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NTP Research Report on

Systematic Literature Review on the Effects of Fluoride on Learning and Memory in Animal Studies

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NTP Research Report on Systematic Literature Review on the Effects of Fluoride on Learning and Memory in Animal Studies

Research Report 1

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This report has been reformatted to meet new NTP publishing requirements; its content has not changed.

About This Report

National Toxicology Program[1]

[1]Division of the National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA

Collaborators

Kristina Thayer, Jean Harry, Andrew Shapiro, Stephanie Holmgren, Mamta Behl, John Bucher, Greg Carter, Pamela Hartman, Cara Henning

Division of the National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA
Contributed to conception or design and contributed to drafting of protocol
Jean Harry, Ph.D., Project Co-lead
Kristina Thayer, Ph.D., Project Co-lead
Mamta Behl, Ph.D.
John Bucher, Ph.D.
Andrew Shapiro, M.S.

Contributed to drafting of report
Jean Harry, Ph.D., Project Co-lead
Kristina Thayer, Ph.D., Project Co-lead

Critically reviewed draft report and figures
Mamta Behl, Ph.D.
John Bucher, Ph.D.
Stephanie Holmgren, M.S.
Andrew Shapiro, M.S.

Retrieved and managed references
Stephanie Holmgren, M.S.

Assessed risk of bias
Jean Harry, Ph.D.
Kristina Thayer, Ph.D.

ICF, Durham, North Carolina, USA
Critically reviewed draft report and figures
Pamela Hartman, M.E.M., Technical Lead
Greg Carter, M.E.M., Co-Lead Work Assignment Manager
Cara Henning, Ph.D., Co-Lead Work Assignment Manager

Extracted data
Pamela Hartman, M.E.M.

Developed visualizations
Pamela Hartman, M.E.M.

Contributors

Division of the National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA
Managed peer review of draft document and critically reviewed draft report and figures
Mary S. Wolfe, Ph.D.

ICF, Durham, North Carolina, USA
Coordinated peer review of draft document
Canden N. Byrd, B.S.

Assisted in document production
Penny Kellar, M.S.
Whitney Mitchell, B.S.

Managed article acquisition
Nicole Vetter, M.L.I.S.

Screened studies
Autumn Bordner, B.S.
Susan Goldhaber, M.P.H.
Maureen Malloy, B.A.

Extracted data
Susan Goldhaber, M.P.H.
Tao Hong, Ph.D.
Bryan Luukinen, M.S.P.H.
Johanna Rochester, Ph.D.
Pam Ross, M.P.H.

Assessed risk of bias
Robyn Blain, Ph.D.
Tao Hong, Ph.D.

University of Edinburgh, Department of Neurology and Translational Neuroscience, Coordinator of the Collaborative Approach to Meta-Analysis and Review of Animal Data in Experimental Studies, Edinburgh, Scotland
Technical advisor
Malcolm Macleod, Ph.D.

Radboud University Medical Centre, Departments of SYstematic Review Centre for Laboratory Animal Experimentation (SYRCLE) and Anesthesiology, Nijmegen, Netherlands
Technical advisor
Carlijn Hooijmans, Ph.D.

Peer Review

The research report, *Systematic Literature Review on the Effects of Fluoride on Learning and Memory in Animal Studies*, was evaluated by the reviewers listed below. These reviewers served as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, reviewers determined if the design and conditions of these NTP studies were appropriate and ensured that this NTP Research Report presented the experimental results and conclusions fully and clearly.

Peer Reviewers

Charles Vorhees, Ph.D.
Division of Neurology
Cincinnati Children's Hospital Medical Center
Cincinnati, Ohio, USA

Russell Carr, Ph.D.
Center for Environmental Health Sciences
Department of Basic Sciences College of Veterinary Medicine
Mississippi State University
Starkville, Mississippi, USA

Protocol Review

The protocol and design were evaluated by the reviewers listed below.

Emily Sena, Ph.D.
Coordinator of the Collaborative Approach to Meta-Analysis and Review of Animal Data in Experimental Studies
Department of Neurology and Translational Neuroscience
University of Edinburgh
Edinburgh, Scotland

Staff
National Industrial Chemicals Notification and Assessment Scheme
Department of Health
Australian Government
Canberra, Australia

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Abstract

Background: Previous systematic reviews of epidemiology studies have found support for a geographical association between high levels of naturally occurring fluoride in water (>1.5 ppm) and lower IQ in children. Most of the evidence from humans is from fluoride-endemic regions having higher background levels of fluoride compared to the fluoride concentrations historically used in community water fluoridation programs (0.7–1.2 ppm). Confidence in this body of evidence is limited, primarily due to poor reporting quality, lack of consideration of confounding (e.g., nutritional status, socioeconomic status, iodine deficiency), and concern for co-exposures to relatively high levels of other known neurotoxicants such as lead or arsenic. A systematic review of experimental animal studies could help in interpreting the human evidence.

Objective: To investigate whether fluoride exposure has detrimental impacts on neurobehavior in laboratory animal studies, prioritizing assessment of learning and memory outcomes. Confidence in the body of evidence was assessed according to one of four statements: (1) High, (2) Moderate, (3) Low, or (4) Very Low/No Evidence Available.

Methods: We included experimental animal studies that used mammalian species (whole organism) exposed during development or adulthood, which compared the effects of oral exposure to various fluoride concentrations to vehicle controls on neurobehavioral responses. The principal outcomes were learning and memory, but other neurobehavioral studies were included (e.g., anxiety, motor activity, aggression, sexual behavior). Studies assessing brain-related cellular, morphometric or histological endpoints were considered beyond the scope of this analysis. A literature search was performed up to January 14, 2016, using PubMed, BIOSIS, EMBASE, Scopus, Web of Science, PsycINFO, and several specialized databases. There were no date or language restrictions, and unpublished data and abstracts were excluded. Risk of bias was assessed regarding randomization, allocation concealment, blinding, exposure characterization, health outcome assessment, incomplete outcome data, selective outcome reporting, and other biases.

Results: The database searches yielded 4,643 unique records and 13 records were identified from other sources. Of the 4,656 studies, we identified 68 studies using mice or rats and testing drinking water or dietary concentrations of 0.45 to 272 ppm fluoride (0.12 to 40 mg/kg-d). Most included studies were published after 2000. Forty-eight studies addressed learning and memory, 16 of which assessed exposure during development.

Synthesis of results: Meta-analysis was not conducted due to the small number of studies that measured endpoints similarly based on study design, that is, dose levels, duration of treatment, lifestage at exposure, species, or differences in measurement of behavioral responses. Relatively few studies provided information on other sources of fluoride (e.g., diet, water source). Most studies were statistically underpowered to detect a <20% change from control groups for behavioral tests. Approximately 30% of the learning and memory studies were considered to have a very serious risk of bias and were excluded from the narrative analysis. Conclusions were reached based on an analysis of 32 studies. Results show **low-to-moderate** confidence for a pattern of findings suggestive of an effect on learning and memory based on developmental and adult exposure studies. The evidence is strongest (moderate level-of-evidence) in animals exposed as adults and weaker (low level-of-evidence) in animals exposed during development. Level-of-evidence conclusions were rated down due to concern for indirectness and risk of bias. The evidence was strongest and most abundant for adult exposure studies using the Morris water

maze. In many cases, across the entire dose range tested, whether the effects were specifically related to learning and memory—versus a possible impact on motor or sensory function that could have impaired the ability of the animal to perform the learning and memory tests as measured—was not possible to discern. This was considered a form of indirectness. Additional studies are required to have higher confidence in the specificity of the responses as learning or memory impairments and in quantitative measures such as the effect sizes, point of departure, identification of no observed effect level or lowest observed effect level doses, or parameters for benchmark dose analysis. Based on control values (means and standard deviations/standard errors) and the number of animals per group, the studies appear statistically underpowered to detect a <10% or <20% change from controls for most behavioral endpoints.

Conclusion: Very few studies assessed learning and memory effects in experimental animals (rats and mice) at exposure levels near 0.7 parts per million, the recommended level for community water fluoridation in the United States. At concentrations higher than 0.7 parts per million, this systematic review found a low to moderate level-of-evidence that suggests adverse effects on learning and memory in animal exposed to fluoride. The evidence is strongest (moderate level-of-evidence) in animals exposed as adults and weaker (low level-of-evidence) in animals exposed during development. Confidence in these findings was reduced primarily based on potential confounding of the learning and memory assessments by deficits in motor function or fear and risk of bias limitations. Additional research is needed, in particular to address potential effects on learning and memory following exposure during development to fluoride at levels nearer to 0.7 parts per million. NTP is conducting laboratory studies in rodents to fill data gaps identified by this systematic review of the animal studies. The findings from those studies will be included in a future systematic review to evaluate potential neurobehavioral effects from exposure to fluoride during development with consideration of human, experimental animal and mechanistic data.

Introduction

Sources of Exposure

In 2010, the US Environmental Protection Agency (EPA) conducted a relative source contribution analysis of fluoride. Sources include drinking water, foods, beverages, dental products (toothpaste, mouth rinses), supplements, industrial emissions, pharmaceuticals, and pesticides (e.g., cryolite, sulfuryl fluoride). Soil ingestion is another source of exposure in young children (US EPA 2010b). The major contributors to exposure are drinking water, beverages, food, and toothpaste. The relative source contribution from drinking water intake was 40–60% after the age of 1 year and 70% in children less than 1 year old.

Table 1. Representative Values for Fluoride Intakes Used in Calculation of the Relative Source Contribution from Drinking Water

Age Group (years)	DWI[a] (mg/d)	BI (mg/d)	FI (mg/d)	TI (mg/d)	SuF (mg/d)	SI (mg/d)	Total (mg/d)	RSC (%)
0.5–<1	0.84	–	0.25[b]	0.07	0.03	0.02	1.2	70
1–<4	0.63	0.36	0.16	0.34	0.05	0.04	1.58	40
4–<7	0.82	0.54	0.35	0.22	0.06	0.04	2.03	40
7–<11	0.86	0.60	0.41	0.18	0.07	0.04	2.16	40
11–14	1.23	0.38	0.47	0.20	0.09	0.04	2.41	51
>14	1.74[b]	0.59	0.38	0.10[c]	0.08	0.02	2.91	60

From Table 7-2 (US EPA 2010b).

[a]Consumers only; 90th percentile intake except for >14 years of age. The >14-year value is based on the Office of Water policy of 2 L/d.

[b]Includes foods, fluoride in powdered formula, and fruit juices; no allocation for other beverages.

[c]Assumed. 50% of the 11- to 14-year-old age group. DWI = drinking water intake; BI = beverage intake; FI = food intake (solid foods); TI = toothpaste intake; SuF = sulfuryl fluoride intake; SI = soil intake; RSC = relative source contribution.

EPA has proposed a reference dose (RfD) of 0.08 mg/kg-d for protection against pitting of tooth enamel (severe dental fluorosis), and this value also is considered protective against fractures and skeletal effects in adults (US EPA 2010a).

Use of Fluoride to Prevent Tooth Decay

Fluoride from community water fluoridation, mouth rinses, gels, and toothpastes is intended to prevent dental caries primarily through topical remineralization of tooth surfaces. Community water fluoridation and fluoride toothpaste are the most common sources of non-dietary fluoride in the United States (US DHHS 2015). Because fluorine is the 13th most abundant element in Earth's crust, fluoride also naturally occurs in water and is present in many non-fluoridated water systems.

Although other fluoride-containing products and sources are available (e.g., mouth rinses, dietary supplements, professionally applied fluoride compounds), community water fluoridation has been identified as the most cost-effective method for delivering fluoride to all members of the community regardless of age, educational attainment, or income level. Consuming fluoridated water and beverages and foods prepared or processed with fluoridated water throughout the day maintains a low concentration of fluoride in saliva and plaque, which enhances remineralization

(US DHHS 2015). Community water fluoridation to minimize the occurrence and severity of tooth decay, which began in 1945, had reached 67% of the US population by 2012. About 25 countries practice community water fluoridation (Iheozor-Ejiofor et al. 2015), and many more provide fluoride through other means such as salt. In 2012, an estimated 200 million people in the United States were served by 12,341 community water systems that added fluoride to water or purchased water with added fluoride from other systems (US DHHS 2015).

The US Public Health Service (PHS) first recommended communities add fluoride to drinking water in 1962. PHS guidance is advisory, not regulatory, which means that although PHS recommends community water fluoridation as an effective public health intervention, state and local governments decide whether to fluoridate water systems. For community water systems that add fluoride, PHS currently recommends a fluoride concentration of 0.7 milligrams/liter (mg/L, ppm).[1] This recommended level provides the best balance of protection from dental caries, while limiting the risk of dental fluorosis. Dental fluorosis is a condition marked by changes in the appearance of tooth enamel most commonly appearing as lacy white markings (US DHHS 2015). Dental fluorosis can result when children regularly consume fluoride from birth through 8 years of age—the time when their permanent teeth (with the exception of the third molars) are developing.

