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Glyphosate and neurological outcomes: A systematic literature review of animal studies

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ABSTRACT

Studies of nervous system effects of glyphosate, a widely used herbicide, have not been critically examined. The aim of this paper was to systematically review glyphosate-induced neurotoxicity literature to determine its usefulness in regulatory decision-making. The review was restricted to mammalian studies of behavior, neuropathology, and neuropharmacology; *in vitro* and other biochemical studies were considered supplementary information. Glyphosate formulation studies were also considered, despite uncertainties regarding toxicities of the formulated products; no studies used a formulation vehicle as the control. Inclusion criteria were developed *a priori* to ensure consistent evaluation of studies, and *in vivo* investigations were also ranked using ToxRTool software to determine reliability. There were 27 *in vivo* studies (open literature and available regulatory reports), but 11 studies were considered unreliable (mostly due to critical methodological deficiencies). There were only seven acceptable investigations on glyphosate alone. Studies differed in terms of dosing scenarios, experimental designs, test species, and commercial product. Limitations included using only one dose and/or one test time, small sample sizes, limited data presentation, and/or overtly toxic doses. While motor activity was the most consistently affected endpoint (10 of 12 studies), there were considerable differences in outcomes. In six investigations, there were no marked neuropathological changes in the central or peripheral nervous system. Other neurological effects were less consistent, and some outcomes were less convincing due to influences including high variability and small effect sizes. Taken together, these studies do not demonstrate a consistent impact of glyphosate on the structure or function of the mammalian nervous system.

KEYWORDS

Glyphosate; herbicide; behavior; neuropathology; neuropharmacology

Introduction

Glyphosate (N-(phosphonomethyl) glycine) is a widely used herbicide with broad-spectrum activity. Glyphosate acid (technical) is used in several salt forms, the most common being isopropylamine. Glyphosate acts on the shikimate pathway in plants, and presumed chemical specificity may be attributed to lack of this pathway in mammals (Richardson et al. 2019); however, glyphosate is not without biological activity in animals (ATSDR 2020). The oral LD_{50} in mice and rats was shown to be >5,000 mg/kg (WHO 2016). Glyphosate is rapidly but incompletely absorbed when exposure is by the dietary or oral gavage routes. This herbicide is subsequently eliminated with limited metabolism with a small fraction by hydrolysis producing the metabolite aminomethylphosphonic acid (AMPA) (WHO 2016; ATSDR 2020; Anadón et al. 2009).

The use of glyphosate products has steadily increased over the years, mostly due to the rising number of crops that are genetically modified to resist its herbicidal activity, as well as increasing weed resistance, which requires greater herbicide usage (Benbrook 2016). Due to its widespread use, glyphosate has been commonly measured in a number of food products and water sources. Human exposures to glyphosate may occur via dietary routes from intake of food products and water with glyphosate residues (Faniband et al. 2021; Gillezeau et al. 2019), but also might involve potential dermal or inhalation exposures to users or close bystanders in agricultural or residential settings or production locations (Solomon, Marshall, and Carrasquilla 2009). It should be noted that glyphosate has a low vapor pressure (1.31×10^{-5} Pa at 25°C) and is not expected to volatilize from soil and plants (EFSA 2015). Dermal absorption is also low, estimated to be 1–3% (WHO

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2016). Therefore, significant inhalation or dermal exposure is likely restricted to immediate exposure from potential aerosolized dispersion during application. A recent literature review (Gillezeau et al. 2019) described papers that quantified urinary and other biological glyphosate levels with only somewhat higher levels being detected in occupationally exposed individuals compared to the general population, as well as a suggestion of increasing levels over time based upon two studies. The widespread use of glyphosate has raised increasing concerns regarding toxicity, including neurological outcomes.

Glyphosate is used commercially only as a component (active ingredient) of formulated products, which are complex chemical mixtures that include intentionally added ingredients such as surfactants, adjuvants, and preservatives, as well as manufacturing byproducts such as impurities. While these are considered "inert" or "other" ingredients, these may have distinct toxicity profiles, and/or may influence the toxicity of the active ingredient (US EPA 2020). The exact ingredient composition, including chemical names and concentrations, is typically withheld as confidential information. Further, different companies produce different formulations with the same active ingredient, and are also likely to change their inert ingredient composition for the same product over time (Mesnage, Benbrook, and Antoniou 2019). A number of investigators found that formulated products, including those of glyphosate, exhibit greater toxicity than the active ingredient alone (Adam et al. 1997; Nagy et al. 2020). Further, studies of the agents contained within the formulation, such as surfactants and metals, demonstrated significant toxicity of these agents, particularly *in vitro* (Defarge, Spiroux de Vendomois, and Seralini 2018; Mesnage, Benbrook, and Antoniou 2019). The differences in specific components, their relative proportions, and toxicity profiles hamper meaningful conclusions regarding toxicities, and study outcomes may or may not reflect effects of glyphosate alone. It is critically important to acknowledge and consider the scientific uncertainties on the contribution of the inert ingredients to the overall results from a formulation study that does not have a concurrent formulation vehicle control group.

Glyphosate toxicity has been reviewed most recently by the US Agency for Toxic Substances and Disease Registry (ATSDR 2020), European Food Safety Authority (EFSA 2015), the European Commission Assessment Group on Glyphosate (AGG 2021), and World Health Organization (WHO 2016), but a complete review of the literature focusing specifically on neurotoxicity has not apparently been conducted. The purpose of this paper was to systematically review the neurotoxicity literature of glyphosate and determine its usefulness in regulatory decision-making. In keeping with the focus on endpoints that are appropriate for current regulatory risk assessments, this review was restricted to mammalian studies both *in vivo* and *ex vivo*. *In vitro* mammalian cell systems were also considered as supportive mechanistic data that informs the evaluation of mammalian studies. Inclusion/exclusion criteria were developed *a priori*, with supplemental criteria specific for neurotoxicity investigations, to evaluate reliability of each paper/report. This was followed by a quantitative evaluation of relevant *in vivo* studies using ToxRTool, a general purpose assessment tool. This review summarizes and evaluates papers ranked as reliable or reliable with restrictions with a goal of developing an overall picture of glyphosate-induced neurotoxicity potential for risk assessment purposes.

Methods

Literature search and initial assessment

To obtain potentially relevant toxicological studies on the association between glyphosate and neurotoxicity, a search of the open literature was conducted, using PubMed, on 22 June 2021 with the following query:

(glyphosate [tiab] OR 1071–83-6 [tiab]) AND (neurotoxic* [tiab] OR neurodevelopment* [tiab] OR neurobehavior* [tiab] OR neurobehaviour* [tiab] OR neurologic* [tiab] OR attention [tiab] OR cognit* [tiab] OR "developmental disability" [tiab] OR social [tiab] OR intelligence [tiab] OR memory [tiab] OR learning [tiab] OR brain [tiab] OR psychomotor [tiab] OR behavior* [tiab] OR behaviour* [tiab] OR "Nervous System"[tiab] OR parkinson* [tiab] OR tremor [tiab] OR "movement disorder" [tiab] OR mental [tiab] OR emotion* [tiab] OR cognitive [tiab] OR cognition [tiab] OR dementia [tiab] OR neuronal [tiab] OR neuropathy [tiab] OR

motor [tiab] OR sensory [tiab] OR neurodegen* [tiab] OR neurocognitive [tiab] OR depression [tiab] OR mood [tiab] OR personality [tiab] OR IQ [tiab] OR autis* [tiab] OR "amyotrophic lateral sclerosis" [tiab] OR Alzheimer* [tiab]) NOT (poisoning [tiab] OR intoxication [tiab]).

An additional supplemental title-only search was performed using the database query site, ProQuest, which included the databases, AGRIS, APA PsycInfo®, BIOSIS® Toxicology, CAB ABSTRACTS, Embase®, GEOBASE, GeoRef, MEDLINE®, Neurosciences Abstracts, SciSearch®: a Cited Reference Science Database, ToxFile®, Toxicology Abstracts, TOXLINE, and Water Resources Abstracts. Duplicate references obtained through this method were eliminated.

Inclusion and exclusion criteria for this systematic literature review were developed *a priori* to ensure consistent identification of relevant *in vivo, ex vivo*, and/or *in vitro* neurotoxicity studies of glyphosate. The inclusion and exclusion criteria were applied in phases for efficiency, with first-pass inclusion criteria for glyphosate studies based primarily on review of titles and abstracts, as described below.

First-pass inclusion criteria:

- Entire paper in English
- Mammalian species
- Only full experimental papers/reports, i.e., not abstracts, conference proceedings, or regulatory agency data evaluations/summaries
- Only original research articles, i.e., not review articles, case studies, meta-analyses, editorials, or commentaries
- Publicly available, including study reports made public through the Glyphosate Renewal Group (GRG), a consortium of companies with registered glyphosate formulations in the EU (https://www.glyphosate.eu/transparency/scientific-dossier/glyphosate-study-reports/). (Typically, these studies are considered business confidential information and are unpublished with only public health agency summaries being publicly available.)
- Study objective and scope to evaluate only neurotoxicity-associated outcomes. This specifically included assessments of neurological/functional (i.e., behavioral), neuropathological, or neuropharmacological (i.e., neurochemical) endpoints.
  - Not included were reproductive or endocrine-focused studies (unless they included behavioral or neuropathological/pharmacological endpoints on parent or offspring animals), with the potential exception of thyroid hormone studies (with neurological and/or neuropathological correlates)
  - Not included were exploratory bioinformatic studies without neuropathology or neurological endpoint correlates
  - Not included were studies of general mechanisms of toxicity, for example, oxidative stress, inflammation; however, such studies with some focus on neurological or neuropathological endpoints were evaluated on a case-to-case basis for inclusion.
- Studies using any route of exposure, except direct brain injection
- Studies providing a direct evaluation of glyphosate
  - Test material limited to technical glyphosate or salt form (most commonly, glyphosate isopropylamine).
  - For glyphosate formulation studies, must include a formulation vehicle control (i.e., the formulation ingredient mixture without the present of glyphosate)

Second-tier full-text assessment

Studies that passed the first-pass criteria were more closely evaluated using the entire paper/report. The inclusion/exclusion criteria for this second-phase assessment were designed to be aligned with US EPA (2012) Open Literature guidance for investigations to be considered for regulatory hazard and dose response assessment. This guidance is intended for food-use pesticides (such as glyphosate) for which many toxicological and metabolism guideline studies are required, including those with neurotoxicity endpoints. The specific considerations for this second-pass evaluation, and reflected throughout this review, are listed below:

Second-pass inclusion criteria:

- Controls
  - Must have concurrent control group given same vehicle as treated groups (if formulation, must use formulation vehicle).

- *In vivo* vehicles with inherent neurological activity (e.g., DMSOs and solvents) considered low quality. For *in vitro*, high vehicle concentrations (e.g., >0.5%) considered low quality.
- Performance, health, and response of controls must be within normal values as established in the laboratory or the general literature.

- Sample size
  - Must have adequate animals per group or replicates (e.g., plates/wells for *in vitro*) per concentration, based on expert judgement and, where applicable, appropriate test guidelines.
  - Small sample sizes ($n \leq 3$/group, *in vivo*) or replicates ($n < 3$, *in vitro*) considered pilot studies of low relevance.
  - Sample size should be clearly presented for each endpoint measured.
  - For cell culture studies, must state number of isolations for primary cells and number of passages/vials of frozen stocks of continual passage cells.
- Statistics
  - Must present measures of central tendencies (e.g., means, medians) for groups as well as measures of variability. Studies without this are low quality.
  - Must include statistical comparison to controls.
  - If developmental study, litter must be accounted for in study design and statistics
  - A clear description and justification of the statistical approach must be provided.
  - Lack of appropriate statistical presentation and analysis considered low quality.
- Test article
  - Must include important test article characteristics, e.g., purity and source.
- Exposures
  - Must clearly specify dose(s) prepared and administered, and exposure/application method including route, frequency, and duration.
  - Studies that involve dietary and drinking water intake of test material should evaluate consumption to verify actual test material intake. An unverified claim of an administered dosage amount was considered low quality.
  - Any confounding exposures must be described and, if present, must be accounted for or discussed during analysis
  - Appropriate routes include oral, dermal, inhalation, subcutaneous, intraperitoneal, intranasal.
  - Intravenous studies considered low relevance unless there are toxicokinetic (TK) data to justify its use.
- Toxicity
  - Neurotoxicity outcomes must be clearly described, including nature, incidence, time of occurrence, severity, and duration of effects.
  - Neurotoxicity outcomes that occur along with overt toxicity (e.g., weight loss, lethality, and cell death) considered low relevance.
- Test system
  - Must be adequately described. *In vivo*, this includes animal species, age, sex, health, life stage, source, and husbandry. *In vitro*, this includes cell cultures source, storage, passages, purity, composition, origin, negative and positive controls.
  - Performance, health, response of controls within normal values established in literature.
- Endpoints
  - Must have full description of test methods and the results must be consistent with the endpoints discussed in the methods
  - Must be relevant to human health (e.g., animal functional, morphologic, and physiological) and/or be reasonable predictive of neurotoxicity outcomes in humans (e.g., *in vitro*, alternative model species).
  - Experimental procedures including time of testing and dosing must be counterbalanced across dose groups.
- Dose-response
  - Two or more doses are preferred.
  - Studies with one dose considered low relevance unless the study is rigorous and there is good justification for that dose.
  - Doses associated with overt toxicity considered low quality.

The *in vivo* studies that included neurobehavioral, neuropathology, and neuropharmacological endpoints were also evaluated with the

European Union's software-based tool ToxRTool (Toxicological Data Reliability Assessment Tool) to determine study reliability per the Klimisch categories (Schneider et al. 2009). Given that ToxRTool is a general-purpose assessment tool that does not necessarily possess criteria that are specific to assessment of neurotoxicity data, the scoring of study reliability integrated the criteria in the software with the second-pass criteria above. For example, some methodological issues specific for developmental studies (testing and/or statistical analyses of littermates) and neuroimaging evaluations (unbiased sampling and counting in stereology) were incorporated into the ToxRTool assessments (see Results).

Studies were scored as 1 (reliable without restrictions), 2 (reliable with restrictions), or 3 (not reliable for risk assessment purposes). Although the results and rationale for ToxRTool scores are tabulated for all investigations that passed the first-pass inclusion criteria (Table 1), our weight-of-evidence evaluation was based upon studies and endpoints with scores of 1 or 2, so that this review depends most heavily on investigations that are reliable for risk assessment purposes. There were some cases in which the overall study rated a score of 1 or 2, but specific endpoints failed to meet the established criteria. In those cases, data from unreliable endpoint(s) were not considered in this review, whereas the rest of the experimental data were included, and the overall study scored as determined by ToxRTool.

Detailed study design characteristics, results, and ToxRTool evaluations for all articles meeting inclusion criteria were extracted into a standardized spreadsheet by one author (KMS) and independently checked by a second author (VCM), with any inconsistencies resolved by discussion among all authors. Additional independent checks by a third author (AAL or JRR) were also provided during detailed review of papers by subject area, resolved through discussion among all authors.

Studies that focused on only mechanistic endpoints and *in vitro* studies were reviewed qualitatively as supplementary supporting data. While these data were evaluated in the context of the second-pass criteria described above, these were not subject to the numerical assessment given from a ToxRTool review.

Third-tier assessment of glyphosate formulation studies

Studies classified as "formulation studies" were those that tested glyphosate as a component of a formulated product, but not glyphosate alone. None of the formulation studies passed the inclusion criteria for evaluation of glyphosate; that is, none of the formulation studies contained a formulation vehicle control group (i.e., formulation without glyphosate) for comparison, and therefore did not meet inclusion criteria for a risk assessment evaluation of glyphosate. Indeed, the ToxRTool software provides a default score of category 3 (not reliable) if a study does not contain the appropriate negative controls. Thus, strict application of the assessment criteria above would have resulted in exclusion of all of the formulation studies.

Nonetheless, the glyphosate formulation investigations were evaluated in this literature review with modified criteria, as described below, because formulations are the end-products used in occupational and residential settings, and therefore have relevance to occupational and public health. Further, if there are similar effects for both glyphosate and glyphosate formulations, this might inform weight-of-evidence considerations. Therefore, these studies were still considered to display potential relevance for further evaluation, and separate appraisals were conducted for investigations evaluating glyphosate alone and for studies evaluating various glyphosate formulations. The following third-pass inclusion criteria and modified ToxRTool approach were adopted to categorize the formulation studies:

- Met all of the first- and second-pass criteria described above for the assessment of glyphosate-alone, except for the criterion regarding the appropriate formulation vehicle control
- Identified the full product name of the formulation and source of formulation
- Identified the total concentration of glyphosate in the formulation
- Identified the dilution vehicle
- Specified the administered dose per body weight (mg/kg) and indicated the dilution status for the test material.

Data summary

The studies/reports that passed the first-, second-, and third-pass criteria were grouped and are presented herein by: 1) glyphosate technical (or salt form) or formulation study, 2) type of endpoint (e.g., behavioral, neuropathological, and neuropharmacological), and 3) exposure duration. Some papers had multiple types of endpoints and/or exposure durations; these papers were included in several groups. Quantitative comparisons were conducted with studies of glyphosate alone, but due to the uncertainties with formulation studies, only qualitative summaries were possible. The neurotoxic effects observed following developmental exposure may differ from those in an adult; therefore, investigations of glyphosate following developmental (gestational, perinatal) exposure are grouped separately from those of exposure at adolescent or adult ages. Studies with *in vitro* assessments were also grouped and reviewed separately.

For this review, the approach for evaluation followed US EPA (1998a) Guidelines for Neurotoxicity Risk Assessment in that "the identification of a critical adverse effect often requires considerable professional judgment and should consider factors such as the biological plausibility of the effect, the evidence of a dose–effect continuum, and the likelihood for progression of the effect with continued exposure." Other neurotoxicity guidance-based considerations included severity and nature of the outcome, overall pattern and consistency of an observed effect, patterns of impairments across tests and functional domains, and reversibility of an effect (OECD 2004; US EPA 1998a; WHO 2001). Where multiple doses were administered, evidence of greater effect with increasing dose was considered a stronger indication of neurological effect. In tests where there are multiple measures, such as the functional observational battery (FOB) with upwards of 20 endpoints, there was less concern for neurotoxicity if only a few unrelated measures were affected or the effects were not consistent with dose. Biological relevance was also considered, such as whether a measured change could be related to an adverse outcome, and whether the effects occurred at doses that did not produce systemic toxicity. In a weight-of-evidence evaluation, data lacking these characteristics may lower concern for neurotoxicity.

