows influencing neurogenesis, neuronal migration, neuronal and glial cell differentiation, myelination, and synaptogenesis. Red No. 3, Red No. 40, Blue No. 1, and Green No. 3 were active for an assay mapped to thyroid peroxidase (TPO), which measures TPO activity as a loss of signal. As stated in the Report, TPO inhibition could impair thyroid hormone synthesis, which ultimately could compromise neurodevelopmental processes. This raises a red flag for use of these food dyes in food materials consumed by pregnant women and infants.

2.5. Effects on Neural Receptor Function

The red and yellow dyes were all active in assays targeting dopaminergic and opioid receptor subtypes, with additional activity on G-protein-coupled receptors. Red No. 40 was also active for muscarinic and nicotinic cholinergic receptors. While these studies reveal the potential neuroactive properties of these substance, the significance of these observations in relation to the health of human subjects cannot be assessed. Few studies examined the effects of dyes on glutamate and GABA receptors, targets that would be associated with effects on the regulation of neuroexcitation and corresponding behaviors. Such studies require the use of positive and negative controls to aid in study interpretation. In general, however, transient effects of neuronal receptors are expected to be readily reversible.

3. FOOD DYE ASSESSMENT

3.1. Developmental Neurobehavioral Toxicology (DNT) Studies

DNT studies typically focus on detecting long-term or permanent effects on brain and brain function that occur after developmental exposure. Food dyes were provided at a fixed concentration in the diet throughout *in utero*, infant, juvenile and adolescent development and extending into adulthood. Studies performed in the 1970s-1980s yielded sparse evidence of adverse behavioral effects for Yellow No. 5, Red No.3, and Red No. 40. Later studies supported the hypothesis that sulfanilic acid, a common metabolite of the azo food dyes Yellow No. 5 and Yellow No. 6, was the effective agent in producing the dye mixture effects on activity. recorded in Japanese studies.

Animal studies performed in Japan investigated the neurobehavioral effects of several food colors, including Blue No 1, Yellow No. 5, Red No. 3, Yellow No. 6 and Red No. 40. These were dose-response studies, and

dosing was continued throughout several generations and life stages. While individual behavioral changes were noted, some related to sex, the Report notes several reservations that compromise the use of these studies for risk assessment.

Another research group administered a mixture of food dyes by gavage to rats during pregnancy and evaluated the offspring for behavioral effects at 90 days of postnatal age. Six of the seven FD&C dyes were represented (no Green No. 3). Three behavioral tests were used for adults. While the results of these three studies cannot be directly compared, they demonstrate long-term effects on behavior from *in utero* exposure at doses of the individual dyes found to have no effects in FDA regulatory reviews. Sensitive areas of brain function included regulation of activity, anxiety and exploration in a novel environment, and persistence in the forced swim test. Notably, no effects on learning and memory were seen. Analysis of brain receptors for neurotransmitters (glutamate, acetylcholine) were inconclusive.

Another study used a mixture of dyes (Red No. 40, Yellow No. 5, Yellow No. 6, Blue No. 1) that was added to drinking water of the male offspring after they had been weaned (PND 22) and throughout adolescence (PND 50). Measures of activity and anxiety were detected at earlier but not later animal ages.

In summary, these animal studies found changes in motor activity with mixed dye treatment but not in the results of learning and memory tests.

3.2. Adolescent/Adult Neurobehavioral Toxicity Studies

3.2.1. Brain Damage Underlying Neurobehavioral Toxicity

In the 1980s, two studies of neurobehavioral toxicity were conducted with dye exposures beginning at puberty or later. Two such studies used the azo dyes Yellow No. 5 and Red No. 40 to examine effects on cognitive function. Noorafshan et al. (2018) treated adult rats with/out Red No. 40. Treated animals showed more reference and working memory errors than controls, while learning the radial arm maze, and also in a retention test. Post-mortem examination of the brain was described as showing evidence of neuroanatomical changes (cell loss, dendritic shortening, reduced dendritic spines) in the medial prefrontal cortex that explained observed deficits in learning and memory tests of animals treated with high doses of Red No. 40. However, the reported neuroanatomical changes are not consistent with chemical-induced brain degeneration (*vide infra*). Similar reservations apply to the study of Rafati et al. (2017) who evaluated Yellow No. 5 using the same methodological design.