Under the Safe Drinking Water Act, EPA sets standards for drinking water quality. Currently, the enforceable fluoride standard is set at 4.0 mg/L to protect consumers from exposure to drinking water sources with naturally high occurrence of fluoride against severe skeletal fluorosis (a condition caused by excessive fluoride intake over a long period that, in advanced stages, can cause pain, crippling damage to bones and joints, or both). EPA also has a secondary drinking water standard of 2.0 mg/L to protect against moderate to severe dental fluorosis. This secondary standard is not enforceable but requires water systems to notify the public. EPA is currently reviewing the drinking water standards for fluoride (US EPA 2013).

Concerns for Potential Fluoride Toxicity

The most commonly cited health concerns about fluoride and water fluoridation focus on bone fractures and skeletal fluorosis, intelligence quotient (IQ) and other neurological effects, cancer, and endocrine disruption. Effects on neurological function, endocrine functions (thyroid, parathyroid, pineal), metabolism (glucose), and carcinogenicity were assessed in the 2006 National Research Council (NRC) report, Fluoride in Drinking Water: A Scientific Review of EPA's Standards (NRC 2006). The review considered adverse effects of water fluoride, focusing on concentrations of 2–4 mg/L,[2] a range higher than the current recommendation for community water fluoridation (0.7 mg/L). At levels below 4.0 mg/L, NRC found no evidence substantial enough to support negative health effects other than severe dental fluorosis. The conclusions from the NRC review were the primary source of information for the potential hazard summary in a 2015 report by the US Department of Health and Human Services (DHHS), Federal Panel on Community Water Fluoridation. The NRC report noted several challenges to evaluating the

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[1]For many years, most fluoridated community water systems used fluoride concentrations ranging from 0.8 to 1.2 mg/L (US DHHS 2015).

[2]EPA's maximum contaminant level goal (MCLG) for fluoride is 4 mg/L and secondary maximum contaminant level (SMCL) is 2 mg/L. MCLGs establish an exposure guideline to prevent adverse health effects in the general population, and SMCLs are intended to reduce the occurrence of adverse cosmetic consequences from exposure to fluoride. Both the MCLG and the SMCL are nonenforceable guidelines (NRC 2006).

literature, including deficiencies in reporting quality; consideration of all sources of fluoride exposure; consideration of potential confounding; selection of appropriate control subject populations in epidemiology studies; demonstrated clinical significance of endocrine effects; and the biological relationship between histological, biochemical, and molecular alterations with behavioral effects. Regarding neurotoxicity and neurobehavioral effects, the main conclusions in the 2006 NRC report are:

"Animal and human studies of fluoride have been published reporting adverse cognitive and behavioral effects. A few epidemiologic studies of Chinese populations have reported IQ deficits in children exposed to fluoride at 2.5 to 4 mg/L in drinking water. Although the studies lacked sufficient detail for the committee to fully assess their quality and relevance to U.S. populations, the consistency of the results appears significant enough to warrant additional research on the effects of fluoride on intelligence." [p. 8] (NRC 2006)

"A few animal studies have reported alterations in the behavior of rodents after treatment with fluoride, but the committee did not find the changes to be substantial in magnitude. More compelling were studies on molecular, cellular, and anatomical changes in the nervous system found after fluoride exposure, suggesting that functional changes could occur. These changes might be subtle or seen only under certain physiological or environmental conditions. More research is needed to clarify the effect of fluoride on brain chemistry and function." [p. 8] (NRC 2006)

Since release of the 2006 NRC report, approximately 10 epidemiological studies of children's IQ have been published. A 2015 systematic analysis of the human literature conducted for the Republic of Ireland's Department of Health (Sutton et al. 2015) reviewed the new literature and concluded no evidence of an association with lowered IQ was apparent in studies of community water fluoridation. The authors based this conclusion primarily on an analysis of a prospective cohort study conducted in New Zealand (Broadbent et al. 2015). For fluoride-endemic areas, there was a strong suggestion that high levels of naturally occurring fluoride in water (>1.5 ppm) could be associated with negative health effects, including lowering of IQ. Overall, these studies were considered low quality, as they did not fully account for known confounding factors with regard to IQ (e.g., nutritional status, socioeconomic status), nor other potential influencing factors (e.g., iodine deficiency, chemical contaminants in the ground water such as arsenic and lead). The conclusions of Sutton et al. (2015) are consistent with findings of a 2012 meta-analysis of 27 epidemiology studies that supported the possibility of an adverse effect of "high" fluoride exposure[3] on children's neurodevelopment, specifically for lowered IQ (Choi et al. 2012). The Choi et al. meta-analysis, however, also identified study quality limitations, primarily related to reporting quality, that limited the strength of the conclusions (Choi et al. 2012). More than 35 experimental animal studies evaluating the neurobehavioral effects of fluoride have been published since release of the 2006 NRC report. A systematic review of experimental studies that focuses on the effects of fluoride on learning and memory would help interpret the human literature on IQ and identify key research/data gaps for additional study.

For cancer and endocrine disruption, the National Toxicology Program (NTP) is analyzing the amount of evidence available and the merit of pursuing systematic reviews given factors such as

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[3]"High" was defined based on drinking water concentration, evidence of fluorosis, exposure related to coal-burning activities, and urine levels.

the extent of new research published since previous evaluations and whether these new reports address or correct the deficiencies noted in the literature (OEHHA 2011; NRC 2006; SCHER 2011).

Objectives and Research Strategy

The overall objective of this evaluation is to undertake a systematic review of the existing animal studies to develop NTP level-of-evidence conclusions (NTP 2015a) about whether fluoride exposure is associated with impairments in learning and memory.

Research Strategy

- Identify literature reporting the effects of exposure to fluoride and neurobehavioral outcomes in experimental animal studies utilizing mammalian species (whole organism).

- Extract data on neurobehavioral outcomes from identified studies. Primary outcomes are learning and memory, but other behavioral measures are summarized (e.g., "anxiety," motor function).

- Assess the internal validity ("risk of bias") of individual studies.

- Summarize the extent of evidence available.

- Synthesize the evidence, and perform quantitative meta-analyses if appropriate, and evaluate sources of heterogeneity.

- Rate confidence in the body of evidence for effects on learning and memory according to one of four statements: (1) High, (2) Moderate, (3) Low, or (4) Very Low/No Evidence Available.

- Translate confidence ratings into level-of-evidence for effects on learning and memory according to one of four statements: (1) High, (2) Moderate, (3) Low, or (4) Inadequate.

- Describe limitations of the evidence base, limitations of the systematic review, and findings in the context of human exposure levels.

- Identify data gaps and key research needs.

Methods

The systematic review was conducted based on guidance outlined in the Office of Health Assessment and Translation (OHAT) *Handbook for Conducting a Literature-Based Health Assessment* (NTP 2015a). Methods are summarized below and presented in more detail in the study protocol available at https://hawcproject.org/assessment/126/.

Development of Research Question

A PECO statement (**P**opulation, **E**xposure, **C**omparator, **O**utcome) was developed to address and understand potential effects of fluoride on neurobehavioral measures in animal models, especially related to learning and memory (Table 2).

Table 2. PECO (Populations, Exposures, Comparators, Outcomes) Statement

PECO	Evidence
Population	Non-human mammalian animal species (whole organism) of all ages
Exposure	Fluoride at any concentration; relevant forms are those used as additives for water fluoridation: *fluorosilicic acid* (also called *hydrofluorosilicate*; CASRN 16961-83-4) *sodium hexafluorosilicate* (also called *disodium hexafluorosilicate* or *sodium fluorosilicate*; 16893-85-9) *sodium fluoride* (CASRN 7681-49-4) other forms that readily *dissociate* into free fluoride ions (e.g., potassium fluoride, calcium fluoride, ammonium fluoride)
Comparator	Animals exposed to vehicle-only treatment
Outcomes	Any *neurobehavioral* outcome Primary outcomes: learning and memory Secondary outcomes: motor and sensory function, and other aspects of behavior that do not fall under learning and memory (e.g., anxiety, territorial aggression)

Literature Search Strategies

Searching Electronic Databases

Systematic search strategies were developed to identify all relevant published evidence on the health effects of water fluoridation by using index terms and text words based on key elements of the research question. Nine electronic databases[4] initially were searched on January 7, 2015, and the search was updated on January 14, 2016, unless noted otherwise:

- BIOSIS (Thomson Reuters – searched 2/18/2015)
- EMBASE (Elsevier)
- European Chemicals Agency (ECHA) Registration dossiers ("REACH") database (searched 3/24–27/2015)

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[4]NLM's Toxline database was not included in the search because recent changes have resulted in significant reductions in search functionality that limits running the search strings for this topic. In addition, the other databases are very likely to identify relevant published and peer-reviewed animal studies.

- Organization for Economic Co-operation and Development (OECD) Existing Chemicals Screening Information Data Sets (SIDS) database (searched 2/18/2015)
- PsycINFO (APA [American Psychological Association] PsycNet)
- PubMed (National Library of Medicine [NLM])
- Scopus (Elsevier)
- USEPA HPV Challenge Program Robust Summaries and Test Plans database ([High Production Volume] searched 3/23/2015)
- Web of Science (Thomson Reuters; Web of Science indexes the journal, *Fluoride*)

No publication date or language restrictions were applied. Full details of the search strategy for each database are presented in Appendix A.

Searching Other Resources

The reference lists of included studies, records that do not contain original data (i.e., reviews, editorials, or commentaries), and the Fluoride Action Network website ("Fluoride affects learning and memory in animals" at http://fluoridealert.org/studies/brain02/) were searched for additional relevant publications.

Selection Criteria for the Evidence

Studies were screened for inclusion using a structured form in DistillerSR (Evidence Partners; https://distillercer.com/products/distillersr-systematic-review-software/). Two members of the evaluation design team independently conducted a title and abstract screen of the search results to identify studies that met the eligibility criteria. Studies that were not excluded based on the title and abstract advanced to full-text review. For citations with no abstract, articles were screened based on all or some of the following: title relevance (title should indicate clear relevance), page numbers (articles two pages in length or less were assumed conference reports, editorials, or letters), and PubMed MeSH (Medical Subject Headings).

Full-text copies of potentially relevant articles were independently assessed by two reviewers to identify studies that satisfied the inclusion and exclusion criteria. Discrepant screening results were resolved by discussion. Assessment of eligibility status of non-English studies was facilitated by native-language speakers at ICF International or National Institute of Environmental Health Sciences (NIEHS).

To be eligible for inclusion, studies had to comply with the criteria specified by the PECO statement (Table 2). Studies that did not meet the PECO criteria were excluded. In addition, the following exclusion criteria were applied:

- Studies did not contain original data, such as reviews, editorials, or commentaries.
- Studies were not peer reviewed (e.g., conference abstracts, technical reports, theses/dissertations, working papers from research groups or committees, white papers).

Data Collection and Presentation

Data Extraction

Data were extracted from individual studies by members of the evaluation team using DRAGON (Dose Response Analytical Generator and Organizational Network) and Health Assessment Workspace Collaborative (HAWC)[5] software. Data extraction elements collected from animal studies are listed in Appendix B Information on concentrations of fluoride measured in brain, blood, urine, or bone also was summarized when available. One member of the evaluation team extracted the data, which a second member checked. Missing data from individual studies were not sought. Extracted data were warehoused using HAWC.

Quality Assessment of Individual Studies

Risk of bias ratings for individual studies were assessed using the OHAT tool (NTP 2015b) (Table 3). Two raters assessed the studies, answering nine risk of bias questions following guidance outlined in OHAT's risk of bias documentation (NTP 2015b). After one reviewer independently determined risk of bias across all questions, a second person went through and agreed or disagreed with the ratings. Differences in ratings were discussed and resolved. Authors were queried to obtain missing information, and responses received were used to evaluate risk of bias. Information that was not reported was assumed not conducted (e.g., randomization, blinding), resulting in an assessment of "probably high" risk of bias.

Table 3. OHAT Risk of Bias Questions for Evaluating Experimental Animal Studies

OHAT Risk of Bias Questions for Evaluating Experimental Animal Studies
Questions
Selection Bias
1. Was administered dose or exposure level adequately randomized?
2. Was allocation to study groups adequately concealed?
Performance Bias
3. Were experimental conditions identical across study groups?
4. Were the research personnel and human subjects blinded to the study group during the study?
Attrition/Exclusion Bias
5. Were outcome data complete with respect to attrition or exclusion from analysis?
Detection Bias
6. Can we be confident in the exposure characterization?
7. Can we be confident in the outcome assessment?
Selective Reporting Bias
8. Were all measured outcomes reported?
Other Sources of Bias
9. Were there no other potential threats to internal validity (e.g., statistical methods were appropriate)?

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[5]ICF International. 2014. From Systematic Review to Assessment Development: Managing Big (and Small) Datasets with DRAGON. http://www.icfi.com/insights/products-and-tools/dragon-online-tool-systematic-review. Health Assessment Workspace Collaborative (HAWC): A Modular Web-based Interface to Facilitate Development of Human Health Assessments of Chemicals. https://hawcproject.org/portal/.