In addition, for glyphosate studies the lowest observed effect level (LOEL) and no observed effect level (NOEL) were summarized for each endpoint and compared across studies. The LOEL is the lowest dose or exposure level at which a statistically and/or biologically significant effect is observed that can be attributed to treatment in the treated group compared with an appropriate unexposed control group, whereas the NOEL is the level at which no treatment-related effect is observed. These NOELs and LOELs provide initial basis for comparisons across studies, while the narrative discussions described above provide more critical evaluation of whether the findings are adverse effects. As discussed above, quantitative comparisons across formulation studies are imprecise and should not be used to determine NOELs/LOELs for glyphosate.

RESULTS

Literature search

The PubMed literature search returned 410 unique hits in the open literature, of which 62 were considered relevant to neurotoxicity (Figure 1). The first-pass cut, based on titles and abstracts, resulted in 22 relevant *in vivo* papers, to which were added 5 regulatory studies that were made available to the public by the Glyphosate Renewal Group (https://www.glyphosate.eu/transparency/scientific-dossier/glyphosate-study-reports/). Another 21 studies were included with *in vitro* endpoints. Of the total 27 *in vivo* studies, 10 studies evaluated glyphosate only, 16 studies evaluated glyphosate formulations, and one study evaluated both glyphosate alone and formulation (Dechartres et al. 2019). These 27 papers and reports were scored using the second-pass criteria in ToxRTool and are presented in Tables 1 (glyphosate alone) and 2 (glyphosate formulations); note that the Dechartres paper appears in both tables.

A total of 11 of the 27 *in vivo* papers/reports received a ToxRTool score of 3 because there were important study methodology deficiencies that limited their use for risk assessment purposes (Tables 1 and 2). The remaining 16

Table 1. Overall methods and reliability for glyphosate-only studies. Summary of endpoints and ToxRTool review.

Reference/ test material/ route / duration/ species / sex	Neurobehavioral endpoints	Neuropathology or Neurochemistry and other biochemistry endpoints	General toxicity endpoints	ToxRTool Review and Score		
				Strengths	Limitations	Score
Brammer (2001) Glyphosate technical Oral (dietary) chronic (52 wk) Rat (Alpk: APfSD) Male and female	MA; FOB: forelimb and hindlimb grip strength; landing foot splay; tail-flick test	H&E microscopy evaluation of brain (cerebrum, cerebellum, brainstem) and spinal cord (cervical, thoracic, lumbar)	Body weight and gain; food consumption; clinical observations; hematology, clinical chemistry, and urinalysis; organ weight and tissue evaluation	GLP study performed in accordance with international guidelines in force at the time of the study's conduct; dose response evaluation; general toxicity indicators; blind observer confirmed	No specific protocol or equipment details are provided for grip strength, foot splay and tail-flick test; the study only cites an unpublished protocol and positive control validation study submitted to the US EPA	2 (reliable with restrictions)
Coullery, Pacchioni, and Rosso (2020) N-phosphonomethyl glycine-monoisopropylamine salt I.p. DNT (GD 8 to GD 20) Rat (Wistar) Male offspring	Righting reflex, negative geotaxis, MA; Morris water maze; contextual fear conditioning	Hippocampus Wnt signaling	Body weight and gain; food consumption; water consumption; litter size; stillbirths; pregnancy duration	Dose response evaluation; sufficient information on behavioral methods	Litter was not clearly confirmed and/or indicated as the statistical unit of measurement in the methods or results for each endpoint; intraperitoneal route is not applicable for direct risk assessment	3 (not reliable)
Dechartres et al. (2019) ^^ Glyphosate isopropylamine salt Oral (dietary) maternal (GD 10 to PND 21) Rat (Sprague Dawley) Dams	Maternal behavior	Hippocampus Ki67 and Doublecortin-positive cell counts (GCL/SGZ); hippocampus synaptophysin and PSD95 immunoreactivity (CA3); hippocampal volume	Maternal weight; litter characteristics; microbiome characteristics; serum inflammatory and clinical chemistry biomarkers	Dose response evaluation; sufficient information on behavioral methods	Significant methodology deficiencies with neuropathology evaluation as reported, including lack of appropriate stereological correction for volume, cell counts, and optical density. Maternal behavior was considered a reproductive not neurological endpoint.	3 (not reliable)
Hernández-Plata et al. (2015) Glyphosate I.p. Acute/subacute (1 d and 2 wk) Rat (Sprague-Dawley) Male	MA	Repeat-dose: D1 and D2 receptor binding in striatum and nucleus accumbens; TH cell count in substantia nigra and ventral tegmental area; monoamine neurotransmitters (and metabolites) determination in striatum and nucleus accumbens; Acute: DA release in striatum and nucleus accumbens	Body weight; aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and gamma-glutamyltransferase (g-GT) serum levels	Dose response evaluated; thorough investigation of neurochemistry, neuropathology and neurological effects within a targeted CNS pathway; includes a recovery period and evaluation following exposure; general toxicity indicators	Intraperitoneal route is not a relevant exposure pathway for risk assessment; no glyphosate purity; not specified if technical or salt; no within-session MA data shown for 3 hr after each dose; small sample size at 16-day test; separate cohorts of rats with MA testing and data not presented separately	1 (reliable)

(*Continued*)

Table 1. (Continued).

Reference/ test material/ route / duration/ species / sex	Neurobehavioral endpoints	Neuropathology or Neurochemistry and other biochemistry endpoints	General toxicity endpoints	ToxRTool Review and Score		
				Strengths	Limitations	Score
Horner (1996a) Glyphosate technical Oral (gavage) acute Rat (Alpk: APfSD) Male and female	MA; FOB; forelimb and hindlimb grip strength; foot splay	Brain weight, length, and width; H&E microscopy evaluation of brain (seven levels), dorsal root ganglia, spinal cord (cervical and lumbar); toluidine blue microscopy evaluation of sciatic nerve, spinal cord, sural and tibial nerve	Body weight and gain; food consumption; clinical observations	GLP study performed in accordance with international guidelines in force at the time of the study's conduct; dose-response evaluation; general toxicity indicators; blind observer confirmed	No specific FOB protocol or equipment details are provided, the study only cites an unpublished protocol and positive control validation study submitted to the US EPA	2 (reliable with restrictions)
Horner (1996b) Glyphosate technical Oral (dietary) subchronic (90 d) Rat (Alpk: APfSD); Male and female	MA; FOB; forelimb and hindlimb grip strength; landing foot splay	Brain weight, length, and width; H&E microscopy evaluation of brain (seven levels), dorsal root ganglia, spinal cord (cervical and lumbar); toluidine blue microscopy evaluation of sciatic nerve, spinal cord, sural and tibial nerve	Body weight and gain; food consumption; clinical observations	GLP study performed in accordance with international guidelines in force at the time of the study's conduct; dose response evaluation; general toxicity indicators; blind observer confirmed	No specific FOB protocol or equipment details are provided, the study only cites an unpublished protocol and positive control validation study submitted to the US EPA	2 (reliable with restrictions)
Luna et al. (2021) Glyphosate technical for *in vitro* N-phosphonomethyl glycine-monoisopropylamine salt for *in vivo* S.c. DNT (PND 7 to PND 27) Rat (Wistar) Male offspring	Novel object recognition; Morris water maze	*In vitro* hippocampus PSD-95 optical density; hippocampus PSD-95, SYN-1, p-CaMKII, and CaMKII protein expression	Body weight gain	Dose response evaluation	Litter was not clearly confirmed and/or indicated as the statistical unit of measurement in the methods or results for each endpoint	3 (DNT not reliable)
Martínez et al. (2018) Glyphosate technical Oral (gavage) subacute (6 d) Rat (Wistar) Male	None	Monoamine neurotransmitters levels (and metabolites) in hypothalamus, midbrain, hippocampus, striatum and prefrontal cortex	Body weight gain; food and water consumption	Dose response evaluated; neurochemistry effects within targeted CNS pathways; sufficient information provided on methodology	No differentiation of adaptive vs adverse outcome or acute vs repeated-effects as neurochemistry was only measured 3-hours after last dose and a later recovery timepoint was not included	1 (reliable)
Nufarm (2009) Glyphosate technical Oral (dietary) chronic (52 wk) Rat (Wistar Han); male and female	MA; FOB: sensory reactivity, grip strength	H&E microscopy evaluation of brain (cerebrum, cerebellum, brainstem) and spinal cord (cervical, thoracic, lumbar)	Body weight and gain; food consumption; clinical observations; hematology, clinical chemistry, and guideline organ weight and tissue evaluation battery	GLP study performed in accordance with international guidelines in force at the time of the study's conduct; dose response evaluation; general toxicity indicators	No verification in the report text that FOB conducted with observer blind to treatment (although the guideline is cited, which requires a blind observer)	2 (reliable with restrictions)
Pu et al. (2021) Glyphosate technical Oral (drinking water) DNT (GD 7 to PND 21) Mouse (ddY) Male offspring	Grooming; three-chamber social interaction	None	None	Dose response evaluation	Litter was not clearly confirmed and/or indicated as the statistical unit of measurement in the methods or results for each endpoint	3 (not reliable)

(*Continued*)

Table 1. (Continued).

Reference/ test material/ route / duration/ species / sex	Neurobehavioral endpoints	Neuropathology or Neurochemistry and other biochemistry endpoints	General toxicity endpoints	ToxRTool Review and Score		
				Strengths	Limitations	Score
SafePharm (2006) Glyphosate technical Oral (dietary) subchronic (90 d) Rat (Sprague-Dawley Crl:CD) Male and female	MA; FOB: grip strength, landing foot splay, tail-flick test	H&E microscopy evaluation of brain (seven levels), dorsal root ganglia, dorsal and ventral root fibers, eyes, optic nerve, sciatic nerve: proximal; tibial nerve: proximal, and spinal cord (cervical and lumbar)	Body weight and gain; food consumption; clinical observations; ophthalmoscopic examination	GLP study performed in accordance with international guidelines in force at the time of the study's conduct; dose response evaluation; general toxicity indicators	No verification in the report text that FOB conducted with observer blind to treatment (although the guideline is cited, which requires a blind observer)	2 (reliable with restrictions)

i.p: intraperitoneal; s.c.: subcutaneous; MA: motor activity; FOB: functional observational battery; H&E: hematoxylin and eosin; TH: tyrosine hydroxylase; DA: dopamine

^^Dechartres (2019) evaluated both Roundup and glyphosate (technical)

studies (7 glyphosate alone, 9 glyphosate formulation studies) were given a score of 1 or 2 and considered "reliable" for risk assessment purposes. Since several papers presented data from different endpoints, each of the behavioral, neuropathology, and neurochemistry endpoints were evaluated on their own merits. In one case, the developmental neurotoxicity (DNT) data in Luna et al. (2021) were excluded (see methodological deficiencies, below), but the *in vitro* portion of the study were acceptable for review. In another case, the clinical observations conducted in de Olivera Joaquim et al. (2012) were considered unreliable since there was no indication that the observer was blind to treatment, but the other behavioral data met criteria and thus the study was acceptable with restrictions. Finally, the Gallegos et al. (2020) paper received an overall ToxRTool score of 3 based upon the behavioral and neuropathology endpoints, but this paper also included biochemical data including acetylcholinesterase (AChE) and glutamate transaminase activity that were considered valid supportive information.

Important study methodology deficiencies

Two critically important methodological deficiencies were frequently encountered in the literature evaluated; specifically, 1) the litter was not the experimental unit in developmental studies; and 2) inadequate methods were used for cell

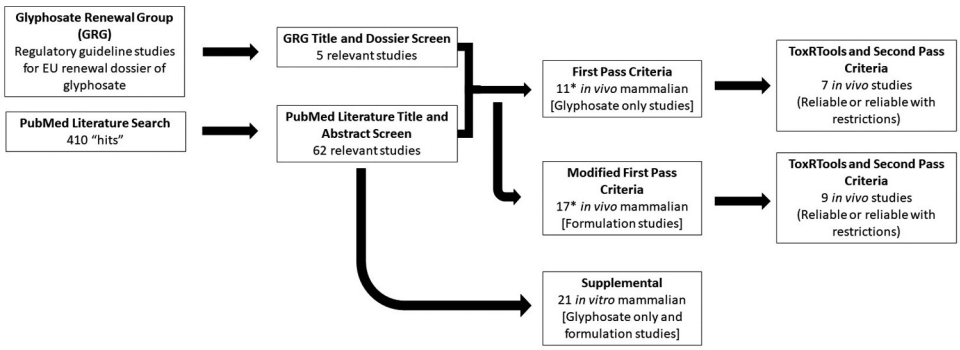

Figure 1. Flowchart of literature review. *Note that one study (Dechartres et al. 2019) tested glyphosate alone as well as a formulation (Roundup) and is counted in both sections. One author (KMS) made the initial criteria determination and was independently checked by a second author (VCM) with any inconsistencies resolved by discussion among all authors. Additional independent checks by a third author (AAL or JRR) were also provided during detailed review of papers by subject area, resolved through discussion among all authors. EU: European Union. GRG: Glyphosate Renewal Group (https://www.glyphosate.eu/).

Table 2. Overall methods and reliability for glyphosate formulation studies. Summary of endpoints and ToxRTool review (modified to account for formulation methodology).

Reference / formulation/ route / duration / species / sex	Neurobehavioral endpoints	Neuropathology or Neurochemistry and other biochemistry endpoints	General toxicity endpoints	Modified ToxRTool Score and Review		
				Strengths	**Limitations**	**Score**
Ait Bali, Ba-Mhamed, and Bennis (2017) Roundup Oral (gavage) Acute (1 d) Subchronic (42 d and 90 d) Mouse (Swiss) Male	MA; elevated plus maze; tail suspension test; splash test	TH and 5-HT immunoreactivity	Body weights	Dose-response is evaluated; investigation of both neuropathology and neurological effects within a targeted CNS pathway	Small sample size (n = 6); within-session MA data not provided; no repeated-measures analysis of within-session data; only took 4 random samples in substantia nigra; immunoreactivity assumed linear DAB development	2 (reliable with restrictions)
Ait Bali et al. (2018) Roundup Oral (gavage) Acute (1 d) Subchronic (42 d and 90 d) Mouse (Swiss) Male	MA; elevated plus maze; tail suspension test; splash test	None	Gut microbiome assay	None	The paper presents the same behavioral data as presented in Ait Bali, Ba-Mhamed, and Bennis (2017) and therefore, does not offer new information	3 (not reliable) – duplicative data
Ait Bali et al. (2019) Roundup Oral (gavage) Acute (1 d) Subchronic (42 d and 90 d) Mouse (Swiss) Male	Novel object recognition; Y-maze; passive avoidance test	AChE activity; superoxide dismutase activity; peroxidase activity	None	Dose-response is evaluated; investigation of both neuropathology and neurological effects within a targeted CNS pathway	Small sample size (n = 6); endpoints manually scored with no confirmation that observers were blind to treatment; control enzyme data not presented	1 (reliable)

(*Continued*)

Table 2. (Continued).

Reference / formulation/ route / duration / species / sex	Neurobehavioral endpoints	Neuropathology or Neurochemistry and other biochemistry endpoints	General toxicity endpoints	Modified ToxRTool Score and Review		
				Strengths	**Limitations**	**Score**
Ait-Bali et al. (2020) Roundup Oral (gavage) DNT (GD 0 to PND 21) Swiss mice: Pregnant dams; male offspring	Offspring pups: Negative geotaxis; righting reflex; cliff avoidance; rotarod tests; Offspring adult: MA; elevated plus maze; three-chambered sociability test; Y-maze; novel object recognition test; passive avoidance test	AChE activity; TH-intensity in substantia nigra pars compacta and ventral tegmental area; GFAP and Iba-1 intensity in prefrontal cortex and dorsal hippocampus	Maternal fertility and reproduction parameters; offspring bodyweight	Dose response evaluation; general toxicity indicator	Litter was not clearly confirmed and/or indicated as the statistical unit of measurement in the methods or results for each endpoint	3 (not reliable)
Baier et al. (2017) Glifloglex Intranasal subacute (4 wk) Mouse (CF-1) Male	MA; elevated plus maze; novel object recognition	None	Body weights	General toxicity indicator	NOR discrimination index not clearly described or presented and does not account for locomotor effects	2 (reliable with restrictions)
Bicca et al. (2021) Zamba Oral (gavage) subacute (30 d) Mouse (Swiss) Male	MA; elevated plus maze test; forced swim test; sucrose preference test	Hippocampus: reactive species, ferric reducing antioxidant potential, reduced glutathione, AChE, H&E stain	None	Behavioral methodology and timeline clearly described	Endpoints manually recorded with no confirmation observer blind to treatment; very short MA session (5 min)	2 (reliable with restrictions)
Cattani et al. (2014) Roundup Oral (drinking water) DNT (GD 5 to PND 15) Rat (Wistar) Pregnant females; male and female offspring	None	Hippocampal slices *ex vivo*: Ca^{2+} uptake, lactate dehydrogenase, glutamate uptake/ release, glutamine synthetase activity, amino acid accumulation, reduced glutathione, lipoperoxidation, AST, ALT, CGT, G6PD	Body weights (pups, dams); water consumption	Extensive evaluation of hippocampus cellular effects	Details on study design and dam group allocation is not sufficiently articulated, sample size greater than number of litters; unclear if hippocampal slice studies used developmentally treated offspring; implies undiluted formulation used *in vitro*	3 (not reliable)

(*Continued*)

Table 2. (Continued).

Reference / formulation/ route / duration / species / sex	Neurobehavioral endpoints	Neuropathology or Neurochemistry and other biochemistry endpoints	General toxicity endpoints	Modified ToxRTool Score and Review		
				Strengths	**Limitations**	**Score**
Cattani et al. (2017) Roundup Oral (drinking water) DNT (GD 5 to PND 15) Rat (Wistar) Pregnant females; male and female offspring	Forced swimming test, MA, rotarod test, sucrose consumption	Hippocampal slices *ex vivo*: Ca2+ uptake, LDH, glutamate uptake, AChE activity; GGT, S100B, total and phosphorylated levels of NF-κB, antioxidants enzymes, catalase activity, superoxide dismutase activity, glutathione peroxidase activity	Body mass gain, mortality	Dose-response evaluated	Litter was not clearly confirmed and/or indicated as the statistical unit of measurement in the methods or results for each endpoint	3 (not reliable)
de Oliveira Joaquim et al. (2012) Roundup Oral (gavage) Transorb Oral acute (1 d) Mouse (BALB/c) Male and female	MA; elevated plus maze; FOB	None	None	Dose-response evaluation	Endpoints manually scored with no confirmation that observers were blind to treatment; clinical observations scored without indication that observer was blind to treatment	2 (reliable with restrictions) (except FOB scored as 3)
de Oliveira Joaquim et al. (2014) Roundup Oral (gavage) Transorb Oral subacute (23 d) Mouse (BALB/c); Male and female	MA; elevated plus maze; forced swim test	None	None	Juvenile exposure paradigm (less studied)	Endpoints manually scored with no confirmation that observers were blind to treatment	2 (reliable with restrictions)

(*Continued*)

Table 2. (Continued).