The claim that Noorafshan and colleagues induced structural brain damage in laboratory animals requires close scrutiny. In this study, adult Sprague Dawley rats received for 6 weeks daily gastric gavages of low doses (7 mg/kg/day) or high doses (70 mg/kg/day) of the azo dye Red No. 40 (purity unstated) dissolved in distilled water (the only control). Taurine was used as a protective compound. Behavioral tests began at 4 weeks and animals were anesthetized terminally at 6 weeks. Short and long-term memory deficits were found on tests of behavior of the high-dose group, and these were partially blocked by co-administration of taurine, an antioxidant and neuromodulator. Brains were fixed by cardiac perfusion (during life or after death?) with 4% (buffered?) paraformaldehyde. Volumetric and stereological studies performed on the fixed prefrontal cortex were reported to show reduced volume, loss of approximately half of the normal population of neurons and glial cells, a 40% reduction of dendritic length, and a 50-63% reduction of dendritic spines, in the high-dose group relative to the control group. Brain atrophy seen in high-dose animals was reportedly prevented by co-administration of taurine.

While this study was hypothesis-based and apparently carefully performed, the anatomical findings are not tenable and suggest study authors had limited neuropathological experience. Brain tissue does not undergo atrophy secondary to cell loss without a plethora of pathological changes that at any one time reflect a plethora of stages of cellular degeneration. Moreover, during a degenerative process, loss of neurons would be accompanied by an increase (not a decrease) in the number of glial cells, whether astrocytes, oligodendrocytes or microglial cells. The authors do not provide illustrations to support the claim of changes in dendritic length or dendritic spines in the brains of animals treated with high-dose Red No. 40 with and without taurine vs the vehicle-treated controls. In summary, the conclusion that neurocellular damage caused the observed behavioral changes is not supported, and evidence of dye-induced brain damage is lacking. In

contrast to the conclusion in the present Report, the reported brain histomorphology attributed to Red No. 40 and Yellow No. 5 does *not* help provide biological plausibility for the behavioral effects. As noted in the Report, other weaknesses of these studies relate to methods used to analyze data generated by the behavior studies.

3.2.2. Other Animal Studies

A rat study of Red No. 3 demonstrated a dose-dependent pattern of diminished activity that reached a low at 2 hours after dye administration and then returned to baseline by 7 hours. Noteworthy, the maximum effect on activity (2 h after administration) corresponded to the peak in Red No. 3 levels in circulation as described in a JECFA review. Post-mortem examination of brainstem, hypothalamus, hippocampus, and striatum revealed serotonin was lowered in a dose-dependent manner in all brain areas except the striatum. In the hippocampus, dose-dependent increases were shown for the serotonin metabolite 5-hydroxyindoleamine and the serotonin metabolizing enzyme monoamine oxidase A. (MAO-A). These results indicate that certain levels of Red No. 3 have reversible neuroactive properties in rats. As noted in the Report, the animal studies parallel challenge studies in children when behavior is measured shortly afterward a single dose of dye or mixture. In both rats and children, the effect of the dye peaks and then dissipates over a few hours post- exposure. However, with repeated exposure to rats, serotonin increased (rather than decreased) and MAO-A activity decreased (rather than increased) in all brain regions studied. These results are difficult to interpret.

Other studies that sought to examine the effects of various mixtures of multiple food dyes on animal behavior had variable experimental designs and results, such that conclusions drawn cannot be assessed with any degree of confidence. As discussed previously (*vide supra*), studies seeking to understand the effects of simultaneous exposure to multiple chemical substances suffer from fundamental problems in design and interpretation.

4. *In Vitro* Studies

4.1. Red No. 3 and Blue No. 1

Red No. 3 added to rat brain homogenates inhibited uptake of neurotransmitters including dopamine, serotonin, gamma amino butyric acid (GABA), and glutamate. Other *in vitro* studies showed that Red No. 3 can inhibit Na+/K+-ATPase, the enzyme activity of which is required to generate chemical energy to support the transport of neurotransmitters across plasma membranes. A proper balance of neurotransmitters is critical for maintenance of physiological homeostasis needed for normal brain function. Evidence that Red No. 3 is a potent inhibitor of glutamate uptake is significant because, in animal and human brains, accumulation of this excitatory neurotransmitter in extracellular space can trigger excitotoxicity leading to acute neuron damage, increased brain activity and, potentially, restlessness and even seizures. This potential neurotoxic phenomenon has not been studied in relation to increased motor activity in animal dye studies. Blue No. 1 showed no evidence of excitotoxicity when compared with glutamate as positive control in neuroblastoma cell cultures.