OHAT Risk of Bias Questions for Evaluating Experimental Animal Studies	
Response Options	
Definitely Low *risk of bias:* There is direct evidence of low risk of bias practices.	++
Probably Low *risk of bias:* There is indirect evidence of low risk of bias practices **OR** it is deemed that deviations from low risk of bias practices for these criteria during the study would not appreciably bias results.	+
Probably High *risk of bias:* There is indirect evidence of high risk of bias practices **OR** there is insufficient information (e.g., not reported or "NR") provided about relevant risk of bias practices.	–/NR
Definitely High *risk of bias:* There is direct evidence of high risk of bias practices.	–

Critical Risk of Bias Domains

Randomization to treatment group, blinding during neurobehavioral outcome assessment, adequate characterization of the administered chemical, and controlling for litter effects in developmental studies were considered key factors in determining whether a study had an overall very serious risk of bias concern, referred to as a "tier 3" study in the OHAT Handbook (NTP 2015a). Studies considered as having "probably high" or "definitely high" risk of bias in several of these domains were classified as tier 3 studies and excluded from the main analysis, although the impact of excluding the studies was considered. Studies also may be considered tier 3 due to a combination of concern for risk of bias in a critical domain(s) and very poor reporting quality (e.g., not reporting the number of animals treated).

Randomization and blinding during outcome assessment were considered especially critical factors for risk of bias assessment because of empirical support that failure to implement these factors can bias results away from the null toward larger effects (Higgins and Green 2011; Krauth et al. 2013). The extent of empirical support documenting these biases in animal studies is better documented compared to other risk of bias factors, for example, blinding during allocation to study groups or during the course of the study (NTP 2015b). In addition, concern for lack of blinding during allocation or the conduct of the study can be attenuated if blinding was implemented at outcome assessment. For these reasons, blinding at outcome assessment was weighed more heavily during risk of bias assessment than blinding during allocation concealment or during the course of the study. In neurobehavioral studies, concern for lack of blinding at outcome assessment is attenuated if behavioral parameters are measured by an automated, computer-driven system.

Adequate characterization of the test compound is necessary to evaluate the purity and stability of the chemical exposure. Independent verification of purity would be considered best practice because the identity and purity as listed on the bottle can be inaccurate. In NTP's experience, about 3% of chemicals purchased are the wrong chemical, and the inaccuracy rate of chemical labeling increases to 10% if inaccurate reporting of purity is included (unpublished, personal communication Brad Collins, NTP chemist). Impurities also might be more toxic than the compound of interest.

Adequate control for litter effects when littermates are used in an experiment is considered essential in developmental studies. In 2000, NTP co-sponsored a workshop with EPA, "Low Dose Endocrine Disruptors Peer Review." As part of the peer review, a group of statisticians

reanalyzed several "low" dose studies (Haseman et al. 2001). Based on studies that used littermates, they determined that litter or dam effects were generally present such that pups within a litter were found to respond more similarly than pups from different litters. The overall conclusion was "[f]ailure to adjust for litter effects (e.g., to regard littermates as independent observations and thus the individual pup as the experimental unit) can greatly exaggerate the statistical significance of experimental findings."

Evidence Synthesis

Endpoint Grouping

Neurobehavioral endpoints were categorized by nature of behavioral domains using the framework below (Table 4). Findings were synthesized at the test/endpoint level to reach conclusions about potential impacts of fluoride at the domain level (e.g., learning and memory, motor, anxiety). The priority domain in this report is learning and memory.

Table 4. Framework for Neurobehavioral Groupings

General Domain	Example Test and Endpoints
Learning and Memory	Maze tests (Morris water maze, T-maze, Y-maze); exploration (novel object recognition, mini-holeboard, activity cage); active and passive avoidance (step-down test, shuttle box); operant behavior
Motor and Sensory Function	Locomotor activity (open field, activity cage); movement coordination (akinesia/catalepsy, plank walking, rotarod, slanted surface, swim test); reflex (auditory startle, negative geotaxis, pain response: tail immersion and Von Frey hair test); developmental motor sensory landmarks (cliff avoidance, surface righting, pivoting/orienting reflex)
Depression	Forced swim; tail suspension test
Anxiety	Elevated plus maze
Other	Grooming; urination/defecation; sexual behavior; territorial behavior

Considerations for Pursuing a Narrative or Quantitative Evidence Synthesis

Heterogeneity within the available evidence was considered when determining whether to calculate an overall estimate of effect (meta-analysis) of fluoride effects on learning and memory. The principal characteristics evaluated for heterogeneity across eligible studies include the following:

- Animal model used (species, strain, sex, genetic background)
- Age of animals (at start of treatment, mating, pregnancy status)
- Dose levels, frequency of treatment, timing, duration, and exposure route
- Behavioral measurements and methodology
- Type of data (e.g., continuous, dichotomous), statistics presented in paper, ability to access raw data
- Concern for risk of bias

Standardizing Results from Behavioral Tests and Dose Levels

Results from behavioral tests were transformed, when possible, to a common metric of percent change from control response to help assess dissimilar but related outcomes measured with different scales. In this project, percent control response was used as the common metric because it is recommended for assessing dissimilar but related outcomes measured with different scales (Vesterinen et al. 2014). Percent control group calculations were based on sample size, means, and standard deviation or standard error values presented in the studies.

For studies in which experimental animals were dosed with sodium fluoride (NaF) or other forms of dissociable fluoride, dose levels were converted to fluoride equivalents (F), for example, 100 ppm NaF = 45.3 ppm (mg/L) fluoride. In studies where F was administered directly (often reported simply as "fluoride"), no such conversions were conducted. Fluoride dose levels were standardized to mg/kg-d and ppm (mg/L). Conversions were made using water consumption rates and body weights for rats and mice reported in the EPA dosimetry (US EPA 1988; 1994). In each case, the "subchronic" values were chosen because this period fit the maternal or single-generation dosing periods in most studies. The strain-specific and sex-specific values were used when available; for strains that were not available, the "other" values were used. For the few studies in which dosing was through the feed, conversion was first made from food ppm to mg/kg-d. Then, the "effective water concentration" was estimated by multiplying the converted dietary dose by body weight and dividing by water consumption rate. The uncertainty in these estimates should be considered higher than in water consumption studies.

Unless otherwise reported by study authors, the fluoride background level was assumed 0 ppm (0 mg/kg-d). Dose levels provided in studies are presented here as mg/kg-d and ppm as available. Dose conversions were made using US EPA (1988; 1994) default food or water consumption rates and body weights (using subchronic age and experiment duration) for the species/strain and sex of the animal of interest. Dose levels in mg/kg-d can vary for a given ppm across different studies if the studies use different species/strain or sex of animals, or both, that are assumed to have different food or water consumption rates.

Assessment of Confidence in the Body of Evidence

The quality of evidence for learning and memory[6] was evaluated using the GRADE system for rating the confidence in the body of evidence (Guyatt et al. 2011a; NTP 2015a). Under the GRADE system, overall confidence in the body of evidence for an outcome is categorized as high, moderate, low, or very low. Like experimental human studies (randomized clinical trials), evidence from animal studies is initially graded as high quality by OHAT. Next, a series of adjustments ("downgrades" or "upgrades") (Table 5) were made to the initial ranking based on the characteristics of the studies constituting the body of evidence after considering factors. The factors included risk of bias across studies, unexplained inconsistency, indirectness, imprecision, publication bias, effect magnitude, dose response, and consistency across different model systems and study designs (Figure 1, see also NTP (2015a) for additional detail). Studies conducted in mammalian model systems are assumed relevant for humans (i.e., not downgraded for indirectness) unless compelling evidence to the contrary exists.

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[6]Although level-of-evidence conclusions focused on learning and memory, studies evaluating other aspects of behavior were identified, and data were extracted and assessed for risk of bias (Appendix D through Appendix R).

Initial Confidence by Key Features of Study Design	Factors Decreasing Confidence	Factors Increasing Confidence	Confidence in the Body of Evidence
High (++++) 4 Features	• **High Risk of Bias**	• **Large Magnitude of Effect**	**High (++++)**
Features • Controlled exposure • Exposure prior to outcome • Individual outcome data • Comparison group used		• **Dose Response**	
Moderate (+++) 3 Features	• **Unexplained Inconsistency**	• **Residual Confounding** − Studies report an effect and residual confounding is toward null − Studies report no effect and resid-ual confounding is away from null	**Moderate (+++)**
Low (++) 2 Features	• **Indirectness** • **Imprecision**	• **Consistency** − Across dissimilar populations − Across study design types	**Low (++)**
Very Low (+) ≤1 Features	• **Publication Bias**	• **Other** − e.g., particularly rare outcomes	**Very Low (+)**

NTP descriptions of confidence in the body of evidence categories:

High: The true effect is highly likely to be reflected in the apparent relationship; further research is very unlikely to change confidence in the apparent relationship. That is, the available evidence usually includes consistent results from well-conducted studies.

Moderate: The true effect may be reflected in the apparent relationship. That is, the available evidence is sufficient to determine an effect is apparent, but our confidence in the estimate is constrained by such factors as:

- The number, size, or quality of individual studies.
- Inconsistency of findings across individual studies.
- Limited generalizability of findings.

As more information becomes available, the magnitude or direction of the observed effect could change, and this change may be large enough to alter the conclusion.

Low: The true effect may be different from the apparent relationship. That is, our confidence is limited and the true effect may be substantially different.

Very low: The true effect is highly likely to be different from the apparent relationship; further research is very likely to have an impact on confidence in the apparent relationship. That is, our confidence is very limited and the available evidence is insufficient to assess effects.
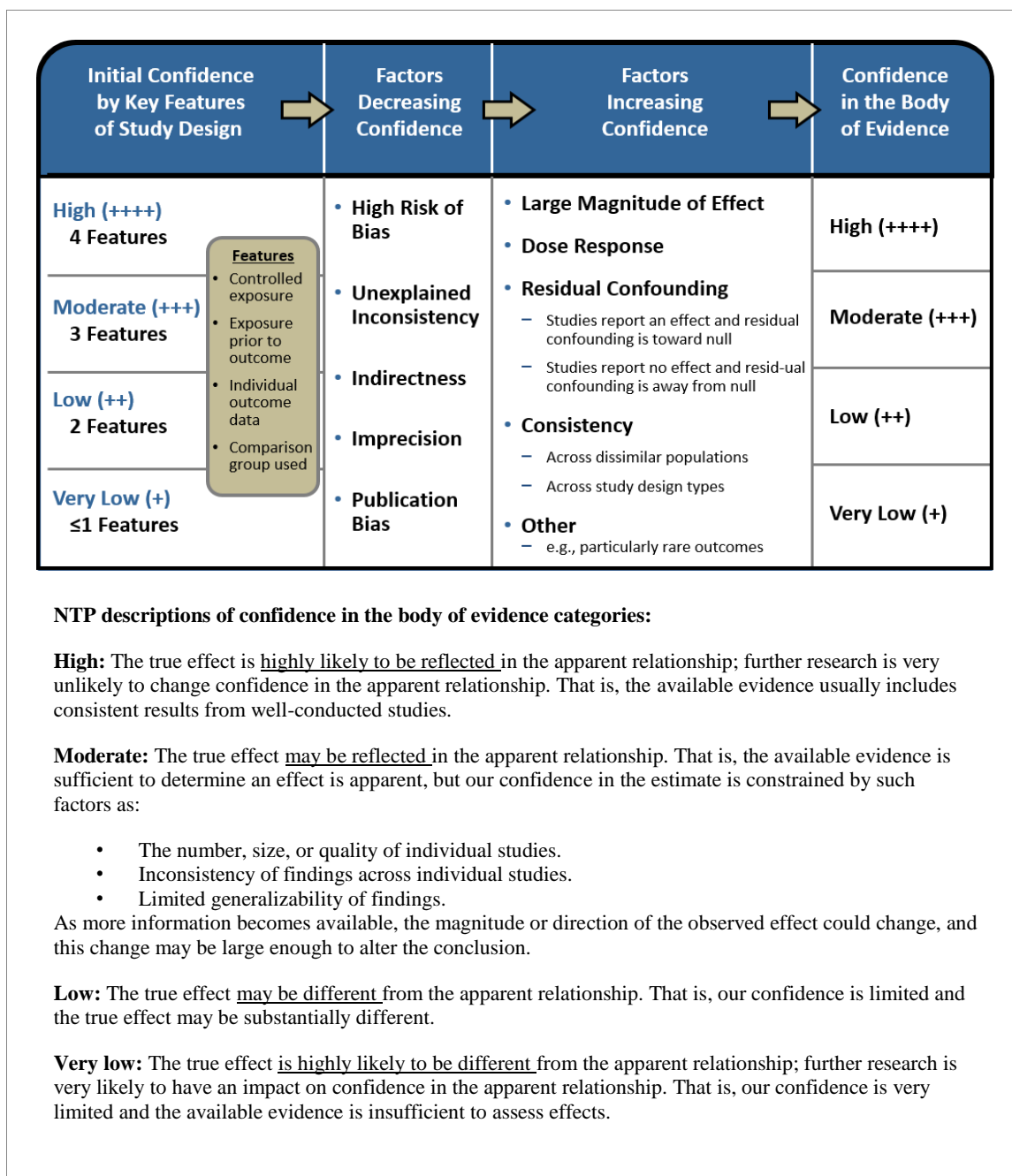
Figure 1. Assessing Confidence in the Body of Evidence ("Quality of Evidence")