Reference / formulation/ route / duration / species / sex	Neurobehavioral endpoints	Neuropathology or Neurochemistry and other biochemistry endpoints	General toxicity endpoints	Modified ToxRTool Score and Review		
				Strengths	**Limitations**	**Score**
Dechartres et al. (2019) ^^ Roundup 3Plus Oral (dietary) maternal (GD 10 to PND 21) Rat (Sprague Dawley) Dams	Maternal behavior	Hippocampus Ki67 and Doublecortin-positive cell counts (GCL/SGZ); hippocampus synaptophysin and PSD95 immunoreactivity (CA3); hippocampal volume	Maternal weight; litter characteristics; microbiome characteristics; serum inflammatory and clinical chemistry biomarkers	Dose response evaluation; sufficient information on behavioral methods	Significant methodology deficiencies with neuropathology evaluation as reported, including lack of appropriate stereological correction for volume, cell counts, and optical density. Maternal behavior was considered a reproductive not neurological endpoint.	3 (not reliable)
Gallegos et al. (2016) Glifloglex Oral (drinking water) DNT (GD 0 PND 21) Rat (Wistar) Pregnant females; male offspring	Offspring pups: righting reflex, cliff aversion, negative geotaxis and eye and ear opening; Offspring adult: MA, elevated plus maze	None	Food and water intake; body weight; litter size; gestation length	Dose-response evaluated	No confirmation that observers were blind when scoring behavior (though measured parameters are non-subjective)	2 (reliable with restrictions)
Gallegos et al. (2018) Glifloglex Oral (drinking water) DNT (GD 0 to PND 21) Rat (Wistar) Pregnant females; male offspring	Novel object recognition	Whole brain: lipid peroxidation; antioxidant enzyme activities; Hippocampus, striatum and prefrontal cortex: glutamate transaminases, alkaline phosphatase activities, AChE activity	None	Dose-response evaluated, automated software for novel object scoring	No concurrent general toxicity indicators (e.g., weight); no raw summary data on novel object parameters including total entries into arms and crossings, only exploratory ratio, not raw data, presented	1 (reliable)

(*Continued*)

Table 2. (Continued).

Reference / formulation/ route / duration / species / sex	Neurobehavioral endpoints	Neuropathology or Neurochemistry and other biochemistry endpoints	General toxicity endpoints	Modified ToxRTool Score and Review		
				Strengths	**Limitations**	**Score**
Gallegos et al. (2020) Glifloglex Intranasal subacute (4 wk) Mouse (CF-1) Male	FOB	Brain regions examined: olfactory bulb, prefrontal cortex, caudate putamen, cortex, hippocampus midbrain; total thiol content; catalase (CAT) activity; AChE activity; glutamate oxaloacetate transaminase (GOT) activity; glutamate pyruvate transaminase (GPT) activity; ChAT, αBTX, TH or GFAP-positive cell count	Body weights and gain; food and water consumption	Methodology and endpoints are sufficiently described; general toxicity indicators	Deficiencies in neuropathology evaluation including only two slides used per animal with no accounting for sectioning/volume variation, and inadequate stereological correction; many of the biochemical markers are indicative of general cellular toxicity and are not neuro-specific, no confirmation that observers were blind when scoring subjective behavior in FOB	3 (not reliable) Biochemistry data acceptable
Gress et al. (2016) Roundup GT Plus (drinking water) Subacute (8 d) Rat (Sprague-Dawley) Male	MA	Brain organ weight	Liver, kidney, heart, and testes weight; liver CYP activity; sex hormone analyses	Sample size is large; general toxicity indicators	Delivered dose not verified with water consumption and body weight; MA measured "at night" but no mention of time of testing or whether groups were tested at same time; within-session MA data not provided	2 (reliable with restrictions)
Pu et al. (2020a) Roundup Maxload (drinking water) Subacute (14 d) Mouse (C57BL/6) Males	None	DA transporter immunoreactivity (striatum); TH-positive cell count (striatum)	Body weight	General toxicity indicator	Significant methodology deficiencies with neuropathology evaluation as reported, including lack of appropriate stereological correction for volume, cell counts, and optical density	3 (not reliable)

(*Continued*)

Table 2. (Continued).

Reference / formulation/ route / duration / species / sex	Neurobehavioral endpoints	Neuropathology or Neurochemistry and other biochemistry endpoints	General toxicity endpoints	Modified ToxRTool Score and Review		
				Strengths	**Limitations**	**Score**
Pu et al. (2020b) Roundup Maxload Oral (drinking water) DNT (GD 5 to PND 21) Mouse (ddY); Pregnant dams; male offspring	MA; novel object recognition; prepulse inhibition (PPI) test; three-chamber social interaction test; grooming test	Prefrontal cortex, striatum, and hippocampus: glutamate, glutamine, glycine, L-serine, D-serine, GABA levels; parvalbumin positive-cell count (mPFC)	Body weight; Short-chain fatty acids rRNA expression in feces; gut microbiota composition	Wide array of neurofunctionality tests; general toxicity indicator	Litter was not clearly confirmed and/or indicated as the statistical unit of measurement in the methods or results for each endpoint	3 (not reliable)

i.p: intraperitoneal; s.c.: subcutaneous; MA: motor activity; FOB: functional observational battery; H&E: hematoxylin and eosin; TH: tyrosine hydroxylase; LDH: lactate dehydrogenase; DA: dopamine; 5-HT: serotonin; AChE: acetylcholinesterase; GABA: γ-aminobutyric acid; GFAP: glial fibrillary acidic protein; DNT: developmental neurotoxicity; GD: gestation day; PND: postnatal day

^^Dechartres et al. (2019) evaluated both Roundup and glyphosate (technical)

counting in neuropathology evaluations. Less commonly encountered were investigations that included subjectively scored behavioral evaluations in which the observer was not blind to treatment.

For developmental studies exposing dams and/or pups, the litter of origin needs to be accounted for in experimental design and statistical procedures. This is a well-established guideline requirement for reproductive, developmental, and neurodevelopmental studies (NAFTA 2016; OECD 2007; US EPA 1998a). It is the litter, not the individual animal, that is the unit of exposure, and it is not scientifically acceptable to use or statistically analyze littermates as if they were independent samples. Thus, studies in which data are collected in littermates and analyzed without accounting for litter may present results that violate statistical assumptions, inflate type I error rates, and are unreliable for risk assessment purposes (Holson et al. 2008; Holson and Pearce 1992).

Of the nine papers where glyphosate (Coullery, Pacchioni, and Rosso 2020; Luna et al. 2021; Pu et al. 2021) or glyphosate formulation (Ait-Bali et al. 2020; Cattani et al. 2014, 2017; Gallegos et al. 2018, 2016; Pu et al. 2020b) was administered during development and offspring were tested, 7 studies collected data from littermates and analyzed without accounting for the litter. In five papers, this was evidenced by having sample sizes that were greater than the number of dams/litters (Cattani et al. 2014, 2017; Luna et al. 2021; Pu et al. 2021, 2020b). In two other papers, it was stated that multiple offspring from the same litter were tested or that some or all of the pups were fostered across litters, yet there was no indication that the litter of origin was tracked (Ait Bali et al., 2020; Coullery, Pacchioni, and Rosso 2020). For these seven studies, the results may not be valid, and are not reviewed here.

For studies involving cell counting, adequate sampling and description of methods to control for bias need to be included such that the results are interpretable and reliable for risk assessment purposes. Stereology is considered the gold standard for cell counting that involves sampling a known fraction of a structural component and generating unbiased estimates of the neuronal number within that region. The preferred stereological approaches for neuropathology include Cavalieri-based volume estimates and optical or physical dissector-based neuron counts of selected brain regions with adequate precision typically being achieved by evaluating 25 to 50 fields in 6 to 8 serial, 3-mm-thick dissectors (where a dissector consists of two adjacent sections) to acquire about 100 object counts (Bolon et al. 2011). Methods need to include the structure and region of interest (ROI), all details of microscopy set up, details on how the tissue was cut (account for variation in thickness), all constants and sampling parameters, precision of the estimates, and section shrinkage correction (if needed) (Bolon et al. 2011; Dorph-Petersen and Lewis 2011).

Three studies (Dechartres et al. 2019; Gallegos et al. 2020; Pu et al. 2020a) contained inadequate methods for neuropathology and were therefore considered unreliable for risk assessment. In these, the reported cell counts had significant deficiencies in methodology and reporting, leading to lower confidence that the numbers were reflective of reliable and reproducible biological values. Dechartres et al. (2019) performed an observer-blinded count analysis on labeled cells in the dentate gyrus cut at 40 μm in a 1:10-series (only one section evaluated per 10 continuous sections). No information was provided on the constants or sampling parameters needed to ensure unbiased measurements nor on the Bregma spatial values of the ROI. There was no provided comparison of the volume and/or area that was measured for each animal and whether their sampling strategy adequately controlled for variance in regions/thickness across different sections. Further, Dechartres et al. (2019) noted that the number of cells counted was simply multiplied by 10 to account for their sampling strategy, but given the irregular shape of the dentate gyrus, and no clear evidence provided of a uniform sampling strategy, this crude adjustment was unreliable. Finally, these investigators reported their values as a total number of cells within the ROI but based upon lack of an unbiased approach to evaluate their ROI, there is insufficient confirmation that these values reflect a true biological estimate of the cell counts in the region. Pu et al. (2020a), lacked similar details as described for Dechartres et al. (2019), and further did not confirm if the observer was blind with respect

to treatment. Finally, Gallegos et al. (2020) provides useful details, including confirmation on observer blindness, series section details, the Bregma spatial values for the ROI as well as the number of brain slides (only 2) for each mouse and ROI. However, Gallegos et al. (2020) normalized the counts to an arbitrary fixed area (0.5 mm^2), which is not an unbiased approach and does not take into account three-dimensional parameters. Since the counts were not normalized to the actual volume of the region, and also because an arbitrary fixed count region was used, the results would not be expected to be reproducible, as the data do not represent a defined biological estimate of the true number of positively stained cells in the ROI.

With regard to behavioral measures, there were two studies in which subjective assessments of behavior were conducted (de Oliveira Joaquim et al. 2012; Gallegos et al. 2020). It is critical that such observations be made with the observer blinded to treatment to prevent bias (Bello et al. 2014; Holman et al. 2015), and it is also required in applicable US EPA and OECD regulatory guidelines (OECD 1997; US EPA 1998b). If the paper/report did not mention that this was the case, it was assumed that the assessments were not conducted appropriately. This was the case in these two studies, and these subjective data were considered unreliable.

Glyphosate review

A total of 11 studies of glyphosate alone (one of which also tested formulation) were found in the open literature (Table 1, Figure 1). Five of these were regulatory study reports that were publicly available. Of the remaining six, which were peer-reviewed scientific articles, four were considered unreliable (ToxRTool category 3), mostly due to the major methodology deficiencies described above and in Table 1. The remaining investigations that were considered reliable (ToxRTool category 1 or 2) are described and evaluated in the following sections.

Adult neurotoxicity: behavior

Acute (single dose) neurotoxicity

The literature search located two studies with single-dose neurotoxicity assessments using glyphosate alone as the test material (Horner 1996a; Hernández-Plata et al. 2015; Tables 1 and 3). Both studies used rats and included multiple dose levels, but utilized different routes of administration: oral gavage (Horner 1996a) and intraperitoneal (i.p.) (Hernandez-Plata et al., 2015).

The Horner (1996a) study was aligned with OECD test guidelines (OECD 1997) for assessment of motor activity (MA) and FOB and was submitted for regulatory purposes. At 6 hr after dosing, 3 out of 10 high-dose females exhibited clinical signs (decreased activity, gait, and/or posture changes), and one died. In addition, at 6 hr, MA was numerically decreased (28% in males, 33% in females), but these effects were not statistically significant. In addition, at 6 hr, high-dose male rats also displayed a significant 17% reduction in landing foot splay compared to controls, but foot splay in that group was 22% lower than controls in the pre-dose test, suggesting pre-existing differences and confounding interpretation. On day 15 test, male rats exhibited a significant fall in MA at the high dose, and an elevated forelimb grip strength at the low dose only. There were no changes on any endpoint at 7 days. The effects at 15 days may be considered spurious given the lack of any effects at 7 days, and the atypical direction of change in grip strength that occurred only at the lowest dose. The clinical signs and no significant alterations in MA suggest acute effects at 6 hr at the high dose only and a LOEL of 2000 mg/kg and NOEL of 1000 mg/kg.

In the Hernández-Plata et al. (2015) investigation, glyphosate was administered i.p. repeatedly at 50, 100, or 150 mg/kg with three injections per week. The experimental design included MA testing immediately before and after each dose administration, including the first dose. Thus, data from the first dose provided an evaluation of an acute (single) dose. Hernández-Plata et al. (2015) reported decreased MA (total distance, vertical activity, and stereotypy counts) over the 3 hr following the first dose; however, while all dose groups were significantly lower than control, the magnitude of effect (in the range of 50%) was similar across doses. Within-session activity data are not provided, so the pattern of change over those 3 hr is unknown. In addition, it was also apparent that two separate cohorts of rats underwent MA testing, and data were combined; while the investigators state that data "were similar," presentation of the

separate datasets would have been helpful for a better understanding of dose-response. Overall, a NOEL could not be determined, as the lowest dose of 50 mg/kg produced a significant effect (LOEL).

In summary, data regarding acute effects of glyphosate are contradictory. There are marked differences in the effective dose response, with 500 or 1000 mg/kg via oral gavage producing no marked effects in the regulatory study, whereas decreased MA was observed at doses as low as 50 mg/kg following i.p. injection (albeit with a flat dose-response). This is an important observation, as there are absorption differences between oral gavage and i.p. routes of administration, although there are limited direct comparisons. Absorption of oral dosing was reported to be ≤35% (depending on dose) (Anadón et al. 2009; ATSDR 2020; Brewster, Warren, and Hopkins 1991; WHO 2016), whereas i.p. absorption was reported in the range of 81 to 90% (WHO 2016). Data suggest that in terms of total absorption, i.p. doses might produce systemic levels similar to oral doses that are approximately 3–4-fold higher. Further, the acute i.p. LD_{50} is 238 mg/kg and the oral LD_{50} is 4300–5600 mg/kg in rats (Rubin 1996). This 20-fold difference in LD_{50} suggests that peak concentrations are likely to be significant contributors to toxicity differences, including the larger differences in the effective doses in these two studies (LOELs of 2000 mg/kg oral compared to 50 mg/kg i.p.). Another consideration is the time of MA testing after the dose. The T_{max} for oral dosing was reported to be 5.16 hr (Anadón et al. 2009), and the timepoint in Horner (1996a) (6 hr) approximates that time. A time-course kinetic study in rats with radiolabeled glyphosate reported a maximum concentration in plasma at 30 min following a single i.p. administration (US EPA 1984). Only total-session activity is given in Hernández-Plata et al. (2015), and key information (i.e., within-session data over the 3 hr) is missing. In addition, these investigators did not specify whether the test material was technical glyphosate or a salt form, or the volume of dosing solution. Since glyphosate is an acid, an i.p. injection might induce peritoneal irritation depending upon the pH of the dosing solution, which might impact behavioral measures. Even considering these potential explanations as well as others (different rat strains or test devices), such large differences in effective doses are notable and present less confidence in the direct comparison of i.p. and oral doses. Overall, oral data from Horner (1996a) are likely more relevant for dietary risk assessment.

Subacute (short-term repeat dose ≤1 month) neurotoxicity

Only one study conducted subacute dosing with glyphosate alone (Hernández-Plata et al. 2015) (Tables 1 and 3). Glyphosate was administered i.p. repeatedly for six injections over two weeks (3 injections/week). MA was measured for 15 min before and for 3 hr after each of the six injections, and also before and after saline injections that occurred 2 and 16 days after the sixth glyphosate dose. Reduced MA (total distance, vertical activity, and stereotypy counts) was reported over 3 hr after each dose, with non-consistent changes in the magnitude of effect as dosing progressed. Significant differences were noted at all doses, but a graded dose-response was not consistently seen over the test days. Beginning after two doses, diminished total distance and vertical activity were also seen in the tests 15 min before each day's dose. In addition to the lack of within-session activity data and apparent merging of data from two separate cohorts of rats mentioned above, the effects of the repeated doses are potentially confounded by carry-over effects of repeated testing plus the effects of that day's dose. All tested doses produced significant effects on MA during the dosing period and at 2 days after, indicating a LOEL of 50 mg/kg/day (lowest dose tested), but attributing this to subacute dosing is uncertain with the study design and data provided. There were no residual effects at 16 days after the last dose, suggesting that the effects observed are transient and reversible.

Subchronic (repeat dose 1-3 months) neurotoxicity

Two subchronic (13 week) neurotoxicity guideline studies using technical glyphosate were identified (Horner 1996b; SafePharm 2006) (Tables 1 and 3). Glyphosate was administered in feed to rats, and both studies reported decreased body weight gain

over time, mostly at the high dose (Horner 1996b: 1547–1631 mg/kg/day; SafePharm 2006: 1499–1555 mg/kg/day).

The earlier subchronic study (Horner 1996b) conducted MA and FOB assessments at monthly intervals. In male rats, MA was increased only at the low dose in week 9: no other changes were recorded. Female rats in the mid-dose group showed elevated forelimb grip strength and reduced latency (faster) tail flick in week 5 only. These changes were not consistent across time with ongoing exposure, did not show a graded dose–response relationship, and the direction of change was not considered adverse (e.g., decreased grip strength indicates muscle weakness, but this study reported elevated grip strength). These were considered incidental changes and not evidence of neurotoxicity; thus, in this study, the highest exposure level (1547 mg/kg/day in males, 1631 mg/kg/day in females) was the NOEL.

A second subchronic study (SafePharm 2006) used a similar protocol and feed concentrations to that of Horner (1996b), but used different test times. It should be noted that the report did not provide direct confirmation in the protocol or results that the observers were blind to treatment for behavioral assessment, though the investigators reported that it was compliant with guidelines, which requires observers to be blind (OECD 1997). In male rats, increased overall MA was recorded at the low dose only at 3 and 5 weeks, with diminished activity in the middle dose only observed at 13 weeks. In contrast, female rats exhibited elevated MA in all treatment groups at weeks 9 and 13 tests, with similar magnitude change at both the 1000 and 5000 ppm and an attenuated response at 20,000 ppm level. The lab also reported "mobile" data, and there were no marked differences from control at any time; however, how this was defined and how it was different from activity was not described. No marked differences were noted in the FOB observations or forelimb and hindlimb grip strength. Thus, these MA differences did not display a clear dose-response or time-course (progression as dosing continued) relationship, the direction of change was not consistent, and there were no correlating changes in "mobile" activity or other endpoints. These transient and inconsistent changes do not support conclusions of MA effects at any dose level, and thus the NOEL was the highest concentration tested (1499 mg/kg/day in males, 1555 mg/kg/day in females).