Whether and how results of *in vitro* studies relate to exposure *in viv*o will depend on the concentration of the free protein-unbound chemical in the bloodstream and its access to the nervous system. While there is a regulatory interface (BBB), between blood and brain tissue, there are several regions in the central and peripheral nervous system where chemical access to neural tissue is unimpeded (*vide supra*). This includes brain that regulate endocrine function, including thyroid hormone status, which appears to be impacted by treatment with Red. No. 3. As stated in the Report, “Studies are needed with oral administration and toxicocokinetics, including distribution and elimination of Red No. 3 and its deiodinated metabolites, di-iodo-fluorescein, mono-iodo- fluorescein, and fluorescein.”

Red No. 3 and Blue No. 1 respectively showed the highest and among the lowest overall activity in ToxCast assays, and Red No. 3 was the only dye with activity for monoamine oxidase. The ToxCast data support the estrogenic activity reported for Red No. 3. However, as noted in the Report: “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.

4.2. Red No. 40

Red No. 40 was found to be the most potent of the azo dyes in an *in vitro* study specifically conducted for risk assessment of developmental neurotoxicity. The study was conducted in neuronal progenitor cells and looked at four of the seven FDA certified dyes, the three azo dyes (Yellow No. 5, Yellow No. 6 and Red No. 40) and the trimethylamine dye Blue No. 1. Red No. 40 stood out from the other FDA certified dyes because it reduced cell viability at micromolar concentrations.

4.3. Yellow No. 6

Yellow No. 6 reversibly inhibited the enzyme activities of human cholinesterase and pseudocholinesterase *in vitro.* Potency was about an order of magnitude lower than that of common organophosphate pesticides. Chemicals that inhibit the enzyme activity of acetylcholinesterase, which is required in physiological levels for normal neural and muscle function, are referred to as cholinesterase inhibitors. These are classified as reversible, irreversible, or pseudo-reversible. Reversible cholinesterase inhibitors are utilized for therapeutic purposes. In contrast, irreversible and pseudo-reversible inhibitors are often used in pesticides and biowarfare nerve agents.

5. Conclusion

Clinical trial studies provide limited evidence for short-term, reversible changes in behavior associated with exposure to food dyes in children. These are attributed to direct or indirect neuroactive effects.

Animal studies provide evidence for variable effects of exposure to food dyes on activity, memory and learning, sometimes with evidence of changes in brain neurotransmitter systems. The structural changes in the brain reported in two Iranian studies are not supported. While there is no evidence that exposure to food dyes at the doses tested elicits brain damage, given the genotoxic properties associated with the three azo dyes, studies are needed to assess the possibility of neuronal DNA damage resulting in genomic instability and long-latent effects require experimental evaluation.

Mechanistic and *in vitro* neurotoxicity studies of certain dyes provide evidence for the induction of oxidative stress, interaction with neurotransmitter receptors and key enzymes, and systems that exert influence on the brain including glucocorticoid pathways, thyroid and estrogen receptors. Of these, the effects of certain food colors on thyroid function, whether mediated by direct action on thyroid tissue or via the hypothalamus, has special importance for the developing human brain.

Data from multiple lines of evidence show that some FD&C batch-certified synthetic food dyes may temporarily impact neurobehavior in children. More evidence is currently available for Red No. 3, Red No. 40, and Yellow No. 5 relative to the other FD&C batch-certified dyes. The three azo dyes (Yellow No. 5, Yellow No. 6 and Red No. 40) are of special concern because of possible long-latency effects linked to genotoxic activity has not been ruled out. Brain and body development may be affected by food colors with potential to perturb thyroid function, and thereby brain development.

Future human and experimental studies should be hypothesis-based, employ an appropriately powered methodological design, and incorporate positive and negative control compounds to assist interpretation of effects observed with test articles.

6. Relevance to Attention Deficit Hyperactivity Disorder (ADHD)

Concerns about possible associations between synthetic food dyes and the exacerbation of ADHD symptoms in children prompted the Legislature to ask OEHHA to conduct this assessment. ADHD is characterized by symptoms of inattention, impulsivity and hyperactivity, and is considered to encompass a spectrum of neurobehavioral symptoms and severity. The percentage of US children and adolescents diagnosed with ADHD reportedly has increased from an estimated 6.1% to 10.2% in the past 20 years. It is postulated that environmental exposures to chemicals, including those used as food additives, may contribute to ADHD symptoms. While the results of key clinical studies of the effects of dyes on infant behavior have unknown relevance to diverse U.S. populations, there is a collection of animal studies that support the proposal that oral exposure to synthetic dyes can reversibly modify behavior in the short-term. The effects of oral exposure to dyes to genetic or chemical-induced animal models of ADHD have not been undertaken. Animal models

suggest of ADHD suggest involvement of dopaminergic, noradrenergic, and serotonergic systems, as well as more fundamental defects in neurotransmission.