Table 5. Key Factors When Considering Whether to Downgrade or Upgrade

Downgrade Factors	
Risk of Bias	Risk of bias across all domains but with priority consideration for these critical factors: Randomization, blinding at outcome assessment, exposure characterization (reporting source, purity, internal dose level), control for litter effects in developmental exposure studies.
Inconsistency	Presence of unexplained inconsistency in studies of similar design. Compared to clinical human data, differences between experimental animal studies could be larger due to factors such as use of different strains (which are often inbred to minimize variability), different exposure or treatment paradigms, and differences in handling or laboratory conditions (Vesterinen et al. 2014).
Indirectness	*Within the animal model:* Directness of the measure as an indicator of learning and memory, for example, ability to rule out impaired motor or sensory functions that might impact ability of animals to perform the learning and memory tests. Also consider route of administration; oral is considered most relevant for fluoride.
	Animal (rodent) to human extrapolation: In vivo mammalian model systems have demonstrated utility for examining autonomic, sensory, and motor system functioning as they relate to human health. Although human cognitive function is not easily assessed in such systems, aspects of learning and memory can be evaluated as based on learning theory that translates across species (Crawley 2007). Some neurobehavioral measures, however, have not been demonstrated to translate readily across species (social behaviors, aggression, risky behaviors) and others (e.g., verbal learning/performance, gender preferences) cannot be evaluated adequately in a nonhuman mammalian model system.
Imprecision	Confidence in quantitative measures such as effect sizes, identification of no observed effect level (NOEL) or lowest observed effect level (LOEL) doses, or parameters for benchmark dose analysis. Typically, 95% confidence intervals are used as the primary method to assess imprecision (Guyatt et al. 2011b). OHAT also considers whether studies are adequately powered when considering whether to downgrade.
Publication Bias	Downgrade if "strongly detected." Publication bias is difficult to assess (Guyatt et al. 2011c), especially when multiple endpoints related to the primary outcome are reported in the same study, few studies are available, and papers lack reporting on funding and conflict of interest. Analytical tools, such as funnel plots or trim-and-fill approaches, can be useful to assess publication bias but also have substantial limitations and should be interpreted with caution (Guyatt et al. 2011c).
Upgrade Factors	
Large Magnitude of Effect	Determining whether the magnitude of the effect is large includes consideration of factors such as the effect being measured and the dose range used. No upgrade considered if serious concern for risk of bias is present.
Dose Response	Patterns of dose response are evaluated within and across studies when considering whether to upgrade for evidence of dose response. No upgrade considered if serious concern for risk of bias.
Consistency across Animal Models or Species	An upgrade may be considered for consistent results reported in multiple experimental animal models or species.

Preparation of Level-of-evidence Conclusions

Confidence levels in the body of evidence conclusions from Figure 1 were translated into statements of health effects from animal studies according to one of four statements: (1) High, (2) Moderate, (3) Low, or (4) Inadequate (Figure 2). The descriptor "evidence of no health effect" is used to indicate confidence that the substance is not associated with a health effect as detected by neurobehavioral outcomes. Because of the inherent difficulty in proving a negative, the conclusion "evidence of no health effect" is reached only when confidence in the body of evidence is high.
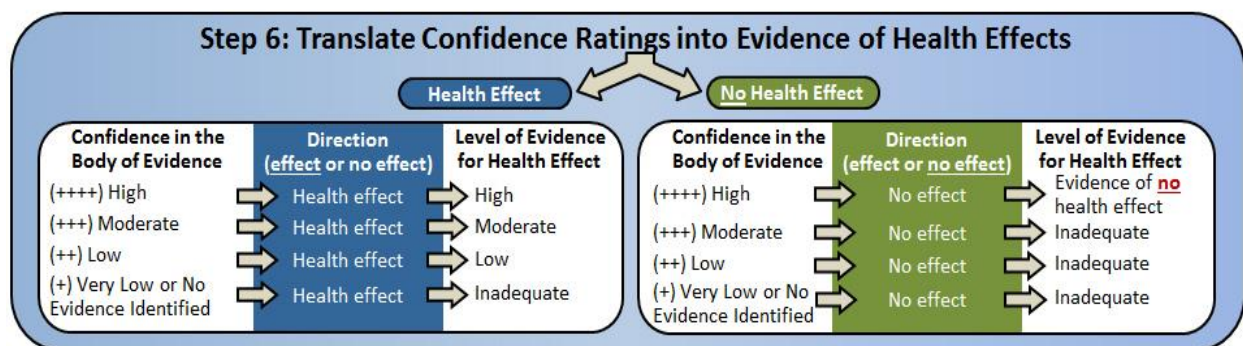
Evidence Descriptors	Definition
High Level-of-Evidence	Confidence is high in the body of evidence for an association between exposure to the substance and the behavioral outcome(s).
Moderate Level-of-Evidence	Confidence is moderate in the body of evidence for an association between exposure to the substance and the behavioral outcome(s).
Low Level-of-Evidence	Confidence is low in the body of evidence for an association between exposure to the substance and the behavioral outcome(s), or no data are available.
Evidence of No Health Effect	Confidence is high in the body of evidence that exposure to the substance is not associated with the behavioral outcome(s).
Inadequate Evidence	Insufficient evidence is available to assess if the exposure to the substance is associated with the health outcome(s).

Figure 2. Translate Confidence Ratings into Evidence of Health Effect Conclusions from Animal Studies

Results

Literature Search Results

The database searches yielded 4,643 unique records, and 13 records were identified from reviewing the reference list of included studies or the Fluoride Action Network.[7,8] Of the 4,656 studies identified, 4,552 were excluded during title and abstract screening, 104 were reviewed at the full-text level, and 68 studies were considered relevant to the PECO statement and were included (Figure 3). Most included studies were published after 2000 (Figure 4). A list of excluded studies is available on the HAWC fluoride project site.

Nineteen of the 68 studies (see Included Studies) were considered to pose a very serious overall risk of bias (Appendix C), primarily based on concern for at least 3 of the following factors: lack of randomization, lack of blinding at outcome assessment in conjunction with not using automated tools to collect information, lack of reporting on what was administered to animals (source, purity, chemical form of fluoride), lack of control for litter effects, lack of expected response in control animals, and lack of reporting of key study information such as the number of animals treated or sex. Across all the studies, information often was not reported and assumed not conducted, resulting in an assessment of "probably high" risk of bias. Authors were queried for missing information, and responses received were used to update risk of bias assessments. Sixteen of the 48 studies that reported findings related to learning and memory were excluded from the evidence synthesis due to concerns for serious risk of bias, which left 32 studies for use in the primary analysis.

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[7]Thirteen studies were identified as potentially relevant during the reference search of included studies and the Fluoride Action Network website. Two of these were not considered relevant based on full-text review (Bai et al. 2010; Vishnevskii and EL Nichnykh 1969), and we were unable to obtain a full-text record for one (Zhang et al. 2015). Thus, 10 of the 13 records identified from other sources were included.

[8]No relevant records were identified from the chemical toxicity databases after review of dossiers identified using the search term "fluoride." USEPA HPV Challenge Program Robust Summaries and Test Plans: The submission package for "Fatty Nitrogen Derived Amines Category" was reviewed for relevance. European Chemicals Agency (ECHA) Registration dossiers ("REACH"): Study summaries for repeated dose toxicity, reproduction, and developmental toxicity/teratogenicity were reviewed for sodium fluoride, potassium fluoride, calcium fluoride, ammonium fluoride, and magnesium sodium fluoride silicate.
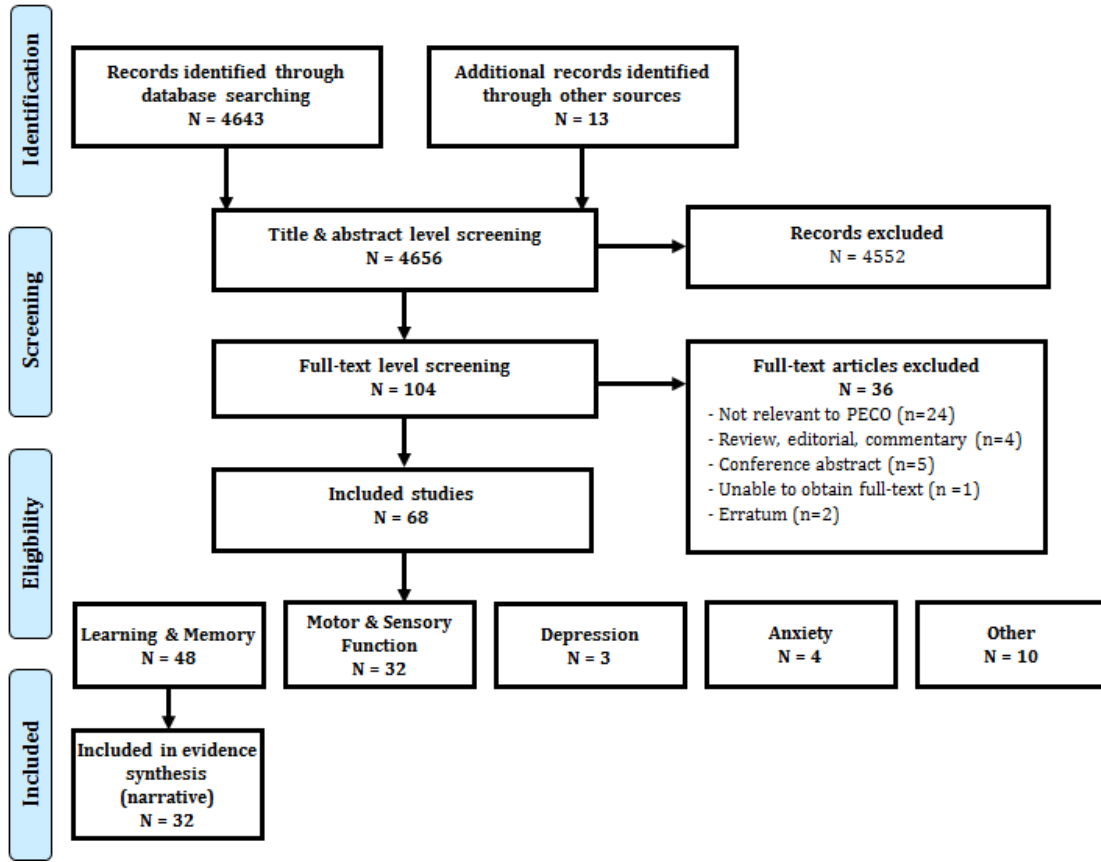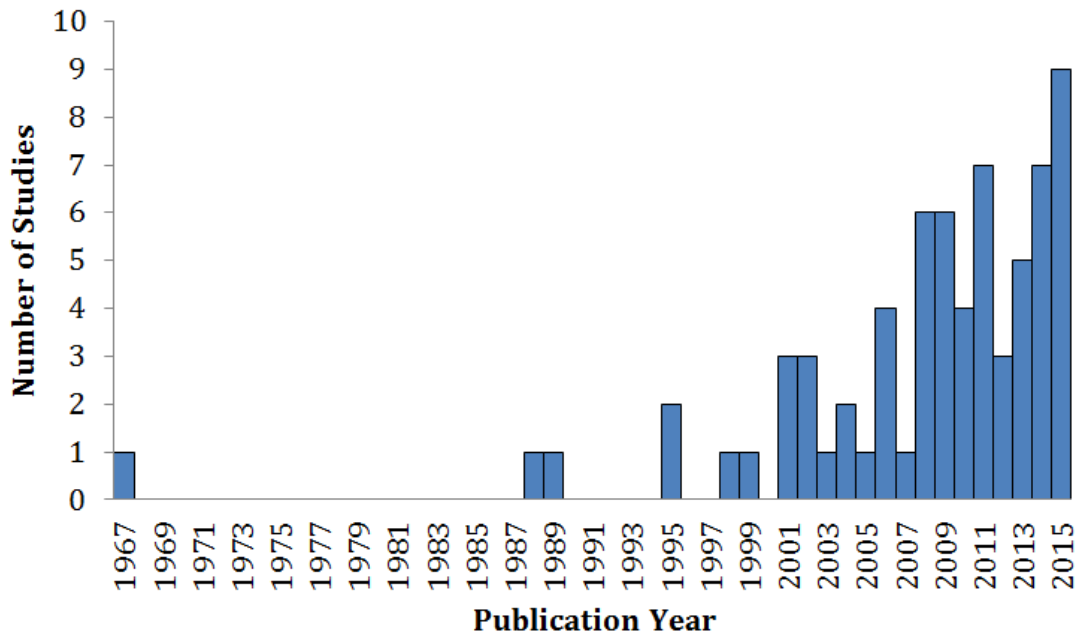
Figure 3. Study Flow Selection Diagram

Figure 4. Publication Time Trend

Included Studies

1. Balaji B, Kumar EP, Kumar A. 2015. Evaluation of standardized Bacopa monniera extract in sodium fluoride-induced behavioural, biochemical, and histopathological alterations in mice. Toxicol Ind Health. 31(1):18-30. http://dx.doi.org/10.1177/0748233712468018

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9. Bera I, Sabatini R, Auteri P, Flace P, Sisto G, Montagnani M, Potenza MA, Marasciulo FL, Carratu MR, Coluccia A et al. 2007. Neurofunctional effects of developmental sodium fluoride exposure in rats. Eur Rev Med Pharmacol Sci. 11(4):211-224.