Chronic (repeat dose 1 year) neurotoxicity

There are two chronic dietary toxicity/carcinogenicity guideline studies available for glyphosate (Brammer 2001; Nufarm, 2009) (Tables 1 and 3). In these 2-year studies, neurotoxicity assessments (MA and FOB) were conducted in a subset of animals at 1 year. Glyphosate was administered in feed to rats, and both studies reported reduced body weight gain over time, mostly at the highest dose (Brammer 2001: 1214–1498 mg/kg/day; Nufarm 2009: 1077–1382 mg/kg/day).

In the earlier study (Brammer 2001), there were no marked effects on MA. In female rats, landing foot splay was decreased at the high dose, and tail flick latency was shorter at the middle dose. The direction of change observed for both foot splay and tail flick was not considered adverse, and the effect on tail flick was evident at the middle dose only. Given the lack of effects on MA or other behavioral endpoints, the NOEL in this study was the highest dose tested (1214 mg/kg/day in males, 1498 mg/kg/day in females).

The second chronic study (Nufarm 2009) reported no significant changes in any of the FOB measures or MA. Thus, the NOEL was the highest dose tested (1077 mg/kg/day in males, 1382 mg/kg/day in females).

Summary of behavioral data

Overall, the regulatory studies showed no biologically significant effects of 90-day or 1-year repeated dosing with glyphosate, and there are no other published behavior studies that tested glyphosate alone to compare. In these studies, the dietary NOEL was greater than 1077–1555 mg/kg/day (range of intake in feed over months) across all studies. While these regulatory studies reported a few significant changes at various doses and time points, these changes were inconsistent and not clear evidence of neurotoxicity.

Acute effects of glyphosate have more support from both the regulatory study (which exhibited activity, gait, and posture changes, as well as trends towards decreased MA) and the published study of both acute and subacute effects (which

showed MA falls, albeit with no dose-response). As discussed above, the large difference in effective doses (NOEL of 1000 mg/kg in the regulatory study, LOEL of 50 mg/kg in the published study) may be the result of different TK profiles of oral vs i.p. dosing, and/or i.p. irritation. Thus, the oral acute NOEL was 1000 mg/kg, and the lower effective i.p. doses are likely less relevant for risk assessment.

Adult neurotoxicity: neuropathology/pharmacology

There were three papers from the published literature and five publicly available regulatory studies evaluating neuropathology and/or neurochemical endpoints (Tables 1 and 4). The neuropathology regulatory studies were conducted on either perfusion-fixed (acute, subacute studies) or immersion-fixed (chronic studies) tissues of brain, spinal cord, and peripheral nerves followed by staining with hematoxylin and eosin (all tissues) and toluidine blue (for peripheral nerves and ganglia embedded in plastics) (Brammer 2001; Horner 1996a, 1996b; Nufarm 2009; SafePharm 2006). One published study received a ToxRTool score of category 3 (unreliable), for reasons described earlier, and was therefore excluded from further consideration (Dechartres et al. 2019).

Acute (single dose) neurotoxicity

Horner et al. (1996a) evaluated the effects of a single oral dose of glyphosate in rats (500 to 2000 mg/kg; males and females; Table 4). Using standard histopathological techniques, there were no microscopic changes in perfusion-fixed sections of brain, spinal cord, and peripheral nerve at 2000 mg/kg. Thus, the NOEL was greater than 2000 mg/kg based upon US EPA neurotoxicity guidelines (US EPA, 1991).

Hernández-Plata et al. (2015) used microdialysis to measure the acute effects of a single acute 150 mg/kg i.p. dose of glyphosate on dopamine (DA) and metabolite levels in the striatum and nucleus accumbens (NAcc) of anesthetized rats. The striatum and NAcc are innervated by the nigrostriatal and mesolimbic pathways, respectively, which are two of the major dopaminergic pathways in the brain. Data revealed that glyphosate produced a transient reduction in the release of dopamine (DA) in the striatum, but not NAcc. Three hr after i.p. injection of glyphosate or saline, rat brains were perfused for 1 hr with a high-potassium (60 mM) Ringer solution to test total evoked DA release. As expected, high potassium increased DA levels in the microdialysate from both control and glyphosate groups, but the rise was significantly attenuated in the glyphosate group (approximately ↑ 1500% glyphosate vs ↑ 3000% saline group from baseline). In contrast, release of DA in the NAcc was similar between glyphosate and control.

In summary, following acute exposures there were no marked neuropathological changes observed at doses as high as 2000 mg/kg following oral gavage in the regulatory study, but some changes in striatal DA release were noted using microdialysis following 150 mg/kg i.p. (only dose given). Thus, there are only sparse data to support a finding of acute neurotoxicity based upon these endpoints, because the neuropathology assessments were negative and the adversity is unclear regarding a transient functional change in DA release in one brain region following a single high dose.

Subacute (short-term repeat dose ≤1 month) neurotoxicity

Martínez et al. (2018) and Hernández-Plata et al. (2015) measured the effects of glyphosate on monoamine levels in brain areas of male rats following repeated oral and i.p. doses, respectively. Both studies generated dose–response data for a large number of neurotransmitter and metabolite levels in different brain areas using 3 or 4 glyphosate dose levels (Table 4). Martínez et al. (2018) administered glyphosate (0, 35, 75, 150, or 800 mg/kg/day) daily by oral gavage for 6 consecutive days (total six doses) and measured monoamine changes three hr after the sixth dose. As this is close to the T_{max} for oral glyphosate, it is difficult to interpret acute vs subacute effects of glyphosate. In contrast, Hernández-Plata et al. (2015) dosed rats i.p. three times a week over two weeks (total 6 doses each for the 0, 50, 100, or 150 mg/kg/day dose groups) and measured levels of monoamines, tyrosine hydroxylase (TH) enzyme (the rate-limiting enzyme for DA and norepinephrine (NE) synthesis), and mesencephalic TH^+ cells 2 and 16 days after the last dose.

Martínez et al. (2018) reported decreased tissue levels of DA, NE, and serotonin (5-HT) and their metabolites in various brain regions. There was a clear NOEL of 35 mg/kg/day, while the next lowest dose of 75 mg/kg/day produced a number of significant changes: specifically, decreases of 5-HT in striatum, DA in prefrontal cortex and midbrain, and NE in striatum and midbrain. The highest dose (800 mg/kg/day) decreased all except one neurotransmitter measurement (NE in hypothalamus). A graded dose-response was evident in some regions (such as, 5-HT in striatum) but not others (such as, NE in midbrain), and metabolite levels were correspondingly altered in some regions but not others. Overall, most of the significant changes were in brain regions that have lower innervation and neurotransmitter levels (i.e., DA in the prefrontal cortex, NE in striatum and hippocampus). In contrast, areas of the brain associated with highest innervation and levels of the neurotransmitter either displayed: (a) no effects (for example, NE in hypothalamus), (b) reduction only at the highest dose level of 800 mg/kg (for example, DA in striatum, and 5-HT in hypothalamus and midbrain), or (c) did not display a dose-response (for example, DA in midbrain).

Hernández-Plata et al. (2015) measured the subacute effects of glyphosate on DA markers, 5-HT and metabolite 5-hydroxyindolacetic acid (5-HIAA) in the NAcc and striatum. Glyphosate did not significantly alter DA, 5-HT or metabolite levels, TH levels (as measured by quantitative western blot) or TH^+ cell counts at either 2 or 16 days after the sixth dose. The only detectable change was a dose-related decrease (significant at 50, 100, and 150 mg/kg/d i.p.) in specific binding to D1, but not D2, DA receptors in the NAcc at 2 days. Binding reduction was significantly correlated with a fall in MA. These changes were not observed 16 days after dosing, and there were no marked changes in D1 or D2 receptor binding in the striatum at either time. Note that in the acute microdialysis study using a single high dose of 150 mg/kg i.p., there were acute effects on DA release in striatum but not NAcc, whilst the receptor binding decrease following subacute exposures was only affected in the NAcc, not the striatum. Although the NOEL for a fall in D1 receptor binding was below 50 mg/kg i.p, the toxicological significance of this isolated and reversible neurochemical effect for risk assessment purposes is uncertain.

In summary, the NOEL for subacute effects on neuropharmacological endpoints was determined to be 35 mg/kg/day based upon neurotransmitter changes measured 3 hr after the last dose in the Martínez et al. (2018) paper. However, these effects are of uncertain toxicological significance due to the single time point, lack of dose-response in some cases, and/or lack of consistency across neurotransmitters and brain regions. Although there are important experimental differences (e.g., dose regimen and timing after sacrifice), it is notable that Hernandez-Plata et al. (2015) found no marked effects on 5-HT or DA in the striatum, in contrast to the effects that Martínez et al. (2018) reported, suggesting the likely reversibility of these neuropharmacological effects.

The NOEL for neuropathology of multiple areas of the dopamine system in the brain evaluated by Hernández-Plata et al. (2015) was greater than 150 mg/kg (i.p.).

Subchronic (repeat dose 1-3 months) neurotoxicity

Two subchronic dietary neurotoxicity studies were conducted in a manner aligned with regulatory guidelines for neurotoxicity testing (US EPA 1998b; OECD 1997) involving perfusion fixation, plastic embedding of peripheral nerves and a survey of sections throughout the central and peripheral nervous system (Horner 1996b; SafePharm 2006). There were no histopathological findings measured at the highest intake levels tested (1547–1631 mg/kg/day for Horner 1996b; 1499–1555 mg/kg/day for SafePharm 2006). Taken together, the NOEL for subchronic neuropathology evaluation is greater than 1590 mg/kg, the highest dose level tested. There was a significant elevation (3%) in total brain weight noted at the highest intake level of 1499–1555 mg/kg/day (SafePharm 2006), but these levels were not considered adverse by the investigators and the brain-to-body ratio was within the historical control range.

Chronic (repeat dose 1 year) neurotoxicity

Two chronic regulatory dietary studies included standard histopathologic evaluation of brains, spinal cord, sciatic and optic nerve in rats following 1 and 2 years of exposure (Brammer 2001; Nufarm 2009). One of these two chronic studies evaluated rats in all four dose groups (0, 121–145, 361–437, and 1214–1498 mg/kg/day) at both sacrifice times (Brammer 2001). The other chronic study evaluated only the high dose (1077–1382 mg/kg/day) and control groups consistent with guideline recommendations to only evaluate lower doses if there were findings at the highest dose level (Nufarm 2009). The sample size for the 1-year timepoint was 21 rats/sex at the high dose in the Nufarm (2009) study. The sample sizes at the 2-year sacrifice times ranged from 34 to 45 in the Nufarm (2009) study, and 16–39 in the Brammer (2001) study. The NOEL for these studies was determined to be the highest intake levels tested, 1077–1498 mg/kg/day, as no marked effects were observed at those levels. The neuropathological evaluations of these studies were limited compared to the experiments conducted according to neurotoxicity guidelines. However, the very large sample size and chronic duration of exposure provide further supportive evidence that glyphosate did not produce standard neuropathological changes following repeated oral exposures at doses up to 1500 mg/kg/day.

Summary of neuropathology/pharmacology data

Taken in concert, data from the studies evaluated indicate an oral NOEL for neuropathology up to 2000 mg/kg based upon acute neurotoxicity rat study that were conducted in a manner consistent with US EPA and OECD neurotoxicity guidelines (US EPA 1998b; OECD 1997) and in which no significant effects were observed at the highest dose tested. One published study reported neuropharmacological effects at a single dose of 150 mg/kg i.p. As discussed above (behavioral section), i.p. doses result in higher absorption and peak levels compared with oral, and equivalent doses cannot be determined.

The NOEL for repeat-dose effects on or neuropathological evaluations consistent with US EPA and OECD neurotoxicity guidelines (US EPA1998b; OECD 1997) is 1077–1555 mg/kg/day in dietary subchronic and chronic studies based upon no marked effects observed at the highest doses tested. A lower NOEL of 35 mg/kg/day by oral gavage was reported by Martínez et al. (2018) based upon neuropharmacological alterations, but there were no behavioral endpoints with which to compare to other studies, as these were only measured a few hr after six daily doses (suggesting acute effects), and biological significance and adversity of relatively small and transient neurotransmitter and metabolite changes is unknown. Further, data in both published studies demonstrated some inconsistencies and lack of dose-response in some measures.

Developmental neurotoxicity

There were two DNT studies of glyphosate alone (Luna et al. 2021; Pu et al. 2021) (Table 1); however, in both papers, the litter was not clearly confirmed and/or indicated as the statistical unit of measurement in the methods or results for each endpoint. Therefore, these studies were considered of low reliability and not further assessed (see methodological deficiencies discussed above).

One paper (Dechartres et al. 2019) administered glyphosate alone (as well as formulated glyphosate) during development, but investigated only maternal behavior of the dams, with no assessments of the offspring. These maternal behaviors (time spent licking pups, building the nest, time off nest, as well as arched-back, passive, and blanket nursing) are greatly influenced by endocrine status and are not comparable to the neurobehavioral endpoints described above, nor are they applicable for neurotoxicological risk assessment purposes. Therefore, this paper was considered not relevant to this review, although it is notable that glyphosate alone exerted no significant effects on these outcomes.

Glyphosate formulations review

Seventeen formulation studies were selected for full-text review based upon the modified third-pass criteria for formulation investigations described in the Methods. Note that one of the studies also tested glyphosate alone and is described above (Dechartres et al. 2019). It is important to note that since none of the glyphosate formulation investigations contained

Table 3. Neurobehavioral locomotor activity (MA) and functional observational battery (FOB) effects for glyphosate studies.

Reference Species	Exposure/observation/sample size	Methodology description	Behavioral observations	General toxicity
Acute dose				
Hernández-Plata et al. (2015) Rat (Sprague-Dawley) Male	0, 50, 100, 150 mg/kg/d via i.p. injection **Acute Exposure**: First injection (total, three injections a wk for two wks) **Observations**: MA session conducted 15 min before and 3 hr period after injection n = 16–17/dose	**MA**: Automatic recording system in square chambers surrounded by horizontal and vertical infrared beams	**50, 100, and 150 mg/kg**: ↓ in total distance, vertical activity, and stereotypy 3 hr after injection	Body weight not reported after a single dose
Horner (1996a) Rat (Alpk: APfSD) Male and female	0, 500, 1000 and 2000 mg/kg via oral gavage **Exposure**: Single dose (young adult) **Obs. timepoints**: 1 week before exposure and 6 hr (day 1), 7 d (day 8) and 14 d (day 15) after exposure n = 10/sex/dose	**MA**: Automated activity recording apparatus; 50 min session in 10 5-min intervals; treatment groups were counterbalanced **FOB**: Standardized observations plus landing foot splay; tail-flick test; fore-and hindlimb grip strength; observations were made by an observer that was blind to animals treatment	**MA**: **500 and 1000 mg/kg**: no difference from controls **2000 mg/kg**: *Day 1*: trend towards decreased overall MA but not statistically significant *Day 15*: ↓ overall male MA **FOB**: **2000 mg/kg**: ↓ male landing foot splay (6 hr) (similar to pre-test data) **500 mg/kg**: [&&] ↑ male forelimb grip strength (15 d)	**500, 1000 mg/kg**: no effect **2000 mg/kg**: ↓ in food consumption; ↑ general clinical signs of toxicity; ↑ mortality (one female rat)
Subacute repeat dose				
Hernández-Plata et al. (2015) Rat (Sprague-Dawley) Male	0, 50, 100, 150 mg/kg/d via i.p. injection **Exposure**: Three injections a week for two weeks **Observations**: MA session conducted 15 min before and 3 hr period after each injection; also 2 and 16 d after last injection n = 16–17/dose/timepoint or n = 6–7/dose (16 d post-treatment)	**MA**: Automatic recording system in square chambers surrounded by horizontal and vertical infrared beams	**50 mg/kg/d**: ↓ in total distance, vertical activity, and stereotypy 3 hr after injection; no differences 15 min before each daily dose or at 16 d after last treatment injection **100 mg/kg/d**: ↓ in total distance, vertical activity, and stereotypy 3 hr after injection and 2 d after last injection; ↓ in total distance, vertical activity 15-min before injection for some later sessions; no difference 16 d after last treatment injection **150 mg/kg/d**: ↓ in total distance, vertical activity, and stereotypy 3 hr after injection and 2 d after last injection; ↓ in total distance, vertical activity 15 min before injection for later sessions; no difference 16 d after last treatment injection	**50, 100, and 150 mg/kg/d**: no bodyweight differences 2 d or 16 d after last treatment injection
Subchronic repeat dose				
SafePharm (2006) Rat (Sprague-Dawley) Male and female	0, 1000, 5000, and 20,000 ppm via diet **Male**: 77, 395, and 1499 mg/kg/d **Female**: 78, 404, and 1555 mg/kg/d **Exposure**: 90 d (young adult) **Obs. timepoints**: 3, 5, 9 and 13th wk of exposure n = 10 /sex/dose	**MA**: Automated infrared beam activity monitors; 30-min test session **FOB**: Standardized observations, forelimb and hindlimb grip strength	**MA**: **77 mg/kg/d**: [&&] ↑ male overall activity (wk 3 and wk 5) **395 mg/kg/d**: [&&] ↓ male overall activity (wk 13) **1499 mg/kg/d**: no change in overall activity **78–1555 mg/kg/d**: [&&] ↑ female overall activity in females (wk 9 and wk 13) (no dose response relationship) **FOB**: No changes	**77–395 mg/kg/d (males) and 78–404 mg/kg/d (females)**: no differences with bodyweights, food consumption, and food utilization **1499 mg/kg/d (males)**: ↓ male bodyweight gain and food intake **1555 mg/kg/d (females)**: ↓ bodyweight gain

(*Continued*)

Table 3. (Continued).