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21. El-lethey HS, Kamel MM, Shaheed IB. 2010. Neurobehavioral toxicity produced by sodium fluoride in drinking water of laboratory rats. J Am Sci. 6(5):54-63.*

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27. Gao Q, Liu Y-J, Guan Z-Z. 2009. Decreased learning and memory ability in rats with fluorosis: increased oxidative stress and reduced cholinesterase activity in the brain. Fluoride. 42(4):277-285.

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*Identified from other sources, [ ] non-English.

Overview of Included Studies

A survey of included studies describing species, lifestage at exposure, and dose levels tested is presented in Table 6. Eighteen of the 68 studies (26%) were in non-English, defined as those for which a publicly available version in English was not available.[9] English-translated versions of several studies were available in the publication *Fluoride* and are not classified as non-English.

Table 6. Description of Relevant Studies

Descriptor	Learning and Memory	Motor and Sensory Function	Depression	Anxiety	Other[1]
Number of Studies	48	32	3	4	10
Non-English	17 (35%)	6 (19%)	0 (0%)	0 (0%)	0 (0%)
Species					
Rats	39	24	0	2	8
Mice	9	8	3	2	2
Lifestage of Exposure[2]					
Adult	33	22	3	2	5
Developmental	16	11	0	2	5
Doses Range Tested[2]	0.45–271 ppm	0.9–226 ppm	0.9–90 ppm	0.9–90 ppm	1–136 ppm
Developmental	1–200 ppm (n = 16)[3]	1–68 ppm (n = 11)	–	2.26–61 ppm (n = 2)	1–45 ppm (n = 5)
≤5 ppm	2 studies	3 studies	–	1 study	2 studies
≤4 ppm	2 studies	3 studies	–	1 study	2 studies
Adult	0.45–271 ppm (n = 33)	0.9–226 ppm (n = 22)	0.9–90 ppm (n = 3)	0.9–90 ppm (n = 2)	40–136 ppm (n = 5)
≤5 ppm	12 studies	2 studies	1 study	1 study	0 studies
≤4 ppm	12 studies	2 studies	1 study	1 study	0 studies
Studies with Very Serious Risk of Bias[4]					
Total	16/48	9/32	0	0	3/10
Developmental	9/16	5/11	–	0	2/5
≤5 ppm	2/2 (100%)	2/3 (66%)	–	–	–
≤4 ppm	2/2 (100%)	2/3 (66%)	–	–	–
Adult	7/33	4/22	0	0	1/5
≤5 ppm	2/12 (17%)	0	0	0	0
≤4 ppm	2/12 (17%)	0	0	0	0
Studies Used for Evidence Synthesis					
Total	32	–	–	–	–
Developmental	7 (11–50 ppm)	–	–	–	–
≤5 ppm	0	–	–	–	–
≤4 ppm	0	–	–	–	–
Adult	26 (0.45–226 ppm)	–	–	–	–
≤5 ppm	10	–	–	–	–
≤4 ppm	10	–	–	–	–

[1]Other includes studies assessing grooming/urination/defecation; sexual behavior, territorial aggression, vocalization.
[2]Expressed in ppm fluoride equivalents. The number of studies might not total because studies often tested multiple dose levels and some studies evaluated effects in multiple lifestages of exposure.
[3]Duplicate data from Dong et al. (2015c) appear to be reported in a second study from this group Dong et al. (2015a).
[4]See Appendix C.

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[9](Chen and Geng 2011; Dong et al. 2015a; Dong et al. 2015b; Gao et al. 2008a; Gao et al. 2008b; Gao et al. 2009b; Hong et al. 2005; Liu 1989; Liu et al. 2011; Liu et al. 2009; Rumiantsev et al. 1988; Shen et al. 2004; Wang et al. 2006; Wei et al. 2014; Xu et al. 2001; Zhang et al. 2009; Zhang et al. 2001; Zhu et al. 2012).

Behavioral Tests for Learning and Memory

Theories of learning stress the significance of drives and incentives or motivating factors in the process of acquisition and in establishing a memory of a task. In animal experimentation, there are a variety of motivating factors, including such incentives as escape from punishment, escape from water, satisfaction of hunger, satisfaction for novel experiences, sexual satisfaction, satisfaction of maternal drive, and satisfaction to return to home cage and cage mates. A relative efficacy exists for different degrees of reward and the degree-of-reward-value inherent in any incentive can be modified by varying the physiological condition of the animal (e.g., periods of deprivation).

All learning paradigms that rely on appetitive reinforcement (food or water) require animals to be placed on deprivation to increase reinforcement value. Once animals have learned to perform the task, with continued training to examine full acquisition or to transition to a more complicated paradigm, body weight is normally maintained at 80–85% of free feeding body weight. Although initially this condition might be stressful to the animals, the restricted caloric intake can be of health benefit. For toxicological assessments, consideration is required with regard to the initial health of the animals and the motivational incentive value of the appetitive reward. The use of aversive stimuli, which for the animal is reinforced by removing itself from experiencing, is by its nature stressful in the initial acquisition phase. The reinforcement value of the stimuli is a variable that requires consideration. For example, altering the pain threshold (diminished or elevated) will influence reinforcement value of a painful stimulus and, thus, acquisition.

The reliance on a motor response can result in issues that require consideration for interpretation. As an example, if latency is an outcome endpoint, absence of general motor performance equivalency could become an issue for concern. In maze-based tasks, latency often determines performance. In these cases, altered motor function or activity levels can significantly influence the outcome endpoint yet not reflect differences in learning. In avoidance tasks, for animals that display lower activity levels, performance on passive avoidance tasks can appear improved while animals with a higher level of activity can give the impression of poor performance, neither of which would directly reflect learning capability. The reverse is true for active avoidance paradigms.

Animals can display differences in response characteristic or level. For example, an animal might respond to the negative stimuli by freezing rather than escaping or might find an alternative method to endure the stimuli. These differences in response do not necessarily mean the animal has not learned the associations between cues—just that it is not performing as the test paradigm dictates. In various tests for learning and memory, the assessment of memory might inadvertently impose an extinction trial that would weaken the conditioned association between cues and influence any subsequent test session. As in all learning assessments, an understanding is required of the learning process and the manifestation of a learned behavior in an individual animal that differs from the defined response of the paradigm.

The following examples of test paradigms used to assess learning and memory in rodents are not intended to be inclusive. Rather, they reflect the tests represented in the literature for evaluating the effects of fluoride exposure on learning and memory.

Morris Water Maze

The Morris water maze is a standardized test used to evaluate aspects of spatial learning and memory in rats and mice (Vorhees and Williams 2006; 2014). The general paradigm is to allow the animal to navigate a circular water-filled tank to find a hidden platform that will allow for escape from the water. The animal accomplishes this task by using various visual cues within the spatial environment. The animal is allowed to search the tank for a defined period (60–120 seconds) to find the platform. Several sequential training trials are conducted (the most common is four trials per day). Several sequential daily training sessions are conducted consisting of multiple trials per day to reach a predetermined criterion of learning. Learning, as defined by acquisition of the task over training sessions, is evaluated by a decrease in the amount of time the animal takes to find the platform (latency), a decrease in the distance traveled to reach the platform, and (with video imaging techniques) a shift in the swimming pattern to achieve a more direct and efficient route to the platform. Additional measures collected include visual capability (seeing a visual platform above the water surface) and swimming speed across multiple sessions to confirm an absence of differences between groups that could affect performance on the Morris water maze and influence data interpretation regarding learning and memory. When the animal reaches a set criterion of performance for the Morris water maze (has learned the task), a spatial memory assessment (probe trial) is conducted. During this probe trial, the platform is removed and the search strategy of the animal is evaluated [time spent in escape platform quadrant (absolute and relative to each other quadrant), latency and distance traveled to the first time crossing the previous platform location, number of times the animal crosses the previous platform location]. These measures are collected across the total time interval for the probe trial (60–90 seconds) to capture the memory of the previous platform location (memory) and within equal time intervals (15-sec epochs) to capture the animal's ability to detect the absence of the platform and to change the search strategy, respectively. To confirm a learning deficit, a "reversal-learning" session can follow the probe trial in which the animal is required to learn a new location of the escape platform. Motor strength, motor coordination, or attention can influence any isolated measures of latency or distance traveled, rather than acquisition (better performance over time), and thus, confound interpretation of change in learning. Many studies examining the effect of fluoride exposure on Morris water maze performance relied on such isolated measures, and the acquisition of the task was not analyzed. In addition, endpoints collected during the memory probe tests often were limited to motor-dependent tasks (e.g., latency to the initial find of the learned location of the previously hidden platform, number of times the animal swam across the platform site during a single trial). Few studies included an assessment of the time an animal spent in the "platform" location.

T-Maze

The T-maze is an apparatus shaped like the letter T that can be used to evaluate exploratory activity (in absence of a reinforcement) and, in a variety of ways, to evaluate learning and memory in experimental animals. Typically, a spontaneous alternation paradigm and a reinforced alternation paradigm (working memory) were used in the fluoride studies. Normal control animals (mice and rats) display a tendency to alternate their choice of an arm of the maze to enter (e.g., an entry into the right arm on trial 1 is highly likely to be followed by an entry into the left arm of the subsequent trial). In the absence of reinforcement, this behavior is termed spontaneous alteration. The normal alternating pattern is examined over trials in quick succession as a measure that the animal remembers the arm visited in the previous session. With reinforcement

(positive or negative), the paradigm is termed a reinforced alternation paradigm. The animal is placed at the start location in the bottom of the T and allowed to traverse the alley of the maze, making a choice of turning right or left. One arm of the maze, which contains reinforcement, is considered the "correct" arm. The animal is removed from the maze and a subsequent trial conducted. Training consists of a defined number of trials within any one session and several sessions per day for a defined number of days, usually until the animals reach a previously defined criterion of the percentage of correct choices. The number of correct choices and latency to run the maze from the start location to the goal box is recorded. Acquisition is normally determined by a decrease in percentage of incorrect choices and a decrease in latency over time. Deficits in motor function can influence latency, and motivation level of the animal can influence latency and percentage of correct choices.

A multiple T-maze test is a complex maze having multiple T-junctions. Performance is measured as a right or wrong choice at each T intersection. Multiple T-mazes are used to address questions of place versus response learning and cognitive maps. A mouse or rat is placed in the maze and allowed to explore freely for a defined period across a few trials. Under food-deprivation conditions, the animal is placed in one T-end of the maze and allowed to explore the maze for a defined period to find the food reinforcement. This reinforced trial is repeated. Acquisition of the task is measured as a decrease in latency to obtain the reward and a decrease in percentage of incorrect choices at junctions. Motivation level and anxiety/fear level of the animal can influence latency and percentage of correct choices.

Y-Maze

The Y-maze is an apparatus with three arms, oriented at 120-degree angles from each other that can be used to assess exploratory behavior (animals tend to enter a less recently visited arm) or can be used, in conjunction with cued reinforcement, to examine learning and memory for selecting arm to enter. A Y-maze paradigm is similar to the T-maze paradigm in that it can record spontaneous alternation of arm entered or arms entered for reinforcement (positive or negative). A sequence of trials consists of placing the animal in the start location at the base of the maze and allowing the animal to traverse the length of the maze. Over trials, the sequence of choices is recorded to identify the pattern of alternation from one arm to the other. In controls, the choice sequence is expected to alternate with each trial under nonreinforced conditions (spontaneous alternation). For reinforced alternation, the animal's entering a specific arm (either the same or different) is reinforced with each trial, depending on the test paradigm. Acquisition of the task is measured as a decrease in the number of choice errors (entering the incorrect arm). Latency from the start box to the goal box or to the choice point also can be recorded. Differences in motor function or motor performance can influence latency. Motivation level and anxiety/fear level of the animal can influence latency and percentage of correct choices.

Classic Maze

Classic maze performance is related to the animal's progression over sessions to increase its performance as measured by latency or number of errors, depending on the maze design and paradigm. Although its design can vary, the classic maze generally requires the animal to learn to make a series of correct choices (turns) to navigate the maze successfully and receive reinforcement. Appetitive reinforcement, such as food reward, can be used with animals maintained at a level of restricted feeding to enhance motivation. Negative reinforcement also can be used. A standard paradigm for a classic maze involves the animal's being placed in the

start location of the maze and allowed to explore the maze freely for a defined period. A series of successive trials follows during which the animal learns to navigate the maze more quickly and correctly. Acquisition of the task is reflected as a decrease over trials in latency (time) to reach reinforcement or the number of times the animal makes an error in selecting the correct direction to take at a choice point, or both. Differences in motor function can influence latency. Motivation level of the animal and anxiety/fear level can influence latency and percentage of correct choices.

Passive Avoidance

Passive avoidance is a fear-motivated test used to evaluate associative learning and memory. An animal is required to learn to withhold a normal preferred response such as moving from a brightly illuminated large chamber into a smaller dark chamber or moving from an elevated platform to a lower floor. A standard paradigm involves the animal's being placed in the nonpreferred side of the apparatus. The animal is allowed to explore the apparatus freely and, upon the first entry in the normally preferred side, a negative stimulus is delivered. The animal undergoes two or three training/test sessions. The strength of the learned association is indicated by an increase in latency to leave the "safe" chamber and enter the "non-safe" chamber over sessions. The strength of the association can be examined by eliminating the adverse stimuli and measuring the number of trials required for the animal to return to a pretraining latency to enter the "non-safe" side. The strength of the reinforcement (e.g., perception of pain from the shock) influences the strength of the learning. Motor activity level can influence latency, with a decreased activity level skewing the response to appear as if a greater level of learning occurred, while hyperactivity would cause the animal to move prematurely and thus, imply a deficit in learning.

Active Avoidance

Active avoidance is a fear-motivated test used to evaluate associative learning and memory. Animals learn to associate a conditioned stimulus (light, tone) with the delivery of an unconditioned negative stimulus (e.g., shock) and to move to a safe location to escape (escape response) or avoid (avoidance response) the negative stimulus. A standard paradigm involves the animal's being placed in the test apparatus and allowed to explore the environment freely. A training trial is initiated by the delivery of a cue (light), followed within a defined time interval (5–10 sec as determined by the test parameters) by the presentation of a second cue (tone) paired with a shock (or other strong negative stimulus). The animal can escape the negative stimulus by moving from the "non-safe" environment upon the delivery of the first cue (avoidance) or with the second cue and shock (escape). With training, the animal learns an association between the cue (tone, conditioned stimulus) and the shock (unconditioned stimulus). Acquisition is demonstrated with an increase in avoidance responses and decrease in escape responses over the session. The number of escape losses (failing to make a response during the full shock delivery period), which is recorded, could reflect an absence of learned association between cue and shock or an increase in freezing behavior with fear. Active avoidance can be evaluated using various test paradigms, for example, one-way, with actively moving from one location to another; two-way, with actively moving back and forth between two locations with appropriate cues; and three-way, with the animal's being allowed to choose between two locations to escape/avoid the stimuli such as occur in a Y-maze. As with any task requiring motor performance and latency, deficits in such ability can influence the outcome. With a fear-motivated test, the characteristics of the fear response can also influence the outcome of the test.

Each factor can influence performance, independent of learning and memory as assessed by the testing paradigm.

Exploratory Behavior/Novelty Tests: Novel Object Recognition, Social Recognition, Social Novelty

These tests, which are based on an animal's natural propensity toward novelty, evaluate an animal's ability to recognize a previously presented stimulus or to adapt (habituate) to a novel environment. Stimuli can be an inanimate object, another animal (social recognition), or changes in the environment. When animals are exposed to a familiar object and a novel object, they frequently approach—and spend more time exploring—the novel object than the familiar object, which suggests the evidence of memory for the familiar object. In general, the paradigm consists of placing the test animal within a defined open arena and allowing it to adapt to the new environment. The animal is removed, and a novel object is placed within the arena. The animal is returned to the arena, and the number of contacts with the novel object and time spent within a defined radius of it are recorded. The animal is removed from the arena and a new object is placed into the arena in addition to the now familiar object. The proportion of time spent with each object and number of contacts are recorded over a defined period. As with any endpoint that relies on activity, deficits in motor function or activity level and exploration can influence the outcome in this paradigm. Although novel object recognition paradigms recently have been used with greater frequency in assessing the interactive nature of an animal with its environment, the range in differential preference between a familiar and a novel object is relatively narrow, seldom showing a preference above 70%. Thus, these results should be interpreted with caution. Differences in the approach to a novel object between rodent strains have been demonstrated that could influence test selection.

Operant Behavior

In this form of learning, the animal displays a behavior for which they receive reinforcement. Multiple test paradigms can be used for operant conditioning, and appetitive or aversive stimuli can be used to define the reinforcement paradigm. Operant conditioning offers additional power in the ability to elicit the behavior under a schedule of required performance (schedule-controlled behavior). This feature permits increasing complexity to evaluate the nature of a learning deficit. In a more standard experimental operant paradigm, animals are placed within an operant chamber containing a bar that can be pressed or an opening to nose poke to deliver a food pellet or fluid reward; the animals then are "shaped" to perform the operant task (bar press, nose poke) on a continuous reinforcement schedule during which every appropriate behavior results in reinforcement. Once the animal is trained on the continuous reinforcement schedule—has reached a defined level of performance—the paradigm can shift to a different schedule of reinforcement. This different schedule enables examination of the animal's ability to learn a temporal association with behavior and reinforcement (interval) or an association of number of behavioral events and reinforcement (ratio), each of which can be set as fixed or variable. These then can increase in complexity or demand on the animal. Additional test paradigms and schedules can be used to test for other aspects of learning that are outside the focus of this document. Operant behavior offers a very powerful tool to assess learning capability and limitation; this test, however, is used rarely in assessments of neurotoxicity, given the demands of time and expertise and the equipment costs.

Effects of Fluoride on Learning and Memory

Evidence Synthesis

Results were analyzed two ways. First, the collection of studies for each general type of behavioral test (Morris water maze, Y-maze, etc.) was compiled. In many cases, the overall quality of the evidence for each type of behavior test clearly would warrant a downgrade based on serious concern for risk of bias. This concern arose because several individual studies were considered to have "probably high risk of bias" in several critical domains: randomization, blinding at outcome assessment (especially when computer-assisted or automated measurements were not made), exposure characterization, and control for litter effects in developmental studies (Appendix C). Thus, these studies were excluded when reaching level-of-evidence conclusions. Almost one third of the learning and memory studies were considered to present a very serious risk of bias. Nevertheless, the results of the excluded studies are summarized below and presented in Appendix D through Appendix J. An assessment on the impact of excluding these studies found that, if included, these studies would not have appreciably altered conclusions. Quality and level-of-evidence conclusions focused on learning and memory, although studies evaluating other aspects of behavior (motor and sensory function, depression, anxiety, grooming/urination/defecation, territorial aggression, and sexual behavior) were identified, data extracted, and assessed for risk of bias (see Appendix K through Appendix R).

No meta-analysis was conducted because few studies measured endpoints similarly in terms of study design, that is, use of same behavioral tests, dose levels, duration of exposure, lifestage at exposure, or species. Level-of-evidence conclusions were based on a narrative analysis of patterns of response across different testing paradigms and types of learning, including maze learning, avoidance learning, novel object recognition, and operant conditioning. The impact of potential confounding factors on the endpoint and subsequent interpretation also was considered. In many cases, the specificity of an effect as learning and memory could not be ascertained given the motor performance dependency of the tasks and the absence of data on motor or sensory functions to help interpret results from learning and memory tests. The extent to which skeletal fluorosis might have occurred is also unclear, especially at the higher concentrations tested; one would expect, however, that skeletal fluorosis more likely would be associated with exposure durations exceeding those that have been used in studies assessing neurobehavioral toxicity. Based on control values and the number of animals per group, most studies appear statistically underpowered to detect <10% or <20% change from controls for most behavioral endpoints.

Findings are summarized below with fluoride exposures expressed as ppm and as mg/kg-d format in Appendix S (individual studies) and Appendix T (range across studies for a given ppm level).

Exposure during Development

A **low level-of-evidence** conclusion was reached based on a pattern of findings suggestive of an effect on learning and memory in rats or mice treated during gestation through postnatal day (PND) 28 or continuing until PND105 at dose levels of ~11 to 45.3 ppm fluoride (Dong et al. 2015c; El-lethey et al. 2010; Gui et al. 2010; Jiang et al. 2014a; Wang et al. 2004; Wei et al.

2014).[10] The level-of-evidence was rated down due to concern for indirectness and lack of control for potential litter effects. Interpretation of behavioral data as a specific effect on learning and memory was hindered by concerns that the effects were secondary due to changes in motor or sensory function that could impair the animal's ability to perform the task. This was considered a form of indirectness. Additional studies are required to have higher confidence in the specificity of the responses as learning or memory impairments and to provide quantitative measures such as the effect sizes, point of departure, identification of NOEL or LOEL doses, or parameters for benchmark dose analysis. No studies evaluated a male/female difference in response to fluoride treatment (male and female offspring were pooled during analysis) and studies used Sprague Dawley or Wistar rats, so discerning any sex or species differences in response was not possible. The group size ranged between 6 and 12 for the behavioral studies. Based on variability in the control groups, these group sizes were considered statistically underpowered to detect <10% or <20% change from controls for most behavioral endpoints. When reported, the fluoride concentrations in the drinking water of control groups ranged from 0.15 to 1.77 ppm (Dong et al. 2015c; Gui et al. 2010; Jiang et al. 2014b; Wei et al. 2014).

Morris Water Maze

Several studies examined Morris water maze performance in animals exposed during development to ~11 to 50 ppm fluoride (Dong et al. 2015c; Gui et al. 2010; Jiang et al. 2014a; Wei et al. 2014). Exposed animals often took longer or traveled farther to find the platform during the acquisition phase (referred to as "escape time") (Dong et al. 2015c; Gui et al. 2010; Jiang et al. 2014a) and magnitudes of the effects were often twice as great or more (Figure 5). Within a study, Morris water maze endpoints typically were internally consistent, that, if a slower escape time was observed at the end of the acquisition phase, the animals also displayed fewer platform crossings and spent less time in the target quadrant during the probe test. A NOEL was not identified, and the LOEL was 11.3 ppm fluoride in F1 generation Sprague Dawley male and female rats treated from gestation day (GD) 0 through PND60 (Jiang et al. 2014a). The one study that tested more than one dose level showed evidence for dose response (Jiang et al. 2014a). Some evidence was shown for dose response across studies, although variations in treatment time and endpoints assessed limited this analysis. Two studies were excluded based on concerns for overall high risk of bias (Wu et al. 2008; Zhang et al. 2009); (Appendix C). Across the remaining studies were "serious" concerns for risk of bias based on a lack of controlling for litter effects, which OHAT considered a critical risk of bias factor in developmental studies (see Figure 7). Failure to adjust for litter effects can exaggerate the statistical significance of experimental findings (Haseman et al. 2001). Also, in most of these studies, acquisition (learning) of the task either was not reported or limited to a latency measurement on the last day of training. This raised concerns for indirectness/confounding because assessment of learning and memory in the Morris water maze highly depends on latency and swimming capability. The longer latency during acquisition could reflect an effect on learning or, alternatively, impaired motor function.

Jiang et al. (2014a) exposed male and female Sprague Dawley rats to ~11.3, 22.6, or 45.3 ppm fluoride beginning at 65 days of age for 10 days, at which time the animals were mated and females continued throughout pregnancy and lactation. The offspring were maintained at the

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[10]Duplicate data from Dong et al. (2015b) appear to be reported in a second study from this group (Dong et al. 2015a).

same dose level until 60 days of age. A single value for latency or total distance traveled showed increased time and distance covered to complete the task in all fluoride exposure groups. A single value of swimming speed showed no effect of fluoride exposure. Acquisition of the task over training days was not analyzed. In the memory probe test, the percentage of time spent in the target zone and the percent distance traveled in the target zone, as compared to the remaining area, were less in the fluoride-exposed rats at all concentrations tested.

Other studies used exposure paradigms designed to create fluorosis in the parental population. Gui et al. (2010) exposed a male and female parental generation of Sprague Dawley rats from 30 to 190 days of age to ~30 ppm fluoride in the drinking water. At 190 days of age, rats were mated and continued on ~30 ppm throughout lactation. The F1 offspring were maintained at the same dose level until postnatal assessment. Across 5 days of training in the Morris water maze, all animals showed acquisition (learning) of the task; across training days, however, latency remained longer for the fluoride-exposed offspring. In the memory probe trial, fluoride-exposed offspring showed less time spent in the escape quadrant of the arena and, concurrently, fewer crossings of the location of the previously hidden platform as analyzed by a paired t-test.

Dong et al. (2015b) exposed the male and female parental generation of Sprague Dawley rats from 30 to 300 days of age to 22.6 ppm fluoride in the drinking water. At 300 days of age, rats were mated and continued on 22.6 ppm throughout lactation. The offspring were maintained at the same dose level until postnatal assessment. Latency to reach the hidden platform was longer in fluoride-exposed rats; data on acquisition (learning) of the task were not presented. In the probe trial to assess memory of the platform location, the number of times the rat crossed the previous location of the hidden platform was less in the fluoride-exposed rats. Similar changes were observed in the parental population. Apparently, identical data were reported in a second study from this group (Dong et al. 2015a).