Reference Species	Exposure/observation/sample size	Methodology description	Behavioral observations	General toxicity
Horner (1996b) Rat (Alpk: APfSD) Male and female	0, 2000, 8000, and 20,000 ppm via diet **Males**: 0, 155.5, 617.1, 1546.5 mg/kg/d; **Females**: 0, 166.3, 672.1, 1630.6 mg/kg/d **Exposure**: 90 d (young adult) **Obs. timepoints**: 1 week before exposure and 5, 9, and 14th week of exposure n = 12 /sex/dose	**MA**: Automated activity recording apparatus 50 min session in 10 5-min intervals; treatment groups were counterbalanced **FOB**: Standardized observations plus landing foot splay; tail-flick test; fore-and hindlimb grip strength[†]; observations were made by an observer that was blind to animals treatment	**MA**: **155.5 mg/kg/d**: [&&] ↑ male MA (wk 9) **FOB**: **672.1 mg/kg/d**: [&&] ↓ female tail flick latency (wk 5); [&&] ↑ female forelimb grip strength (wk 5)	**155.5–617.1 mg/kg/d (males) and 166.3–1630.6 (females)**: no differences with bodyweights, food consumption, and food utilization **1546.5 mg/kg/d (males)**: ↓ male bodyweight gain and food utilization
Chronic repeat dose				
Brammer (2001) Rat (Alpk: APfSD) Male and female	0, 2000, 6000, and 20,000 ppm via diet **Males**: 0, 121, 361, 1214 mg/kg/d; **Females**: 0, 145, 437, 1498 mg/kg/d **Exposure**: 52 wk **Obs. timepoints**: 52nd wk n = 11–12 /sex/dose	**MA**: Automated activity recording apparatus; 50 min session in 10 5-min intervals; treatment groups were counterbalanced **FOB**: Standardized observations plus landing foot splay; tail-flick test; fore-and hindlimb grip strength; observations were made by an observer that was blind to animals' treatment	**MA**: No changes **FOB**: **437 mg/kg/d**: [&&] ↓ female landing foot splay **1498 mg/kg/d**: [&&] ↓ female tail flick	**121–361 (males) and 145–437 (females) mg/kg/d**: no differences from controls on bodyweight, food consumption, organ weights, hematology, clinical chemistry, organ weights or histopathology **1214 mg/kg/d (males)**: ↓ bodyweights and food consumption; ↓ liver relative weights; ↑ kidney and liver histopathological effects **1498 mg/kg/d (females)**: ↓ bodyweights and food consumption
Nufarm (2009) Rat (Wistar Han) Male and female	0, 1500, 5000, and 15,000/24,000 (increased over the first yr) ppm glyphosate (97.6% purity) via diet **Males**: 85.5, 285.2, 1077.4 mg/kg/d **Females**: 104.5, 348.6, 1381.9 mg/kg/d **Exposure**: 52 wk **Obs. timepoints**: weekly; Week 51 n = 12 /sex/dose	**MA**: Automated infrared beam activity monitors; 30-min session **FOB**: Observations weekly for first yr, plus sensory observations, forelimb and hindlimb grip strength at 51 wk	**MA and FOB**: **85.5–1381.9**: no difference from controls	**1077.4 mg/kg/d;** ↓ in male body weight gain (9%); ↑ kidney histopathological effects

i.p.: intraperitoneal; MA: motor activity; FOB: functional observational battery

[&&] Considered spurious, non-treatment related finding as the effect was transient, there was no dose–response relationship, unexpected direction of change, and/or small magnitude of change (see text for more details)

Table 4. Neuropathology and neurochemical effects for glyphosate studies.

Reference Species	Exposure/observation/ sample size	Methodology description	Observations	General toxicity
Acute dose				
Hernández-Plata et al. (2015) Rat (Sprague-Dawley) Male	0, 150 mg/kg/d via i.p. injection **Acute Exposure**: First injection (total, three injections a wk for two wks) **Observations**: 5–6/dose	DA release through simultaneous microdialysis in striatum and NAcc	**150 mg/kg**: ↓ DA in striatum at 20 min, 2 hr after i.p. dose; ↓ DA release when stimulated with high K + solution **LOEL < 150 mg/kg i.p.**	Body weight not reported after a single dose
Horner (1996a) Rat (Alpk: APfSD) Male and female	0, 500, 1000 and 2000 mg/kg via oral gavage **Exposure**: Single dose (young adult) **Obs. timepoints**: 14 d after dose (day 15) 5/dose/sex	Perfusion fixation brain, spinal cord, gasserian ganglia, sciatic, sural, tibial nerves, dorsal root ganglia, and spinal root	No changes in any group **NOEL >2000 mg/kg**	**500, 1000 mg/kg**: no effect **2000 mg/kg**: ↓ in food consumption; ↑ general clinical signs of toxicity; ↑ mortality (one female rat)
Subacute repeat dose				
Hernández-Plata et al. (2015) Rat (Sprague-Dawley) Male	0, 50, 100, 150 mg/kg/d via i.p. injection **Exposure**: Three injections a wk for two weeks **Observations**: 2 and 16 days after last treatment 5–10/dose	2 and 16 days post-treatment: TH, DA, DOPAC, 5-HT, 5-HIAA in striatum and NAcc (n = 9–10) D1 and D2-DA receptor binding in striatum and NAcc (n = 6)	2 days: **50, 100, 150 mg/kg/d**: decreases in specific binding of antagonist to D1-DA receptors in NAcc, but no changes in other neurochemical or neuropathology endpoints No changes in other endpoints 16 days: No changes **LOEL<50 mg/kg/d i.p. 2 d NOEL>150 mg/kg/d i.p. 16 d**	No bodyweight differences 2 d or 16 d after last treatment injection
Martínez et al. (2018) Rat (Wistar) Male	0, 35, 75, 150, 800 mg/kg/d via oral gavage **Exposure**:6 daily doses **Observations**: Sacrifice 3 hr after last dose. N = 6/dose	DA, NE, 5-HT, and their metabolites in hypothalamus, midbrain, hippocampus, striatum, prefrontal cortex	**800 mg/kg/d**: ↓ 5-HT, DA, MHPG (striatum, hippocampus, prefrontal cortex, hypothalamus, midbrain), ↓ 5-HIAA (hippocampus), ↓ DOPAC (hippocampus, hypothalamus), ↓ HVA (prefrontal cortex, hypothalamus, midbrain), ↓ NE (striatum, hippocampus, prefrontal cortex, midbrain) **150 mg/kg/d**: ↓ 5-HT (striatum, hippocampus, prefrontal cortex), ↓ DA (prefrontal cortex, midbrain), ↓ DOPAC (hypothalamus), ↓ HVA (hypothalamus, midbrain), ↓ NE (striatum, hippocampus, midbrain), ↓ MHPG (hippocampus) **75 mg/kg/d**: ↓ 5-HT (striatum), ↓ DA (prefrontal cortex, midbrain), ↓ HVA (hypothalamus), ↓ NE (striatum, midbrain) **NOEL 35 mg/kg/d oral gavage**	Not evaluated
Subchronic repeat dose				
SafePharm (2006) Rat (Sprague-Dawley) Male and female	0, 1000, 5000, and 20,000 ppm via diet **Male**: 77, 395, and 1499 mg/kg/d **Female**: 78, 404, and 1555 mg/kg/d **Exposure**: 90 d (young adult) **Obs. timepoints**: end of exposure n = 5 /sex/dose	Perfusion fixation brain, spinal cord, peripheral nerves, dorsal root ganglia, and spinal root	No effects on any measure **NOEL >1555 mg/kg/d dietary**	**77–395 mg/kg/d (males) and 78–404 mg/kg/d (females)**: no differences with bodyweights, food consumption, and food utilization **1499 mg/kg/d (males)**: ↓ male bodyweight gain and food intake **1555 mg/kg/d (females)**: ↓ bodyweight gain

(*Continued*)

Table 4. (Continued).

Reference Species	Exposure/observation/ sample size	Methodology description	Observations	General toxicity
Horner (1996b) Rat (Alpk: APfSD) Male and female	0, 2000, 8000, and 20,000 ppm via diet **Males**: 0, 155.5, 617.1, 1546.5 mg/kg/d; **Females**: 0, 166.3, 672.1, 1630.6 mg/kg/d **Exposure**: 90 d (young adult) **Obs. timepoints**: end of exposure n = 6 /sex/dose	Perfusion fixation brain, spinal cord, gasserian ganglia, sciatic, sural, tibial nerves, dorsal root ganglia, and spinal root	No effects on any measure **NOEL >1630.6 mg/kg/d dietary**	**155.5–617.1 mg/kg/d (males) and 166.3–1630.6 (females)**: no differences with bodyweights, food consumption, and food utilization **1546.5 mg/kg/d (males)**: ↓ male bodyweight gain and food utilization
Chronic repeat dose				
Brammer (2001) Rat (Alpk: APfSD) Male and female	0, 2000, 6000, and 20,000 ppm via diet **Males**: 0, 121, 361, 1214 mg/kg/d; **Females**: 0, 145, 437, 1498 mg/kg/d **Exposure**: 2 yr **Obs. timepoints**: 1 and 2 yr 1-yr: n = 11–12/dose/ sex 2-yr: n = 16–39/dose/ sex	Immersion fixation brain, spinal cord, sciatic nerve	No effects on any measure **NOEL >1498 mg/kg/d dietary**	**121–361 (males) and 145–437 (females) mg/kg/d**: no differences from controls on bodyweight, food consumption, organ weights, hematology, clinical chemistry, organ weights or histopathology **1214 mg/kg/d (males)**: ↓ bodyweights and food consumption; ↓ liver relative weights; ↑ kidney and liver histopathological effects **1498 mg/kg/d (females)**: ↓ bodyweights and food consumption
Nufarm (2009) Rat (Wistar Han) Male and female	0, 1500, 5000, and 15,000/24,000 (increased over the first yr) ppm glyphosate (97.6% purity) via diet **Males**: 85.5, 285.2, 1077.4 mg/kg/d **Females**: 104.5, 348.6, 1381.9 mg/kg/d **Exposure**: 2 yr **Obs. timepoints**: 1 and 2 yr 1-yr: 12/sex control; 21/sex high dose 2-yr: 34–45/sex/dose	Immersion fixation brain, spinal cord, sciatic nerve	No effects on any measure **NOEL >1230 mg/kg/d dietary**	**1229.7 mg/kg/d;** ↓ in male body weight gain; ↑ kidney histopathological effects

i.p.: intraperitoneal; NOEL: no observed effect level; LOEL: lowest observed effect level; NAcc: nucleus accumbens; DA: dopamine; TH: tyrosine hydroxylase; NE: norepinephrine; 5-HT: serotonin; DOPAC: dihydroxyphenylacetic acid; 5-HIAA: 5-hydroxyindolacetic acid; HVA: homovanillic acid; MHPG: 3-methoxy-4-hydroxyphenylglycol

a formulation vehicle group (i.e., formulation ingredients without glyphosate), none of them provide reliable scientific assessments of glyphosate alone. In most papers, investigators described the formulation dilutions and administered doses as glyphosate without specifying technical or salt form, and in a few papers preparation was not completely clear. In this review and tables, the administered doses are cited as described by the investigators. It should also be noted a variety of different formulations were used as the test material in these papers, including Roundup® (Roundup Original®), Roundup MaxLoad®, Roundup Transorb®, Gligloflex®, and Zamba®. Within the context of the limitations of formulation studies, the overall objective for reviewing these investigations was to determine if there was any clear neuropathology or behavioral pattern consistent with the active glyphosate literature, and whether the formulation data could be informative for potential human health outcomes evaluated in the epidemiological literature.

During review, it was noted that one formulation study, Ait-Bali et al. (2018), presented the same behavioral results that were presented in a prior study, Ait-Bali et al. (2017): while the data were transformed in the 2018 paper, some statistical values (F- and p-values) were

Table 5. Neurobehavioral locomotor activity (MA) effects for glyphosate formulation studies.

Reference Species	Exposure/observation/sample size	Methodology description	Behavioral observations	General toxicity
Acute dose				
Ait Bali, Ba-Mhamed, and Bennis (2017) Mouse (Swiss) Male	0, 250, 500 mg/kg glyphosate-based herbicide (Roundup: 360 g/L glyphosate) via oral gavage **Exposure**: Single dose (PND 30) **Obs. timepoints**: PND 31 n = 6/dose	**MA**: OF arena equipped with automated video-based tracking software; 20-min session	**250, and 500 mg/kg**: no difference from controls in males and females	No effect on body weight
de Oliveira Joaquim et al. (2012) Mouse (BALB/c) Male and female	0, 25, 50 mg/kg glyphosate (males only) 100 mg/kg glyphosate (males and females) (Roundup Transorb: 480 g/L glyphosate) via oral gavage **Exposure**: Single dose (PND 60) **Obs. timepoints**: every 15 min from 15 to 2 hr following treatment (males) 15 min following treatment (males and females) n = 8–12/dose	**MA**: Circular arena subdivided with floor markings; 3-min session	**Males only**: **25 mg/kg**: ↓ locomotion and rearing in first 15 min; ↑ locomotion at 120 min; ↓ immobility at 45–120 min **50 mg/kg**: ↓ locomotion at 15 and 30 min; ↓ immobility 45 min **100 mg/kg**: ↓ locomotion at 15 min; ↑ immobility at 30 min **Males and females**: **100 mg/kg**: ↓ female locomotion	Clinical observations scored but data not reliable
Subacute repeat dose				
Baier et al. 2017 Mouse (CF-1) Male	0, 50 mg/kg/d glyphosate (Glifloglex, 35.6% w/v glyphosate) via intranasal route **Exposure**: 3 times per week for 4 wk **Obs. timepoints**: 3 d before and once a week during administration as well as 3 and 10 d after last treatment (recovery period)	**MA**: OF automated video-based tracking software; 15-min session	**50 mg/kg/d**: ↓ distance traveled on day 1 and day 8; no significant changes at any other timepoint	**50 mg/kg/d**: weight and weight gain was similar to controls throughout treatment although an apparent decrease in bodyweight gain seems to occur after dosing ended (no statistical evaluation)
Bicca et al. (2021) Mouse (Swiss) Male	0, 50 mg/kg/d glyphosate-based herbicide solution (Zamba, 365 g/L glyphosate) via oral gavage **Exposure**: 30 d (young adult) **Obs. timepoints**: 26th day of exposure (before dosing) n = 10/dose	**MA**: OF arena subdivided with floor markings; 5-min session	**50 mg/kg/d**: no difference on number of crossings or rearing differences from controls	Not evaluated
de Oliveira Joaquim et al. (2014) Mouse (BALB/c) Male and female	0, 50 mg/kg/d glyphosate (Roundup Transorb, 480 g/L glyphosate) via oral gavage **Exposure**: 23 d (PND 23 to PND 45) **Obs. timepoints**: 10 d after end of treatment n = 11–14/sex/dose	**MA**: Circular arena subdivided with floor markings; 5-min session	**50 mg/kg/d**: ↓ locomotion in males; ↑ immobility in females	Not evaluated
Gress et al. (2016) Rat (Sprague-Dawley) Male	0, 135 mg/kg/d glyphosate-based herbicide (Roundup GT Plus: 450 g/L glyphosate) via drinking water **Exposure**: 8 d (PND 60 to PND 68) **Obs. timepoints**: End of treatment n = 24/dose	**MA**: Automated infrared photocell activity recording; 30-min session.	**135 mg/kg/d**: ↓ total, horizontal, and vertical activity	**135 mg/kg/d**: no differences with body weight, and food and water consumption
Subchronic repeat dose				
Ait Bali, Ba-Mhamed, and Bennis (2017) Mouse (Swiss) Male	0, 250, 500 mg/kg/d glyphosate-based herbicide (Roundup: 360 g/L glyphosate) via oral gavage **Exposure**: 42 d (PND 30–72); 90 d (PND 30–120) **Obs. timepoints**: 42 d (PND 73); 90 d (PND 121) n = 6/dose	**MA**: OF arena equipped with automated video-based tracking software; 20-min session	**42 d** **250 and 500 mg/kg/d**: ↓ total distance traveled and velocity **90 d** **250 and 500 mg/kg/d**: ↓ total distance traveled and velocity	**42 d** **250 mg/kg/d**: ↓ bodyweight gain **500 mg/kg/d**: ↓ bodyweight gain; ↓ liver rel. weight **90 d** **250 mg/kg/d**: ↓ bodyweight gain; ↓ kidney, liver and lung weights **500 mg/kg/d**: ↓ bodyweight gain; ↓ liver rel. weight; ↓ kidney, liver and lung weights

OF: open field; MA: motor activity; PND: postnatal day

Table 6. Neurobehavioral anxiogenic and depressive outcomes for glyphosate formulation studies.