Wei et al. (2014) established fluorosis in 30-day-old male and female Sprague Dawley rats by drinking water exposure to 22.6 ppm fluoride for 150 days. Rats were mated, and exposure continued until PND30. The fluoride-treated offspring took longer to find the hidden platform in the Morris water maze probe test.

Classic Maze
Acquisition but impaired performance in the classical maze test was reported in Wistar rats exposed to 45.3 ppm fluoride via drinking water from GD8 to PND105 (El-lethey et al. 2010) (Figure 6, see "maze test" responses). In this study, rats were exposed to 22.6 or 45.3 ppm during GD8 to PND30 or GD8 to PND105 to assess the impact of dose and duration of exposure. The study states an overall effect of fluoride exposure on longer latency and number of errors in locating food. **[Variance was not presented for each data point and significance determined by post-hoc analysis for individual days was not clearly indicated.]** Performance on the first training day indicated that animals receiving 45.3 ppm fluoride showed longer latencies and greater errors to locate feed. During the 5 days of maze testing, all fluoride-exposed rats displayed learning of the task as demonstrated by acquisition from training day 1 to day 5 and a decrease in the time required and number of errors in locating the feed at the end of the maze. Rats exposed to 45.3 ppm from GD8 to PND105 displayed significantly longer latency to complete the maze with more errors (blind entries) compared to the control group across the entire training session. The change in performance on training day 5 as compared to training day 1, however, suggested that choice errors no longer differed across exposure groups while latency

remained longer and errors were more frequent only in the 45.3-ppm group exposed at PND105. No effects were reported for the 22.6-ppm dose group exposed until PND30. Duration of exposure was a significant influence on latency only to complete the maze test. **[Based on text in the results and discussion, the authors do not appear to have considered effects in the 22.6-ppm dose groups or the 45.3-ppm GD8 to PND30 group significant.]** One other study evaluating maze test performance was excluded based on concerns for overall high risk of bias (Bǎran-Poesina et al. 2013) (Appendix C).

Passive Avoidance

Wang et al. (2004) treated 30-day-old Wistar rats with fluoride (controls <0.6 ppm; fluoride 43.5 ppm) in the drinking water for 3 months, which were then impregnated and continued on fluoride in the drinking water throughout lactation. Offspring were continued on fluoride for a minimum of 30 days post-weaning. Animals were maintained on a dry food diet containing 25.57 mg/kg fluoride. Learning and memory were assessed by a "step-down" passive avoidance test at 30, 60, or 90 days following cessation of fluoride. The number of errors in the initial training trial was not altered by fluoride exposure. The time to first error in test session showed 30–50% variance, and less time was observed only in the 30-day test group (Figure 6). In the test session, at the 30-day time point only, the number of errors was greater in the fluoride-exposed group (0.666 ± 0.21) as compared to the total absence of errors in the controls. **[Results for shock number are not shown in Figure 6 due to inability to calculate a percent change in number of shocks as compared to 0.]** Two studies assessing passive avoidance (Bera et al. 2007; Hong et al. 2005) and two studies assessing active avoidance (Bera et al. 2007; Liu 1989) were excluded based on concerns for overall high risk of bias (Appendix C).

Exploration

Differences in exploratory activity were reported in adult rats developmentally exposed to fluoride. In this study, female Wistar rats were exposed to 22.6 or 45.3 ppm in the drinking water during GD8 to PND30 or GD8 to PND105 to assess the impact of dose and exposure duration (El-lethey et al. 2010). All rats demonstrated habituation to the open-field arena over 3 days of 3-minute testing; percent change over time representing habituation was not calculated. Rats exposed to 43.5 ppm fluoride via drinking water showed higher activity levels and an increase in the number of freezing instances (time spent not moving). Exploratory activity in an arena allowing for head exploration into holes in the floor (head dipping) showed no significant difference in the change in performance from day 1 to day 2 between dose groups (Figure 6). Across the 2 days, rats exposed to 43.5 ppm until PND150 showed a higher level of exploratory activity measured by rearing and head-dipping as compared to controls. Variance was not presented for each data point, and significance determined by post-hoc analysis at individual days was not indicated. No effects were observed in animals exposed to 22.6 ppm fluoride. **[Based on text in the results and discussion, the authors do not appear to have considered effects in the 22.6-ppm dose groups or the 45.3-ppm GD8 to PND30 group significant.]** Two studies were excluded based on concerns for overall high risk of bias (Bera et al. 2007; Niu et al. 2014) (Appendix C).

Findings from High Risk of Bias Studies

Nine developmental studies were excluded based on concerns for overall high risk of bias (Table 7, see also Appendix D through Appendix J). Of these, three developmental studies were identified that tested concentrations of fluoride at 10 ppm or lower (Bera et al. 2007; Wu et al.

2008; Zhang et al. 2009). In the Bera et al. (2007) study, Harlan Wistar female rats received daily gavage doses of fluoride (7 and 14 ppm) from GD1 throughout lactation to PND9. Control rats received deionized water. Fluoride-exposed offspring showed no differences in multiple endpoints assessing open-field activity. Males showed decreased performance on the roto-rod, passive avoidance, and active avoidance with significance observed at 14 ppm. All animals showed a normal habituation to a novel object. The authors state that female rats receiving 14 ppm showed no shift from trial 1 to trial 2 of the initial novel object exploration test or the preference novel object test; however, data were not presented showing the variance of all data sets. Although the percent change in median response from trial 1 to trial 2 in the 14-ppm exposure group was less for novel object habituation, for novel object preference it was similar to that observed in controls and greater than that seen in the 7-ppm group. No effects were observed in the 7-ppm group. In Morris water maze tests, no statistically significant effects on escape latency were observed following maternal drinking water ingestion at 4.5 ppm fluoride in ICR rats treated from GD7 to birth (Zhang et al. 2009). In Wistar rats exposed to 5 ppm fluoride from GD0 to PND45, Wu et al. (2008) reported no differences in training trials ("learning correct path") or time required to reach the platform in a water maze task. At 25 ppm fluoride, an increase was observed in latency, but variance was >40%.

Table 7. Learning and Memory Findings from Developmental Exposure Studies Excluded Based on Concern for High Overall Risk of Bias

Reference	Treatment	NOEL	LOEL
1. Băran-Poesina et al. (2013)	NMRI mice (F1, n = not reported, ♂♀); drinking water during development with 18.5 or 37.1 ppm (5 or 10 mg/kg-d) [P0 generation from GD8 to PND0; F1 dosing duration unclear]	–	18.5 ppm (5 mg/kg-d): <u>maze test on PND21</u>: ↑ completion time in ♂♀
2. Basha et al. (2011)	Wistar rat (F1–F3, n = not reported, ♂♀); drinking water during development with 100 or 200 ppm (16 or 32 mg/kg-d) [GD0 to lactation, exposure throughout life in F1 and F2 generations]	–	100 ppm (16 mg/kg-d): <u>T-maze on PND30</u>: ↑ errors in retention phase and ↑time to reach learning standard (F1–F3 generation)
3. Bera et al. (2007)	Wistar rat (F1, 6–12 ♂♀); gavage to dam with 7 or 14 ppm (1.13 or 2.26 mg/kg-d) [GD0 to PND9]	7 ppm (1.13 mg/kg-d)	14 ppm (2.26 mg/kg-d): active avoidance on PND60: ↓percentage of animals displaying conditioned avoidance response in 4th session (no effect in 1st session) passive avoidance on PND60: ↓avoidance latency (no effect on approach latency) <u>novel object test on PND40:</u> ↑time spent exploring familiar object and both objects in trial 2

Systematic Literature Review on the Effects of Fluoride on Learning and Memory in Animal Studies

Reference	Treatment	NOEL	LOEL
4. Hong et al. (2005)	Wistar rat (F1, 6 ♂♀); gavage development with 45 ppm (6.9 mg/kg-d) [GD0 to PND90]	–	45 ppm (6.9 mg/kg-d): <u>passive avoidance on PND60–120:</u> altered response in step down test
5. Liu (1989)	Wistar rat (F1, 7–8 ♂♀); drinking water during development with 13.6 or 27.2 ppm (2.2 or 4.4 mg/kg-d) [GD0 to PND0]	–	13.6 ppm (2.2 mg/kg-d): active avoidance on ~PND35: ↑latency of conditioned avoidance response on day 9 (no effect on day 2)
6. Niu et al. (2009)	Wistar rat (F1, n = 8, sex not reported); drinking water during development with 68 ppm (11 mg/kg-d) [GD0 to PND21, then directly dosed to 12 weeks]	–	68 ppm (11 mg/kg-d) <u>Y-maze at 6 or 12 weeks:</u> ↑time to reach learning standard at 12 weeks and total reaction time at 6 weeks
7. Niu et al. (2014)	Kunming mouse (F1, 15 ♂); drinking water during development with 68 ppm (18 mg/kg-d) [GD0 to PND56]	68 ppm (18 mg/kg-d) [no effects in novel object test on PND56]	–
8. Wu et al. (2008)	Wistar rat (F1, 40 ♂♀); drinking water during development with 1, 5 or 25 ppm (0.2, 1 or 5.3 mg/kg-d) [birth to PND45]	5 ppm (1 mg/kg-d)	25 ppm (5.3 mg/kg-d): <u>MWM on PND45:</u> ↑numbers of errors during acquisition phase and ↑time to find platform during probe test
9. Zhang et al. (2009)	ICR rat (F1, 9–10 ♂♀); drinking water during development with 1.13, 2.26 or 4.52 ppm (0.18, 0.36 or 0.71 mg/kg-d) [GD7 to PND0]	4.52 ppm (0.71 mg/kg-d) [no effects in MWM assessed at 1 month of age]	–
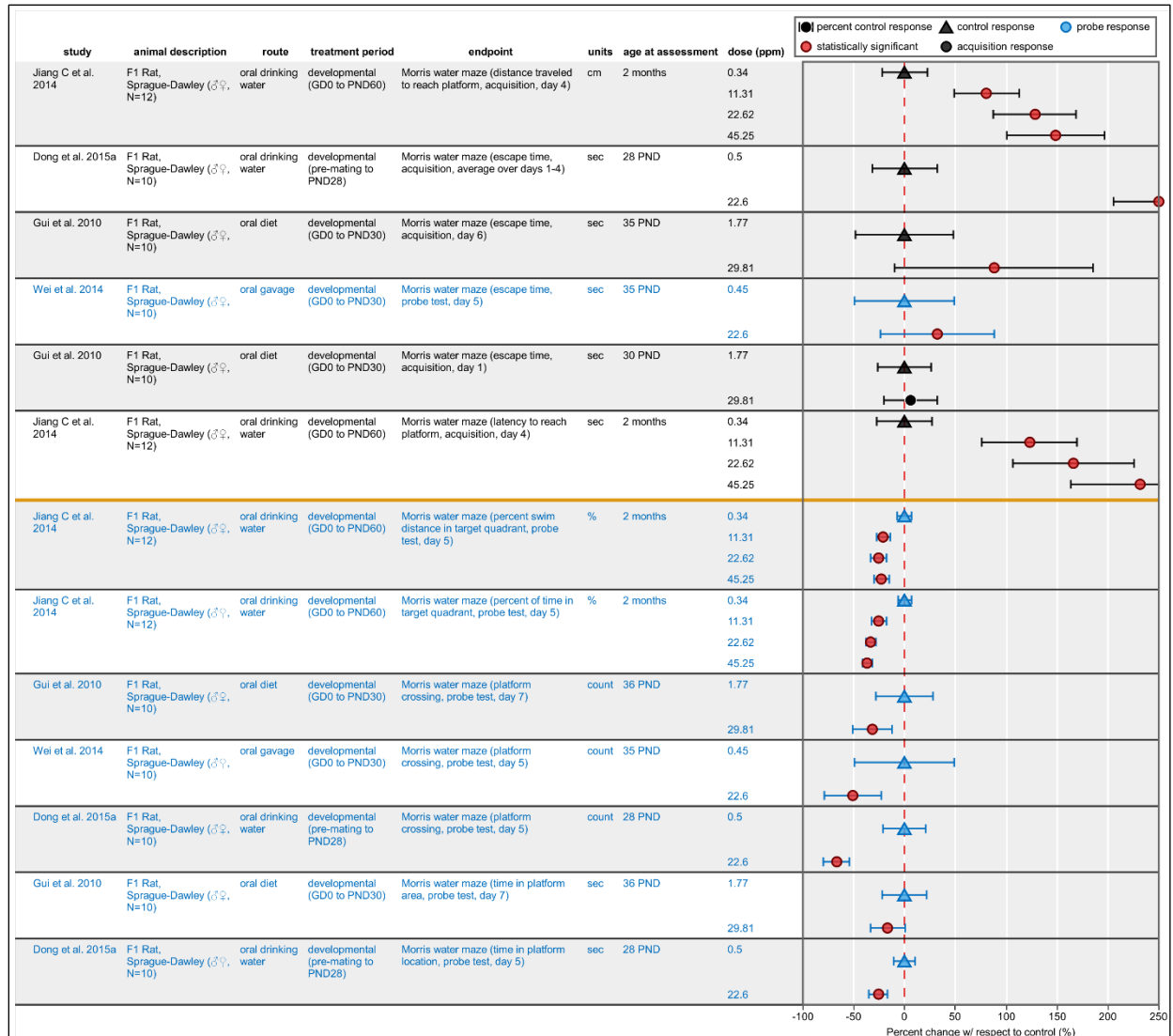
Figure 5. Results from Developmental Exposure Studies Using Morris Water Maze

Ordered by direction of expected adverse effect.
Click here for interactive graphic.
Apparently identical data were reported in Dong et al. (2015b) and Dong et al. (2015a); data only from Dong et al. (2015b) are presented in figure. Effects are grouped by a solid orange horizontal line by expected adversity direction (increase from reference/control group, then decrease from reference/control group).