Reference Species	Exposure/observation/sample size	Methodology description	Behavioral outcomes	General toxicity
Acute dose				
Ait Bali, Ba-Mhamed, and Bennis (2017) Mouse (Swiss) Male	0, 250, 500 mg/kg/d glyphosate-based herbicide (Roundup: 360 g/L glyphosate) via oral gavage **Exposure**: Single dose (PND 30) **Obs. timepoints**: PND 32–34 n = 6/dose	Endpoints were scored with automated video-based tracking software; **MA**: Activity in center of arena recorded; 20-min session **EPM**: 5-min session **TST**: suspended by the tail for 6- min session	**250 and 500 mg/kg**: no difference from controls	No effect on body weight
de Oliveira Joaquim et al. (2012) Mouse (BALB/c) Male and female	0, 100 mg/kg glyphosate (Roundup Transorb: 480 g/L glyphosate) mg/kg/d via oral gavage **Exposure**: Single dose (PND 60) **Obs. timepoints**: 15 min following treatment n = 8 /dose	**EPM**: 5- min session **TST**: suspended by the tail for 6-min session	**EPM**: **100 mg/kg**: no difference in anxiety index; ↓ male closed arm entries and central crosses **TST**: **100 mg/kg/d**: ↑ immobility	Clinical observations scored but data not reliable
Subacute repeat dose				
Baier et al. (2017) Mouse (CF-1) Male	0, 50 mg/kg/d glyphosate (Glifloglex, 35.6% w/v glyphosate) via intranasal route **Exposure**: 3 times per wk for 4 wk **Obs. timepoints**: 3 d before and once a wk during administration as well as 3 and 10 d after last treatment (recovery period) for OF; 12 d after last treatment for EPM n = 10/dose	Endpoints were scored with automated video-based tracking software; **MA**: Thigmotaxis in activity arena; 15-min session **EPM**: 5-min session	**MA**: **50 mg/kg/d**: ↑ in thigmotaxis in OF at some timepoints after administration but effect attenuated 10 d after last treatment **EPM**: **50 mg/kg/d:** ↓ total arm entries; ↑ in percentage of entries and time spent in closed arms	**50 mg/kg/d**: weight and weight gain were similar to controls throughout treatment although an apparent decrease in bodyweight gain seems to occur during the recovery period (no statistical evaluation)
Bicca et al. (2021) Mouse (Swiss) Male	0, 50 mg/kg/d glyphosate-based herbicide (Zamba, 365 g/L glyphosate) via oral gavage **Exposure**: 30 d (young adult) **Obs. timepoints**: 27th and 28th day of exposure (before dosing) n = 8/dose	**EPM**: 5-min session **FST**: placed mouse in cylinder containing water for 6 min (analysis was last 4 min)	**EPM**: **50 mg/kg/d**: ↓ open arms entries and time spent in open arm (no report of total entries or overall activity on assay) **FST**: **50 mg/kg/d**: ↑ immobility	Not evaluated
de Oliveira Joaquim et al. (2014) Mouse (BALB/c) Male and female	0, 50 mg/kg/d glyphosate mg/kg/d (Roundup Transorb, 480 g/L glyphosate) via oral gavage; **Exposure**: 23 d (PND 23 to PND 45) **Obs. timepoints**: 10 d after end of treatment n = 11–14/sex/dose	**EPM**: 5-min session **FST**: training session for five min in container filled with water and 24 hours later mouse placed back in container for 5 min and evaluated	**EPM**: **50 mg/kg/d**: no difference in time spent in open or closed arms; ↓ male entries in open and closed arms, center crossings **FST**: **50 mg/kg/d (female)**: ↑ latency to float; ↓ time of float **50 mg/kg/d (male)**: no difference from controls	Not evaluated
Subchronic repeat dose				
Ait Bali, Ba-Mhamed, and Bennis (2017) Mouse (Swiss) Male	0, 250, 500 mg/kg/d glyphosate-based herbicide (Roundup: 360 g/L glyphosate) via oral gavage **Exposure**: 42 d (PND 30–72); 90 d (PND 30–120) **Obs. timepoints**: 42 d (PND 73–74); 90 d (PND 121–122) n = 6/dose	Endpoints were scored with automated video-based tracking software **MA**: Time in center of activity arena; 20-min session **EPM**: 5-min session **TST**: suspended by the tail for 6-min session	**EPM and MA, 42 and 90 d** **250 and 500 mg/kg/d**: ↓ time spent in center in OF; ↓ ratio of time spent in open arms with EPM; ↑ anxiety index **TST**: **42 d, 250 and 500 mg/kg/d**: no change **90 d, 250 and 500 mg/kg/d**: ↑ immobility	**42 d** **250 mg/kg/d**: ↓ bodyweight gain **500 mg/kg/d**: ↓ bodyweight gain; ↓ liver rel. weight **90 d** **250 mg/kg/d**: ↓ bodyweight gain; ↓ kidney, liver and lung weights **500 mg/kg/d**: ↓ bodyweight gain; ↓ liver rel. weight; ↓ kidney, liver and lung weights

OF: open field; MA: motor activity; EPM: elevated plus maze; TST: tail suspension test; FST: forced swim test; PND: postnatal day

Table 7. Neurobehavioral cognitive outcomes for glyphosate formulation studies.

Reference Species	Exposure/observation/ sample size	Methodology description	Behavioral observations	General toxicity
Acute dose				
Ait Bali et al. (2019) Mouse (Swiss) Male	0, 250, 500 mg/kg/d glyphosate-based herbicide (Roundup: 360 g/L glyphosate) via oral gavage **Exposure**: Single dose (PND 30) **Obs. timepoints**: PND 31–36 n = 6/dose	**NOR**: 10 min familiarization session with 1 hour interval before 5 min recognition session **SA**: Y-maze for 8 min (no confirmation observer was blind to treatment) **PA**: First day was training exploratory session for 3 min; secondary shock training with dark box followed by a test session 2 and 24 hr after first shock trial	**250 mg/kg**: no difference on NOR discrimination index or SA; ↓ in latency for PA at 2 hr **500 mg/kg**: no effect on NOR discrimination index or SA; ↓ in latency for PA at 24 hr	Not evaluated (see Ait Bali, Ba-Mhamed, and Bennis 2017)
Subacute dose				
Baier et al. (2017) Mouse (CF-1) Male	0, 50 mg/kg/d glyphosate (Glifloglex, 35.6% w/v glyphosate) via intranasal administration **Exposure**: 3 times per wk for 4 wk **Obs. timepoints**: 4 d after last administration n = 10/dose	Endpoints were scored with automated video-based tracking software; **NOR**: 10 min familiarization session with 6 and 24 hr intervals before 5 min recognition session	**50 mg/kg/d**: marginal ↓ in time (<1 s) spent exploring novel object after 6 hr session; no difference at 24 hr	**50 mg/kg/d**: weight and weight gain were similar to controls throughout treatment although an apparent decrease in bodyweight gain seems to occur during the recovery period (no statistical evaluation)
Subchronic dose				
Ait Bali et al. (2019) Mouse (Swiss) Male	0, 250, 500 mg/kg glyphosate-based herbicide (Roundup: 360 g/L purity) via oral gavage **Exposure**: 42 d (PND 30–72); 84 d (PND 30–114) **Obs. timepoints**: 42 d (PND 73–78); 84 d (PND 115–120) n = 6/dose	**NOR**: 10 min training sessions with 1 hr interval before 5 min novel session **SA**: Y-maze for 8 min **PA**: First day was training exploratory session for 3 min in light-dark box; secondary shock training with dark box followed by a test session 2 and 24 hr after first shock trial	**42 d** **250 mg/kg/d**: ↓ NOR discrimination index; no difference on SA; ↓ in latency in PA (2 and 24 hr) **500 mg/kg/d**: ↓ NOR discrimination index; no difference on SA; ↓ in latency in PA (24 hr) **90 d** **250 mg/kg/d**: ↓ NOR discrimination index; ↓ on SA; ↓ in latency in PA (24 hr) **500 mg/kg/d**: ↓ NOR discrimination index; ↓ on SA; ↓ in latency in PA (2 and 24 hr)	Not evaluated (see Ait Bali, Ba-Mhamed, and Bennis 2017)

NOR: novel object recognition; SA: spontaneous alterations; PA: passive avoidance; PND: postnatal day

identical to those of the 2017 paper. Therefore, only the original data from Ait-Bali et al. (2017) were reviewed here, and the 2018 was not considered because it is duplicative data. In addition, another seven papers were excluded, mostly for the methodological deficiency issues described above (Table 2). While the behavioral and neuropathology endpoints in Gallegos et al. (2020) paper were considered unreliable (Table 2), the biochemical data including acetylcholinesterase (AChE) and glutamate transaminase data are adequate and are considered below. The remaining nine papers are reviewed here.

Adult neurotoxicity: Behavior

Acute (single dose) neurotoxicity

Three published papers included single-dose assessments of the formulation (Ait Bali, Ba-Mhamed, and Bennis 2017; 2019; de Oliveira Joaquim et al. 2012). All three studies used mice (males only in Ait Bali, Ba-Mhamed, and Bennis 2017; 2019, both sexes with some studies in de Oliveira Joaquim et al. 2012) and administered the dose orally. The two Ait Bali, Ba-Mhamed, and Bennis (2017); (2019)) experiments used higher doses (250 or 500 mg/kg), and although there were reportedly no acute effects on body weight, it

is notable that when those doses were administered repeatedly, there were significant effects on body weight gain, suggesting an overt toxic effect. There are no data in de Oliveira Joaquim et al. (2012) (doses: 25–100 mg/kg) regarding body weight. The behavioral tests were conducted shortly after the acute dose (15–120 min) in de Oliveira Joaquim et al. (2012), whereas testing started the day after dosing and continued for several days in Ait Bali, Ba-Mhamed, and Bennis (2017); (2019)).

Two of the studies conducted MA tests, with differing results (Table 5). There were no marked effects in Ait Bali, Ba-Mhamed, and Bennis (2017) on the day after dosing, which may not be surprising given the rapid recovery from a single dose of glyphosate (ATSDR (Agency for Toxic Substances and Disease Registry) 2020; Brewster, Warren, and Hopkins 1991). In contrast, de Oliveira Joaquim et al. (2012) reported decreased locomotion in male mice at 15 min and reduced immobility (which actually reflects higher activity) beginning at 30 min. A dose–response relationship was not evident in terms of group means, pattern of effect, or direction of change. de Oliveira Joaquim et al. (2012) repeated the MA test in both male and female mice (100 mg/kg only), but in this second study, the fall in MA at 15 min was only seen in females and not males.

The same two studies evaluated anxiety with the elevated plus maze (EPM), and depression using a tail suspension test (Table 6). Based upon a calculated anxiety index, de Oliveira Joaquim et al. (2012) reported no signs of anxiety. Ait Bali, Ba-Mhamed, and Bennis (2017) also noted no marked effect on EPM. In the tail suspension test, de Oliveira Joaquim et al. (2012) found increased immobility in both sexes, suggesting a depressive state, whereas Ait Bali, Ba-Mhamed, and Bennis (2017) reported no effect.

Cognitive behavior was evaluated only in one study (Ait Bali et al. 2019); (Table 7). Following acute exposure, there were no significant effects on novel object recognition test (NOR) or Y-maze performance. In contrast, when tested 4–6 days after the dose, the latency to cross in the passive avoidance (PA) test was shortened in the low-dose group at 2 hr after initial training, and in the high-dose group at 24 hr. While this suggests some cognitive effect after a single dose, there are no other PA studies with which to compare or integrate these data. Since the same rats had been tested with the NOR and Y-maze in the preceding days, with no treatment-related effects, the singular finding of a selective cognitive effect needs to be independently replicated.

A series of spontaneous behavioral observations were recorded in de Oliveira Joaquim et al. (2012), but there is no mention that the observer was blinded with respect to the treatment, which is necessary for evaluations consisting of subjective scores. For this reason, the data are considered unreliable and not discussed here.

In summary, the report of decreased MA shortly after dosing (de Oliveira Joaquim et al. 2012) is reminiscent of the acute MA depressive effects following a single dose of glyphosate alone; however, the formulation paper also noted increased activity at later times after dosing. Given the lack of dose-response and consistency of effects in the de Oliveira Joaquim et al. (2012) data, and no other supporting papers, there is less confidence in those findings. There is also no support to suggest anxiogenic actions, and only one paper showed data suggestive of depressive behaviors. Although the one paper (Ait Bali et al. 2019) reported a specific cognitive change, replication is needed to verify this selective effect.

Subacute (short-term repeat dose ≤1month) neurotoxicity

There were five studies of glyphosate formulation administered repeatedly for a month or less (Baier et al. 2017; Bicca et al. 2021; de Oliveira Joaquim et al. 2014; Gallegos et al. 2020; Gress et al. 2016), 4 of which used mice and only one of which used both sexes (Table 2). The de Oliveira Joaquim et al. (2014) investigation focused on sexual dimorphism in some behaviors, but such comparisons are not relevant here and only the results for treatment compared with control of the same sex are discussed in this review. The only behavioral evaluation in Gallegos et al. (2020) was a FOB conducted 2 weeks after dosing. There is no mention of the observers being blinded to treatment (necessary for

subjective scoring), and while there were no marked findings, data are considered unreliable and not reviewed here. In the remaining four studies, there was either no significant impact on body weight or else it was not mentioned by the investigators. Comparisons across these studies were made difficult by the differing exposure scenarios (intermittent or continuous doses over 8, 23, or 30 days), routes of administration (i.p., intranasal, drinking water, oral gavage), age (adolescents, adults), and formulation products.

MA was assessed in four of these studies (Table 5), all of which used different formulations. One study used rats (Gress et al. 2016) that received diluted formulation in drinking water for 8 days (approximately 135 mg/kg/day); treated rats exhibited decreased horizontal and vertical activity at the end of treatment. The remaining studies used mice at the same dose level (50 mg/kg/day), but various routes of administration. Bicca et al. (2021) reported no marked effect on MA near the end of 30 days of oral gavage. In de Oliveira Joaquim et al. (2014), adolescent mice were tested 10 days after 23-day oral gavage, resulting in diminished locomotion in males and enhanced immobility in females. Using intranasal instillation, Baier et al. (2017) noted that distance traveled was reduced in the first 2 weeks of dosing and immobility was enhanced in the last week of dosing. Thus, there was general but not complete agreement in the report of lowered MA following various routes of administration of glyphosate formulations. Since there were differences in exposure scenarios, formulations, and use of one or both sexes, and all studies employed only one dose, conclusions regarding effective doses or time course over subacute dosing cannot be made.

Three of these studies evaluated anxiety behaviors, all in mice (Table 6). de Oliveira Joaquim et al. (2014) noted reduced center crossings in males only, and no effect on anxiety behavior, suggestive of motor effects (also seen as diminished locomotion in the open field). Bicca et al. (2021) found diminished time and entries in the open arms but did not include a measure of general activity. Baier et al. (2017) reported decreased % time and entries into the open arms time and entries as well as enhanced thigmotaxis in the activity testing; however, these effects are confounded by fewer total arm entries and lower activity levels. Thus, while two studies suggest anxiogenic effects, there was also evidence of motor effects that might influence and confound these conclusions.

Depressive behaviors were evaluated in two studies using a forced swim test (Table 6). While Bicca et al. (2021) reported increased immobility time, the opposite result was found by de Oliveira Joaquim et al. (2014), who noted reduced float time and elevated latency to float in male but not female mice.

Only one study included a test of cognitive function (Table 7). Using a NOR paradigm, Baier et al. (2017) reported a decreased tendency to explore a novel object at 6 hr; however, the effects were marginal, and exploration differences were less than 1–2 sec different. A preference for the novel object was seen equally in both groups at 24 hr. This finding, where recognition memory is impaired at six but not 24 hr, is not expected and precludes any strong conclusions regarding cognitive function.

Overall, there was consistency in findings of decreased MA with subacute dosing of various glyphosate formulations, but differences in exposure, formulations, use of one or both sexes, etc., confound further interpretation. There is less confidence in conclusions regarding anxiety, depression, or cognitive changes.

Subchronic (repeat dose 1-3 months) neurotoxicity

Two papers from the same lab evaluated effects of glyphosate formulation in male mice with exposures that lasted more than a month (Ait Bali, Ba-Mhamed, and Bennis 2017; Ait Bali et al. 2019). These experiments used mice dosed once (see acute exposures), or else daily for 6 or 12 weeks. There is, however, an inconsistency in the longest duration, which is stated in Ait Bali, Ba-Mhamed, and Bennis (2017) to be 12 weeks but the experimental design figure shows 90 days, which is actually 13 weeks. The similar figure in Ait Bali et al. (2019) showed 84 days, which is indeed 12 weeks. For simplicity, in this review the

longest duration was considered to be 12 weeks, although the exact duration is unclear. In both papers, behavioral testing took place over a few days after the last dose. The two papers did not measure the same behaviors, so there can be no direct comparisons.

Ait Bali, Ba-Mhamed, and Bennis (2017) reported body weight effects with both doses, suggesting an overt toxic effect. There was weight loss within the first two weeks of dosing with both dose levels. This reversed somewhat with continued dosing, such that at the end of subchronic dosing (6 week) the weight gain was 53 and 21% of control values in the 250 and 500 mg/kg/day dose groups, respectively, and with longer dosing (12 week) weight gain was 67 and 64% of control values for the 250 and 500 mg/kg/d groups, respectively. Thus while there was some recovery in body weight, all treated mice remained significantly lower than controls. The Ait Bali et al. (2019) investigation did not report body weight, but similar effects may be assumed based upon similarities in test article and dosing paradigm. Such a significant effect on body weight indicates general toxicity that may impact subsequent behavioral, neuropharmacological and pathological assessments.

Ait Bali, Ba-Mhamed, and Bennis (2017) measured MA, EPM, and a tail suspension test (Tables 5 and 6) and generally found reduced activity at both doses and both testing times. Data from the EPM demonstrated a decreased ratio for time in the open arms at both times and both doses, but the number of open arm entries was not altered. The time immobile in the tail suspension test was elevated at both doses only at 12 weeks. Thus, the finding of lower MA was similar to reports following subacute exposures. While anxiogenic and depressive effects were noted in this study, similar findings were not consistently seen with subacute dosing.

Cognitive function was the focus of the Ait Bali et al. (2019) experiment. Using the NOR test, there was less relative time with the novel object at both doses at 6 and 12 weeks. Spontaneous alternation (%) was decreased in the Y-maze at both doses at 12 weeks only. In a PA test, there was reduced latency to cross 2 hr after training in the low-dose group at 6 weeks, and in the high dose at 12 weeks. The day after training, the diminished latency was evident at both doses at 6 and 12 weeks. On most of these measures there was no dose–response evident. It is important to note that these tests have a motor component, and a generalized decrease in activity may confound the data. Activity measures were not reported in these tests (e.g., total time exploring in the NOR), resulting in some uncertainty in the specificity of the cognitive changes.

Chronic (repeat dose 1 year) neurotoxicity

There were no formulation studies with exposure lasting longer than 3 months.

Summary of behavioral data

Across these formulation studies, all doses exerted marked effects on these measure at some time. The lowest dose used (50 mg/kg/day) produced a number of changes when administered repeatedly. However, given the numerous experimental differences including formulation product, route, strain/species, and test times as well as the potential influences of formulation vehicles on the potency and kinetics of glyphosate, such dose specification is not helpful for a quantitative assessment of glyphosate alone.

MA was the most frequently used test in these glyphosate formulation papers, and the changes were mostly seen as decreased activity. After a single dose, MA changes were transient in that effects were seen shortly after dosing but not the next day (Ait Bali, Ba-Mhamed, and Bennis 2017; de Oliveira Joaquim et al. (2012). When administered repeatedly, MA was lowered, regardless of test species, dose, route, dose frequency, or time of testing, in most studies (Ait Bali, Ba-Mhamed, and Bennis 2017; Baier et al. 2017; Gress et al. 2016; de Oliveira Joaquim et al., 2014), with one exception (Bicca et al. 2021). Thus, the most consistent finding across the formulation studies from most labs was reduced MA.

Several papers included tests intended to assess anxiety (EPM, open field central tendencies; Ait Bali, Ba-Mhamed, and Bennis 2017; Baier et al. 2017; Bicca et al. 2021; de Oliveira Joaquim et al. 2014) and/or depressive behaviors (forced swim, tail suspension, splash test, sucrose preference; Ait Bali, Ba-Mhamed, and Bennis 2017; Bicca et al.

2021; de Oliveira Joaquim et al. 2012; 2014). The only reports of increased anxiety behavior involved repeated exposures, although in several of these there was concurrent decreased MA, which confound the maze behavior. In contrast, acute studies and another repeated dose study displayed no marked effects on the EPM (Ait Bali, Ba-Mhamed, and Bennis 2017; de Oliveira Joaquim et al. 2014). Depressive behaviors were included in five papers, but four different tests were used and no more than two papers assessed the same behavior. As such, comparisons across studies are more difficult. Most investigations suggested increased depressive behaviors, except for one paper that reported less depression, and again motor decreases may have impacted the results.

Learning and memory was examined using several different tests (NOR, PA, Y maze), each of which tap somewhat different cognitive processes and are not fully comparable. Cognitive data were only evaluated in two labs and effects were reported on all tests. Effects were noted with PA after a single dose, and all three tests with repeated exposures (Ait Bali et al. 2019; Baier et al. 2017). In all cases, however, these changes were evident in animals that exhibited decreased MA, suggesting that at least some of the outcomes might be influenced by lowered activity and not cognitive function *per se*. In addition, in some cases, the changes were not in the expected direction or the magnitude of change was extremely small. Thus, there is low confidence in conclusions regarding cognitive changes.

Adult neurotoxicity: Neuropathology/pharmacology and other biochemical endpoints

Acute (single dose) neurotoxicity

There were no studies of neurochemical or neuropathological evaluations following a single dose of glyphosate formulation.

Subacute (short-term repeat dose ≤1 month) neurotoxicity

Two studies evaluated neurological endpoints following repeated doses of a month or less. Gallegos et al. (2020) dosed mice intranasally with glyphosate formulation three times per week for 4 weeks (approximately 50 mg/kg/day glyphosate acid based on authors' estimate). Fifteen days after the last day of exposure, AChE activity, total thiol content, catalase (CAT) activity and glutamate oxaloacetate transaminase (GOT), and glutamate pyruvate transaminase (GPT) activities were measured in six brain regions. AChE activity was decreased in olfactory bulb, the brain area with the lowest control AChE activity level, but not in any of the other five brain regions measured. Total thiol content, a crude measure of oxidative stress was highly variable and demonstrated no significant alterations in certain regions. Similarly, CAT activity was highly variable and not significantly affected in several areas. Of note, these regions did not entirely overlap and CAT generally increases, rather than decreases, in an oxidative stress environment. GPT was reduced in the prefrontal cortex and elevated in the caudate putamen, whereas GOT was reduced in the prefrontal cortex and hippocampus. Note that the neuropathological assessments in this study failed to meet the inclusion criteria and were excluded from review (see methodological deficiencies above). This study is limited because only one dose level was tested and statistical significance was based upon a crude comparison without correction for a large number of tests conducted. Further, many of the reported statistically significant differences are based upon highly variable data with small differences of unknown biological significance.

Bicca et al. (2021) dosed mice orally with 50 mg/kg glyphosate formulation for 30 days, and on the next day hippocampi were removed for measurements of AChE activity and reactive oxygen species (ROS) levels. In addition, hippocampal sections were stained with hematoxylin and eosin and analyzed for histopathologic findings. There were no marked changes in AChE activity and no histopathologic findings. With regard to ROS levels, there was a significant rise in 2, 7-dihydrodichlorofluorescein diacetate fluorescence associated with glyphosate formulation treatment that was accompanied by a decrease in reduced glutathione (GSH), as measured with Ellman's reagent. There was no significant effect on total antioxidant status based upon the ferric reducing antioxidant potential assay. It should be noted that the use of these assays

for ROS detection with *ex vivo* samples from treated animals are relatively inconsistent and not state of the art for detecting ROS production (Dikalov and Harrison 2014).

Subchronic (repeat dose 1-3 months) neurotoxicity

Two papers from the same lab measured brain AChE and antioxidant enzyme activities (Ait Bali et al. 2019) as well as performed TH and 5-HT immunostaining (Ait Bali, Ba-Mhamed, and Bennis 2017) in mice following 6 and 12 weeks of daily oral gavage doses of 250 or 500 mg/kg/day glyphosate formulation. AChE activity was reduced at both dosing durations in whole brain and prefrontal cortex, and after 12 weeks of dosing only in hippocampus, whereas superoxide dismutase (SOD) and peroxidase activities were decreased mostly by both doses at both durations (Ait Bali et al. 2019). A limitation of this paper is that the data was only expressed as % control without providing the actual control enzyme activity levels. Ait Bali, Ba-Mhamed, and Bennis (2017) reported a dose-related decrease in TH densitometry in the substantia nigra pars compacta (SNpc) after 12 weeks of exposure using immunohistochemical techniques. 5-HT immunoreactivity was lowered in both rostral and caudal parts of the dorsal raphe nucleus (DRN), the medial prefrontal cortex (mPFC) and amygdala. These serotonergic decreases occurred consistently in all areas at the 500 mg/kg/day dose level following both 6 and 12 weeks of exposure, but also were detected at the 250 mg/kg/day in the DRN and amygdala. Both doses also produced significant evidence of general toxicity as evidenced by marked reduction in body weight gain (described above) and organ weights (brain, kidneys, liver, and lung). This makes interpretation of any specific effects exceedingly difficult.

Summary of neuropathology/pharmacology/biochemistry Data

In one study (Ait Bali, Ba-Mhamed, and Bennis 2017), dose-related decreases of TH^+ and 5-HT immunoreactivity were measured following subacute or subchronic exposures to high dose (250 and 500 mg/kg/day) glyphosate formulation. In another experiment (Ait Bali et al. 2019), a dose-related decrease in AChE activity in the brain were observed at the same doses. This, however, has less relevance since severe toxicity was seen at the same doses (Ait Bali, Ba-Mhamed, and Bennis 2017). On the other hand, another repeated oral dosing study with a lower dose did not significantly alter AChE activity (Gallegos et al., 2020).

Developmental neurotoxicity

Of the six developmental-exposure studies of glyphosate formulations, four exhibited behavioral, neuropathology, and/or neuropharmacological endpoints in which litter was not clearly confirmed and/or indicated as the statistical unit of measurement in the methods or results for each endpoint (Ait-Bali et al. 2020; Cattani et al. 2014, 2017; Pu et al. 2020b) (Table 2). Therefore, these studies were considered of low reliability and not further assessed (see methodology deficiencies, above).

The remaining two formulation developmental studies, from the same lab, were considered acceptable (Gallegos et al. 2018, 2016), as one male and one female from each litter were randomly assigned to the tests. The ANOVAs used sex as a factor, which implies that littermates were not kept separate; however, these investigators clearly state that the litter was used as the statistical unit, and it was decided to accept that at face value.

Behavior

In the two Gallegos studies (Gallegos et al. 2018, 2016), formulation was diluted to 0.2% and 0.4% in drinking water and given to female Wistar rats from conception to weaning of the pups at postnatal day 21. These investigators stated that this is equivalent to doses of 100 and 200 mg/kg/day in the dam. Although it was only indicated that the pregnant dams were exposed, it was not stated if pups were restricted from the drinking water source which was available *ad libitum* to the dams in the days leading up to weaning. Further, Gallegos et al. (2016) showed that food/water intake and body weights (dams and pups) were measured and that there were no treatment-related differences.

In these investigations, one male and one female from each litter were used for behavioral tests after weaning: MA at PND45 and 90, EPM at PND45 and 90, and NOR at PND90. In addition, Gallegos et al. (2016) included several tests of ontogeny of physical (eye and auditory canal opening) and behavioral/sensorimotor (righting reflex, cliff aversion, negative geotaxis) development, using all pups. Gallegos et al. (2016) reported no marked effect on gestation length or litter size. In the pups (both sexes, both dose groups), there was an early onset of cliff aversion and early ear opening. For both measures, acceleration was by approximately one day and there was no significant difference between dose groups. These acceleration differences were significant and indicated altered maturation of specific brain function; however, there is low confidence in these findings since the magnitude of difference was small and there was no dose-response.

MA and EPM were measured in offspring of both sexes, with different rats used at PND45 and PND90 (Gallegos et al. 2016). MA was mostly decreased at both doses in both sexes, although this depended somewhat upon the parameter (number of squares crossed number of rears, number of grooming episodes) and age. At PND45, only high-dose females displayed reduced MA. At PND90, males and females in both dose groups displayed decreased MA, and at the high dose both sexes exhibited fewer rears. Only males demonstrated increased grooming episodes, at both doses. These findings are similar to the MA depressive effects in formulation studies in adult animals, although this was measured long after exposure had ended.

In the EPM, effects were again mostly seen in both sexes, both doses, but dependent upon age. At PND45, females in both dose groups exhibited elevated % time in the open arms. This was again seen in females at PND90, as well as increased % entries in the open arms (high dose only). Males only demonstrated effects at PND90, and both doses altered the same parameters. There were no marked differences in total arm entries, which suggests that the difference was not due to motor differences. Overall, these data suggest an anxiolytic effect, i.e., decreased anxiety. In both MA and EPM, females only were affected at the younger age but both sexes were affected at the older age. This suggests sex-related differences in the age of occurrence of these behavioral effects, but testing the same rats at both ages would provide more support for such a conclusion.

The NOR was conducted in offspring at PND90, with the memory test occurring 24 hr after familiarization with the objects (Gallegos et al. 2018). There were effects in both sexes and both doses; however, data are presented only as a ratio so the actual amount of time spent at each object is not given. Gallegos et al. (2018) indicated that females in both dose groups showed a lower exploratory ratio, but data were similar to controls. While high-dose males did not exhibit a difference in exploratory ratio between the familiarization (pre-test) phase and the test, there appeared to be an unexpected dose-related variation in exploration during the familiarization test itself that accounted for this difference. Thus, while these findings were interpreted as evidence of decreased recognition memory, data are not convincing.

Overall, the formulation studies show credible observations of reduced MA, decreased anxiety, and questionable report of diminished recognition memory. While other studies used some of these same tests to provide comparative data, those investigations did not properly allocate littermates for testing and therefore cannot be used to support or refute these findings.

Neuropathology/pharmacology and other biochemical endpoints

Gallegos et al. (2018) also included neurochemical measures in offspring that were sacrificed at PND90, and reported decreased activities of AChE and glutamate transaminase, and both elevated and reduced brain antioxidant status in brain prefrontal cortex, striatum, and/or hippocampus. The biological significance of these effects as a mechanism of action (MOA) is uncertain especially where glutamate transaminase activity was reduced at the low dose but there was no or less of an effect at the high dose. In summary, the alterations in neurochemical and biochemical endpoints might serve as a basis for developing hypotheses requiring further testing at lower exposure levels and additional timepoints using

reliable measures of oxidative stress (Dikalov and Harrison 2014). At present, these findings cannot be linked as a MOA to any adverse neurotoxicity finding for glyphosate formulation, and certainly not to glyphosate alone.

In vitro glyphosate and formulation studies

Although the primary focus of this review was on *in vivo* studies, a total of 21 *in vitro* studies were also evaluated to gather general information regarding toxicity and potential mechanisms of toxicity. These were not ranked with ToxRTool, but some of the second-pass criteria (relevant to *in vitro* studies) were applied to ensure that papers were systematically identified for consideration. Two papers used a commercial formulation of glyphosate but no formulation control; these studies were deemed to be unreliable because of the potential overt toxicity of the surfactants to cells (Costa Reis et al. 2021; Neto de Silva et al. 2020). This conclusion is supported by a short report (correspondence) from Szepanowski et al. (2018) that demonstrated no significant effect of glyphosate or the combination of glyphosate and isopropylamine on myelination in cultured dorsal root ganglia, while a commercial glyphosate formulation produced significant effects.

More than half of these *in vitro* studies focused on ongoing development and validation of specific techniques, specifically multi-electrode array (MEA) and high-content imaging of neural development. These investigations are mostly from the same lab, and are being developed for use in first-tier screening of chemicals for acute or developmental neurotoxicity. Technical glyphosate was routinely used as one of many chemicals in training sets as a negative control (Aschner et al. 2017). Six were MEA studies in which glyphosate exerted no marked effects (Alloisio, Nobile, and Novellino 2015; Frank et al. 2017; Mack et al. 2014; McConnell et al. 2012; Valdivia et al. 2014; Wallace et al. 2015). These MEA investigations used primary cortical neurons and assessed changes in electrophysiological parameters including mean firing rate, mean burst rate and duration, % spikes in each burst and the inter-spike interval. While not all studies investigated all parameters, across these multiple studies glyphosate did not markedly alter any these measures at concentrations up to 30–100 μM. One paper (LeFew et al. 2013) did not contain new data but performed novel statistical analyses on already published MEA data, and there were no differences in conclusions with glyphosate. There were also six manuscripts that tested glyphosate on cell viability and neurite development. Radio et al. (2008; 2010) exposed NS1 cells, a sub-clone of PC12 cells, and primary cerebellar granule cells to concentrations of glyphosate up to 100 μM and observed no significant effects on viability or neurite outgrowth. Similarly, Mundy, Radio, and Freudenrich (2010) exposed PC12, NIC-115 and SH-SY5Y cells to concentrations of glyphosate up to 30 μM and noted no significant effects on cell proliferation, as measured by BrdU incorporation nor viability, as measured by ATP concentration. Harrill et al. (2011) found no marked effects on neurite outgrowth at concentrations of glyphosate up to 30 μM in rat primary cortical neurons and human embryonic stem cell-derived neurons. Using a human neural progenitor cell line, glyphosate displayed no marked effects on proliferation or viability up to 100 μM (Breier et al., 2008). Similarly, Culbreth et al. (2012) found no significant effects on cell proliferation, viability, or caspase-3 activation in mouse- or human-derived stem cells. Breier et al. (2008) noted an effect on P53 activation at 30 μM, but only in the neurons derived from mouse stem cells. Thus, in the development of these test methods for *in vitro* neurotoxicity screening, it is important to note that glyphosate was consistently inactive.

Other studies used a variety of cell systems and test measures to provide more mechanistic data. Martínez et al. (2020) reported effects of glyphosate on gene expression related to neuronal development (i.e., Wnt and CamK) in SH-SY5Y cells, but these effects occurred at a concentration (5 mM) that significantly reduced cell viability. Chorfa et al. (2013) noted a fall in viability (MTT assay) in SH-SY5Y cells following 72-hr exposure with an IC_{50} of 9 μM glyphosate, but there were no changes in α-synuclein levels up to that concentration. Coullery, Ferrari, and Rosso (2016) found that exposure of primary hippocampal neurons to glyphosate reduced a variety of measures of axonal outgrowth; however, the concentration of glyphosate required to produce this effect was over 20 mM. Similarly,

Luna et al. (2021) used primary hippocampal cultures to explore the effects of glyphosate on synapse formation. Morphometric analysis revealed effects including decreased dendritic development and spine morphogenesis at two very high concentrations (0.5 and 1 mg/ml; 2.96 and 5.92 mM). Martínez and Al-Ahmad (2019) demonstrated that glyphosate treatment increased permeability and associated reduction in occludin expression at 1–10 μM using an *in vitro* brain microvascular endothelial cells monolayer. Martínez and Al-Ahmad (2019) also noted decreased cell metabolic activity at 10–1000 μM in undifferentiated neurons (iPSC-derived), and diminished differentiated neuron density at 1000 μM after a 24-hr exposure. The relevancy of these concentrations, findings, and assays are unclear at predicting *in vivo* outcomes, and these investigators indicated their findings are relevant to a "high level" of glyphosate from acute exposure.

Most recently, Masood et al. (2021) exposed primary neural stem cells isolated from the subventricular zone of postnatal mice to various concentrations of glyphosate. Cell viability, measured with a live-dead assay using Calcein-AM and propidium iodide, was not altered at 0.1 or 700 μg/l glyphosate, but was significantly reduced at 7000 μg/l (41.4 μM) glyphosate. The concentration of 700 μg/l (4.1 μM) exerted no effect on proliferation as measured by BrdU and Ki67 staining but did produce a significant decrease in cellular migration and actually increased neurite outgrowth, in contrast to the other papers cited above that consistently found no effect on neurite outgrowth. Masood et al. (2021) reported significant effects of a lower concentration (0.1 μg/L; 0.59 nM) on glial fibrillary acidic protein (GFAP) and S100β mRNA expression and astrocyte morphology assessed by GFAP staining; however, on the latter measures, the high concentration exerted no effects. It is possible that the reduced mRNA expression confounded assessment of astrocyte morphology assessed by GFAP staining and it is not clear whether the effects on mRNA are transient.

Taken together, these studies demonstrate that glyphosate produced few changes in these various *in vitro* systems. Where effects were observed on *in vitro* neurological parameters, these mostly occurred at high concentrations (high μM to mM levels) which also decreased several measures of cell viability, with the exception of Masood et al. (2021), which needs to be independently replicated, and Chorfa et al. (2013), which was not replicated in other studies with SH-SY5Y cells. Future *in vitro* studies also need to include measures of pH since glyphosate is an acid. This will better ensure that effects exerted by the compound itself can be differentiated from potential glyphosate-induced acidification (Schweizer et al. 2019).

Discussion

The primary focus of this paper is an assessment of the neurotoxicity potential of glyphosate alone for risk assessment purposes. Formulation studies were included as supportive information because of potential relevance to occupational and public health, as well as the interpretation of some epidemiological studies, and because of their potential to provide additional information on a range of neurotoxicological endpoints not available for glyphosate. Due to the possible qualitative and quantitative differences in the study outcomes of glyphosate alone compared with the formulations, these papers were summarized and evaluated separately.

It is important to emphasize that the *a priori* criteria used to evaluate studies was specifically developed for purposes of quantitative or qualitative risk assessment for food-use pesticides, which require a large number of toxicology and metabolism studies including those with neurotoxicity endpoints (US EPA 2012). This distinction is important because studies considered to be unreliable or of low reliability for quantitative or qualitative risk assessment purposes may serve an important role in the neurotoxicology research community to generate hypotheses for further investigations or to better understand mechanisms of toxicity at high doses independent of human health risk assessment purposes. Our intent is to provide a review that transparently evaluates the quality of neurotoxicity data within the context of risk assessment for food-use pesticides.

Consideration of TK data and human exposure levels

When considering toxicity studies for risk assessments, understanding of the environmental and biological relevance of exposure levels used in *in vitro* to *in vivo* studies to human exposures provides important context. The TK and metabolism of glyphosate have been recently summarized within the context of risk assessment (ATSDR2020; AGG 2021; Bus 2015, Bus 2017). Briefly, dermal absorption of glyphosate is low (<1%), and absorption after oral administration is ≤35%, with 20% considered to be a representative absorption level (AGG 2021). Glyphosate is excreted predominantly unchanged in the urine, with virtually no excretion in the bile. Only very low amounts of radiolabeled glyphosate (<0.01% of administered dose) were detected in brain tissue for hr to days after oral or i.v. exposure (AGG 2021), suggesting that it does not readily pass the blood–brain barrier. Further, an *in vitro* diffusion investigation using iPSC-derived blood–brain barrier epithelial cells found that only 1.67% of the applied dose (100 µM glyphosate) had crossed the cells in a 2-hr period (Martínez et al. 2018). Mose et al. (2008) found low (15%) placental transfer of glyphosate in an *ex vivo* placental perfusion study. Finally, glyphosate does not bioaccumulate based upon comparisons of acute and repeat dose TK studies (Bus 2015).

Some studies documented glyphosate levels in various groups of the population. For example, occupational exposures were measured in a glyphosate biomonitoring study for farmers and their families (Acquavella et al. 2004). The maximum systemic dose for farmer applicators was estimated to be 0.004 mg/kg, based upon maximum urinary concentration of 233 ug/l, with a geometric mean of 0.0001 mg/kg. These values are higher than average urinary levels of 0.26 to 73.5 µg/l reported by Gillezeau et al. (2019) in a comprehensive review of glyphosate human exposure studies, suggesting that the estimate of 0.004 mg/kg as a high exposure level is reasonable to compare with oral doses used in animal studies. Another study estimated dietary intake in a small group of pregnant women to be 0.001 mg/kg/day, based upon food frequency questionnaires and measured glyphosate in composite food samples (McQueen, Callan, and Hinwood 2012). The TK data described above further indicate that, of the absorbed glyphosate, considerably less glyphosate reaches the brain and/or the fetus.

Glyphosate

Eleven studies of glyphosate alone were identified, with seven that were considered reliable with or without restrictions. Five of these were regulatory studies that followed specific testing guidelines for neurotoxicity (OECD 1997; US EPA 1998b), and the endpoints were limited to MA, observational assessments (FOB), and neuropathology (Brammer 2001; Horner 1996a, 1996b; Nufarm 2009; SafePharm 2006). While these tests represent only a few of the many neurological tests used in neurotoxicity studies, these are considered to be a broad screen, i.e., evaluations that may be triggered by chemicals acting through many different mechanisms and therefore able to detect a range of neurotoxic chemicals (OECD 2004; NAFTA 2016). In addition, neuropharmacological endpoints were assessed in two acceptable published papers (Hernández-Plata et al. 2015; Martínez et al. 2018).

Glyphosate administered orally produced activity, gait, and posture changes as well as trends towards decreased MA at 2000 mg/kg (Horner 1996a). Hernández-Plata et al. (2015) found MA was decreased following each repeated dose (50–150 mg/kg/day i.p., three times a week for two weeks) as well as two days after dosing ended: all doses were effective so there was no NOEL. Thus, the primary behavioral finding was one of reduced MA and toxicity at high oral doses and lower i.p. doses (noting the absorption and irritation issues with i.p. dosing). MA is an apical behavior that reflects a number of underlying processes including motor capacity, sensory function, emotional processing, and non-associative learning (habituation); thus, changes in MA may reflect alterations in any of these functions as well as non-specific toxicity. Further, MA cannot be considered a specific measure of motor function *per se*, and changes cannot be directly and definitively related to changes in function or structure of specific brain regions (MacPhail, Peele, and Crofton 1989). There were no studies of glyphosate alone on cognitive, anxiety, or depressive behaviors.

Neuropathology regulatory studies of technical glyphosate were conducted using standardized methods which are considered adequate for first-tier evaluation of nervous system tissue damage for risk assessment purposes (OECD 1997; US EPA 1998b). There were no observed effects of glyphosate in any study, even at the highest doses (dietary intakes >1500 mg/kg/day), on the structure of the nervous system.

Neuropharmacology endpoints were assessed in two published papers, which reported effects of glyphosate alone on monoamine levels following acute or repeated treatment (Hernández-Plata et al. 2015; Martínez et al. 2018), and TH levels and immunostaining (Hernández-Plata et al. 2015). Acute changes in DA levels (but not metabolites) in striatum were noted using microdialysis, whereas there were no significant changes in any monoamine or TH levels, or TH^+ cell counts, 2 and 16 days after repeated dosing (Hernández-Plata et al. 2015). On the other hand, Martínez et al. (2018) reported changes in several monoamines in specific brain regions 3 hr after short-term (6 days) repeated dosing, with a NOEL across all measures of 35 mg/kg/day. There was, however, no clear pattern of affected brain regions, and in some cases no clear dose-response. These alterations in monoamine levels occurred just hours after the last dose, and may represent acute effects that are transient/rapidly reversible (suggested by no effects at 2 days reported by Hernández-Plata et al. 2015). Thus, while a lower NOEL was provided by Martínez et al. (2018), the toxicological relevance and permanence of these effects are unknown (US EPA 1998a; WHO 2001).

To evaluate further weight of evidence, it can be advantageous to integrate behavioral and other neuropathological/neuropharmacological findings when study designs allow (i.e., same animals used). For technical glyphosate, the regulatory studies measured behavior (MA, FOB) in the same rats that were then used for neuropathological assessments (Brammer 2001; Horner 1996a, 1996b; Nufarm 2009; SafePharm 2006). There were only transient and inconsistent changes in some studies for behavioral assays across those five studies, and no microscopic histopathology changes were observed; thus, no correlative analysis were possible. Hernández-Plata et al. (2015) tested MA in the same rats that were eventually used for monoamine levels and for receptor-binding studies. Data demonstrated no marked changes in neurotransmitters/metabolites at 2 days after the last of six doses, even though the animals exhibited decreased MA at that time. On the other hand, there was diminished DA1-receptor binding in nucleus accumbens (but not striatum) that correlated with MA levels in that subgroup. However, this statistical correlation cannot be considered evidence of a causal effect, especially given the inconsistent findings on the dopaminergic system within and across these two studies.

In vitro studies have been conducted at exposure levels ranging up to 5 mM glyphosate (5000 μM), with the majority of studies conducted between 30 and 100 μM. These *in vitro* glyphosate exposure levels may be related to *in vivo* levels based upon a study by Anadón et al. (2009), which measured a glyphosate plasma Cmax concentration of 4.6 μg/ml (27.3 μM) after a single oral dose of 400 mg/kg. If the oral TK is linear, 100 μM is equivalent to an approximate oral glyphosate dose of 1465 mg/kg, which is comparable to the highest dose levels (i.e. limit dose) of 1000–2000 mg/kg for regulatory guidelines for toxicity testing. Further, as described in the TK section above, an exceedingly small amount of administered glyphosate reaches the brain, making it even more unlikely that even very high administered doses might achieve brain concentrations similar to those that were typically used in the *in vitro* studies.

Most *in vitro* studies were performed with technical glyphosate, which demonstrated no significant changes in electrophysiological parameters in primary cells. There were some reports of effects on neurite outgrowth and other parameters related to neuronal development, but these effects were detected for mM concentrations or levels that resulted in overt toxicity, with the exception of Masood et al. (2021) who showed that glyphosate actually increased neurite outgrowth in neural stem cells derived from the sub-ventricular zone of early postnatal mice. As mM concentrations are roughly equivalent to *in vivo* levels that far exceed the limit dose required for toxicity testing, the relevance of these findings to neurotoxicity in animals or humans is extremely unlikely. Given the few

in vivo neurological effects in these studies of glyphosate alone, and the few *in vitro* outcomes at biologically relevant concentrations, there are insufficient findings for correlative evaluations.

Glyphosate Formulations

There were more studies of glyphosate formulations than glyphosate alone, although none of the formulation studies contained an adequate formulation control to directly evaluate the neurotoxicity effects of glyphosate alone. Although this shortcoming would typically have resulted in exclusion of these papers as unreliable, these papers were nevertheless considered to provide additional insight on potential glyphosate effects, with the understanding that these formulations may have both qualitative and quantitative differences compared to glyphosate alone. However, none of these studies can be used for risk assessment purposes for glyphosate technical. Due to differences in formulations, no attempt was made to compare effective doses across the formulation studies.

A variety of behavioral assessments, including MA and tests of anxiety, cognition, and depression, were conducted in the studies of glyphosate formulations. Comparisons across studies are complicated by a number of experimental factors including commercial product, dosing scenarios, experimental designs, and species and differences in the outcomes may be due to any of these.

The most consistent finding across the formulation studies from several different labs was decreased MA. There were some differences in response that might be due to a number of factors including dosing paradigm, testing time, testing apparatus, or route of administration. There was less consistency in effects of the other behavioral measures. Where there were changes in anxiety, depressive, or cognitive behaviors, these were mostly confounded by concurrent motor decreases that interfere with the interpretation of the effects observed. Thus, the formulation studies provided indications of potential effects, but data are not sufficiently rigorous to be considered for risk assessment evaluations.

The potential for developmental neurotoxicity is important for assessing glyphosate risk; however, most of the studies could not be evaluated (see excluded studies, above) and only one lab appeared to follow appropriate litter allocation (Gallegos et al. 2018, 2016). While these studies reported effects on activity, recognition memory, and anxiety, additional studies, especially using glyphosate alone, are needed to provide confidence in the data.

A few studies of glyphosate formulations evaluated AChE activity, with inconsistencies in outcomes and/or effects in unexpected brain regions (Ait Bali et al. 2019; Bicca et al. 2021; Gallegos et al. 2020). These general findings, however, are supported by Larsen et al. (2016), who reported glyphosate to be a weak AChE inhibitor in rat brain *in vitro* (IC_{50} = 17.4 mM). This is in contrast to prototypical AChE-inhibiting insecticides and/or their metabolites, such as chlorpyrifos oxon, which has an IC_{50} in the 2–10 nM (Mortensen, Hooper, and Padilla 1998). Thus, although glyphosate may be considered an organophosphorus (OP) compound based upon chemical structure, it does not share the properties of inhibiting AChE similar to typical insecticidal OP compounds. Glyphosate is more correctly classified as an organophosphonic acid because it has one carbon substituent attached directly to phosphorus, which many insecticidal OPs lack. Consequently, it is unlikely that AChE inhibition by glyphosate is a relevant neurotoxicological pathway.

Two studies evaluated dopaminergic and serotonergic neural integrity, which may inform an understanding of potential neurotoxicity. Immunostaining of TH^+ and $5\text{-}HT^+$ neurons was decreased, but only in one study at doses of a glyphosate formulation producing body weight changes during dosing (Ait Bali, Ba-Mhamed, and Bennis 2017). In addition, as described in detail above, the experimental sampling design in that study was not sufficient to assess whether there was truly loss of neurons or expression of the neurochemical markers assayed. In contrast, a study of glyphosate alone at doses that were behaviorally active found no change in TH^+ cell counts (Hernández-Plata et al. 2015), and in that study, there were no body weight changes measured after dosing (no bodyweights during dosing were reported). Thus, alterations in immunostaining observed by Ait Bali, Ba-Mhamed, and Bennis

(2017) at doses of a formulation that produced overt toxicity is not appropriate to be used for human health risk for glyphosate.

A few *in vivo* and *in vitro* studies tested the hypothesis that oxidative stress is a mechanism of neurotoxicity for glyphosate formulations (Ait Bali et al. 2019; Bicca et al. 2021; Gallegos et al. 2018). Taken together, the findings are inconsistent and/or based upon non-specific methods, cytotoxic/toxic test concentrations and doses, and technically limited oxidative stress biomarkers. Several of the same experimental design issues identified by Bus (2017) for consideration of oxidative stress as a key mode of action for carcinogenicity are directly applicable to testing the hypothesis on mode of action for neurotoxicity. For example, studies need to avoid cytotoxic test concentrations/doses, avoid *in vitro* concentrations that are excessively high as described above, employ adequate dose–response data using reliable biomarkers of oxidative stress, and avoid any claims about glyphosate based on formulation studies that do not control for the formulation (Bus 2017). At present, the studies do not provide sufficient evidence that oxidative stress is a mechanism of neurotoxicity for glyphosate or glyphosate formulations.

Comparisons to epidemiology studies

There are growing concerns that exposures to certain types of pesticides may be linked to risks for developing a number of human neurological disorders (Richardson et al. 2019). Epidemiological studies are pivotal for detecting such effects; however, due to the observational nature of such studies, these often suffer from shortcomings including exposure and outcome measurement error, confounding, as well as selection bias and recall bias. Animal studies may generally provide more specific, quantitative information on exposures and simplification of confounding factors, and therefore may inform some of the uncertainties and limitations inherent in human studies. A complement of both animal and human studies can generally best inform health risk assessments.

From a public health perspective, *in vivo* and *in vitro* formulation studies may be relevant to epidemiology studies with agricultural or residential usage of pesticide products. Given the caveats regarding components in those various formulated products, and differential exposure patterns (e.g., pesticide handler vs. bystander), even those comparisons are not definitive. Despite the uncertainties, it may be potentially constructive to compare epidemiology outcomes with the findings of toxicological investigations of both glyphosate alone and formulations.

The available epidemiological database for potential neurological effects of glyphosate has recently been reviewed by Chang et al. (2022). Reports of case studies, some of which describe effects in only a single person and therefore cannot be used to estimate exposure-disease associations, were excluded from their review. Overall, their systematic review found no established causal effect, or even a consistent statistical association, between glyphosate exposure and any specific neurological outcome in humans.

Some of the specific nervous system disorders addressed in those human studies have also been examined in the animal studies reviewed here, and integration of these two lines of research may be helpful for providing weight of evidence for certain results. Exposures to certain pesticides, including glyphosate, have been linked in some epidemiologic studies to increased risk for Parkinson disease (PD), developmental disorders, such as autism and attention deficit hyperactivity disorder (ADHD), cognitive problems, anxiety, and depression (Arab and Mostafalou 2021; Vaccari et al. 2019). These outcomes were also addressed, to some extent, in animal studies, so it was of interest to evaluate those correlations.

The identification of specific pesticides contributing to PD risk has remained elusive, even as mechanistic pathways have recently been proposed for PD development (Baltazar et al. 2014). Case reports suggested that glyphosate might produce a parkinsonian syndrome, but these reports lack characterization of exposures and alternative causes, symptoms appear years after a purported exposure, do not include a comparison group, and there is a lack of data demonstrating a positive levodopa response (characteristic of PD). On the other hand, several comparative epidemiological studies found no significant associations between glyphosate usage and PD (Dhillon et al. 2008; Kamel et al. 2007; Shrestha et al. 2020). Only one

study reported a significant relationship (Wan and Lin 2016), but that study was based upon an ecological design for the exposure metric, relying on county-level glyphosate application data and PD rates.

Some studies in the animal glyphosate literature evaluated neurological features of PD, including decreased DA levels, receptors, and neurons in substantia nigra and striatum; however, these are mostly inconsistent, incomplete, and/or of poor quality. Two studies used glyphosate alone and measured aspects of the DA system (Hernández-Plata et al. 2015; Martínez et al. 2018), but these were not comparable in experimental design. Decreased DA levels (but not DA metabolites) were measured in striatum after a single acute i.p. dose (150 mg/kg), but when the same dose was administered repeatedly, there were no marked changes in levels of DA and only a transiently diminished DA1-receptor binding in nucleus accumbens, but not striatum (Hernández-Plata et al. 2015). In contrast, when glyphosate was given orally for 6 days, striatal DA was reduced only at the highest dose (800 mg/kg/day), and there were no corresponding changes in DA metabolites (Martínez et al. 2018). Another study using glyphosate formulation noted an apparent reduction in TH^+ immunoreactivity in the substantia nigra with repeated dosing; however, toxicity was evidenced by lowered weight gain (Ait Bali, Ba-Mhamed, and Bennis 2017). As described above, the image analysis methodology did not provide appropriate resolution on the pathological outcome, i.e., cell loss vs. decreased protein expression. Based upon these factors, data are of low confidence and cannot be interpreted as cell loss that would be relevant to PD. In summary, neither the epidemiological data nor limited experimental data support a role for glyphosate in contributing to PD or a PD phenotype.

The prevalence of autism spectrum disorder and ADHD have been rising in recent decades (Gurney et al. 2003; Xu et al. 2018), leading some to implicate increasing use of pesticides and/or other environmental chemicals in the etiology of these developmental disorders (Dietert and Dietert 2008; Dietert, Dietert, and Dewitt 2011; Symeonides et al., 2014). Some epidemiological studies evaluated such outcomes related to glyphosate; however, these investigations suffer from inadequate measures of exposure (e.g., residential proximity) and/or outcomes (e.g., self-reports). Here, it was found that animal studies of offspring exposed during gestation and lactation were also mostly inadequate to address this concern, since most suffered from inadequate methodology and inappropriate testing of littermates. The only studies with adequate sampling procedures (Gallegos et al. 2018, 2016) do appear to support developmental effects including cognitive deficits and anxiety, although behaviors that are representative of autism or ADHD were not evaluated. Because exposure was to formulations, the effects cannot be clearly attributed to glyphosate alone. Both human and animal confirmatory studies, using appropriate methodologies and outcome assessments, are needed for better understanding of the potential for glyphosate to initiate developmental neurological disorders.

A human cross-sectional study (Fuhrimann et al. 2021), in which Ugandan farmers were administered a battery of neurobehavioral tests including measures of learning, memory, attention and motor ability, reported an association of glyphosate with poorer visual retention. This singular finding has not been replicated in other studies, and simultaneous assessment of exposures and neurobehavioral outcomes precluded this investigation from identifying causal effects. Animal studies using various formulations reported cognitive deficits, including recognition memory (NOR). These findings, however, (1) were not consistent across studies, (2) might be influenced by lowered activity levels, (3) did not include comparison with formulation alone, and (4) in some cases the magnitude of change was not biologically significant. Interestingly, two epidemiological studies (Beard et al. 2013, 2014) reported no significant association of glyphosate use with depression in women and men, respectively, while animal studies reviewed herein mostly showed effects on tests considered to measure depressive behaviors, although these were confounded by reduction in MA.

Summary

In conclusion, this systematic analysis of the available literature provided no clear evidence of neurobehavioral, neuropathological, or

neuropharmacological outcomes following exposure to glyphosate. The evidence from the regulatory studies, conducted under standardized test guidelines, concluded essentially no marked effects (other than spurious behavioral changes) with acute, subchronic, and chronic exposures, while published studies that did report effects were limited in terms of route of exposure, dosing regimens, and interpretability of endpoints. Although there were a few investigations that addressed other outcomes important for public health, e.g., developmental, cognitive, or other functional changes, these were not acceptable for inclusion according to the criteria set forth in this review.

The studies of glyphosate formulations included greater variety of endpoints such as neuropharmacological measures and tests evaluating more complex behaviors. However, these need to be tempered with uncertainties of testing various commercial formulations which may not reflect effects of glyphosate alone. Further, many of the studies were methodologically weak and findings were not consistent and/or of uncertain toxicological relevance. Agreements between animal data and epidemiological studies are also not evident. Future neurotoxicity studies that evaluate glyphosate or its formulations would be more useful for risk assessment purposes if these use relevant routes of exposures (e.g., oral and not i.p.), specify the test material used (e.g., glyphosate technical, salt form, or formulation), and include: (1) formula-based controls in formulation studies, (2) control for acidity of glyphosate acid in *in vivo* and *in vitro* studies, and (3) use accurate testing methodologies including litter allocations, and sound scientific principles in neurological and neuropathological assessments.

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