Figure 6. Results from Developmental Exposure Studies in Other Maze, Exploration, and Passive Avoidance Tests

Ordered by direction of expected adverse effect.

Click here for interactive graphic. El-lethey et al. (2010) does not report standard errors/standard deviations, so confidence intervals on percent control response cannot be calculated. Effects are grouped by a solid orange horizontal line by expected adversity direction (increase from reference/control group, then decrease from reference/control group, then any change from reference/control group).
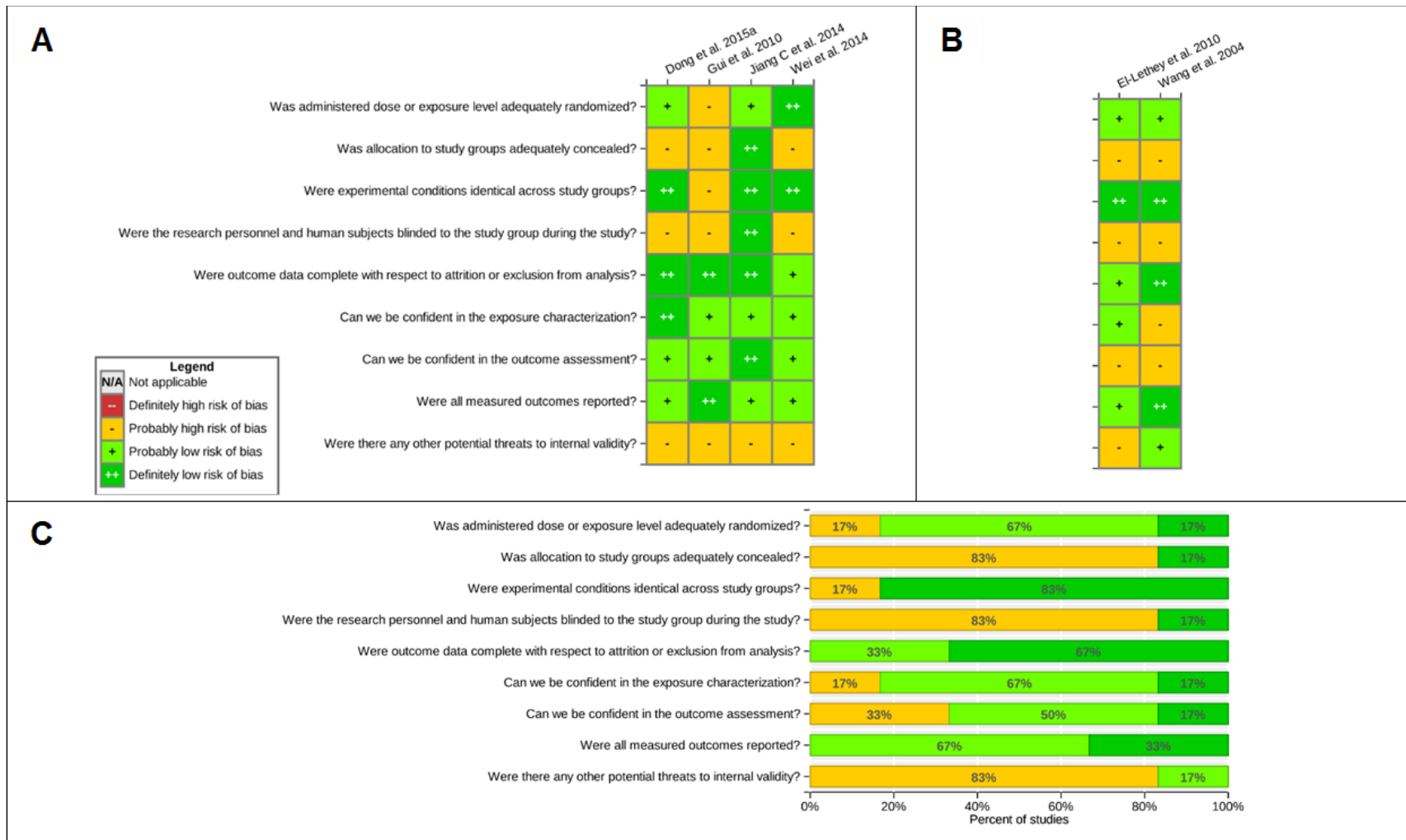
Figure 7. Risk of Bias in Studies in Studies Assessing Developmental Exposure to Fluoride

A. Risk of bias in individual studies using the Morris water maze; B. Risk of bias in individual studies assessing classic maze performance, passive avoidance or exploration; C. Risk of bias across developmental exposure studies

Apparently identical data were reported Dong et al. (2015b) and Dong et al. (2015a) [data only from Dong et al. (2015b) are presented in figure]. Studies at high overall risk of bias excluded (Băran-Poesina et al. 2013; Basha et al. 2011; Bera et al. 2007; Hong et al. 2005; Liu 1989; Niu et al. 2014; Niu et al. 2009; Wu et al. 2008; Zhang et al. 2009).

Exposure during Adulthood

Results show a pattern of findings that suggests an effect on learning and memory when animals were exposed shortly after weaning or in adulthood at dose levels of 2.26 to 226.24 ppm fluoride. Evidence was most abundant and strongest based on findings from the Morris water maze with effects reported at 2.26–62.7 ppm (Dong et al. 2015b; Dong et al. 2015c; Gao et al. 2009a; Gao et al. 2008a; Gao et al. 2008b; Gao et al. 2009b; Jiang et al. 2014b; Li et al. 2015; Liu et al. 2014; Liu et al. 2011; Liu et al. 2010; Liu et al. 2009; Zhu et al. 2012). Support for an effect on learning and memory from the Morris water maze findings was considered **moderate-level-of-evidence** due to concern for indirectness. The same issues raised in developmental-exposure studies hinder interpretation of behavioral data from adult exposure, that is, secondary effects due to changes in motor or sensory function that could impair the animal's ability to perform the task. Findings from T-maze, Y-maze, passive avoidance tests, and active avoidance tests supported an effect on learning and memory at 2.71 to 100 ppm but were considered **low-level-of-evidence** due to concerns for indirectness described above and risk of bias (lack of blinding at outcome assessment and characterization of the test compound)(Chen and Geng 2011; Chioca et al. 2008; Li et al. 2015; Niu et al. 2008; Raghu et al. 2013; Shen et al. 2004; Sun et al. 2008; Wang et al. 2006; Wu et al. 2006; Xu et al. 2001; Zhang et al. 2013; Zhang et al. 1999). Additional studies targeted to address these issues are required to achieve higher confidence in learning or memory impairments. Studies also are required that provide quantitative measures such as the effect sizes, point of departure, identification of NOEL or LOEL doses, or parameters for benchmark dose analysis. Most of the studies that indicated an effect of fluoride exposure on learning and memory were maze or avoidance studies. As in all learning tests, assessment requires measuring the acquisition of the task (increase in performance over time). When such data were available, fluoride-exposed animals often demonstrated acquisition similar to the controls. In these studies, a pattern emerged suggesting the need for (1) further evaluation of an effect on learning and memory using paradigms that are not influenced by motor functions or anxiety/fear reactions or (2) design of studies to interrogate the underlying effect influencing the performance more closely.

Most studies used rats (Sprague Dawley, Wistar); use of mouse models (BALB/c, ICR, Kunming) was more limited. No studies specifically evaluated a male/female difference in response to fluoride exposure, often only males were examined, males and females were pooled or, when sex was considered, unequal group sizes were compared. Thus, determining sex or species/strain difference in response to fluoride exposure was not clear. From the available data, the lowest concentration effects were observed in the 2.26- to 2.71-ppm range in mice (BALB/c in Morris water maze; Kunming in Y-maze) (Liu et al. 2014; Xu et al. 2001) and rats (Sprague Dawley in Morris water maze) (Liu et al. 2011; Liu et al. 2010; Liu et al. 2009). The group size used in most studies ranged between 6 and 10 animals with a limited number of studies using as few as 3 or as many as 30. Based on variability in the control groups, these group sizes were considered statistically underpowered to detect <10% or <20% change from controls for most behavioral endpoints. When measured, the baseline concentration of fluoride in control drinking water ranged from 0.23 to 1 ppm (Chioca et al. 2008; Gao et al. 2009a; Gao et al. 2008b; Liu et al. 2011; Liu et al. 2010; Liu et al. 2009; Raghu et al. 2013).

Morris Water Maze

The Morris water maze has been used to assess effects of adult exposure to 2.26–62.68 ppm fluoride (Figure 8). One study was conducted in BALB/c mice (Liu et al. 2014), while the others used Sprague Dawley rats (Dong et al. 2015c; Gao et al. 2009a; Gao et al. 2008a; Gao et al. 2008b; Gao et al. 2009b; Jiang et al. 2014b; Liu et al. 2011; Liu et al. 2010; Liu et al. 2009; Zhu et al. 2012) or Wistar rats (Li et al. 2015). In general, effects observed were related to latency (time) for the animal to reach an escape platform. Within a study, Morris water maze endpoints were typically internally consistent. If a slower escape time was observed at the end of acquisition phase, the animals also displayed fewer platform crossings and spent less time in the target quadrant during the probe test. With latency at the end of training serving as a critical effect, the NOEL was 0.9 ppm fluoride (Liu et al. 2014) and the LOEL was 2.26 ppm fluoride following 30 days of treatment in BALB/c male mice (Liu et al. 2014) and 6 months of treatment in Sprague Dawley rats in several studies from the same research team (Liu et al. 2011; Liu et al. 2010; Liu et al. 2009). None of the adult exposure Morris water maze studies were excluded based on having a high overall risk of bias and no serious concerns were present (Figure 9). In two instances, identical data appear to have been reported in separate publications[11] [Dong et al. (2015a); Dong et al. (2015b) and Liu et al. (2011); Liu et al. (2009)]. For these studies, results as reported in the earliest publication are discussed below.

The one study conducted in BALB/c male mice examined effects following a 30-day exposure (4–8 weeks of age) to 0.9, 2.26, and 4.52 ppm fluoride (Liu et al. 2014). In the pretest, no significant differences were noted in the initial swimming speed of the mice. In mice exposed to 2.26 and 4.52 ppm, acquisition was demonstrated but the shape of the acquisition curve was altered with an overall higher latency. In the probe test, mice exposed to 2.26 and 4.52 ppm spent less time in the target escape quadrant and showed fewer crossings over the learned escape platform location. No effects were observed at 0.9 ppm fluoride. At 4.52 ppm, authors reported clinical signs in the animals (curled up bodies, less movement, slightly wrinkled fur, and fluorosis symptoms in incisor teeth manifest as pale yellow plaques).

Ten studies assessed the effects of adult exposure in rats (primarily Sprague Dawley) lasting for 2 to 6 months at concentrations of 2.26 to 62.68 ppm fluoride (Dong et al. 2015c; Gao et al. 2009a; Gao et al. 2008a; Gao et al. 2008b; Gao et al. 2009b; Jiang et al. 2014b; Li et al. 2015; Liu et al. 2011; Liu et al. 2010; Liu et al. 2009; Zhu et al. 2012). Seven of 11 studies appear to have represented a body of work conducted by a research team in the Department of Pathology and Molecular Biology at Guiyang Medical College (Dong et al. 2015c; Gao et al. 2009a; Gao et al. 2008b; Liu et al. 2011; Liu et al. 2009). Fluoride concentrations in the control drinking water ranged from 0.23–1 ppm (Gao et al. 2009a; Liu et al. 2011; Liu et al. 2009).

The lowest fluoride drinking water concentrations (2.26, 22.6 ppm) were examined in studies by the Guiyang Medical College team [(Gao et al. 2009a; Liu et al. 2011; Liu et al. 2010; Wu et al. 2008); 2.26 and 22.6 ppm] and by Zhu et al. (2012)(2.26, 4.52, and 9.05 ppm). Gao et al. (2008b) exposed male and female (pooled for analysis) Sprague Dawley rats to 2.26 or 22.6 ppm fluoride via drinking water for 3 months and reported a significantly longer escape latency at the

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[11]Duplicate data from Dong et al. (2015a) for the P0 generation appear to be reported in Dong et al. (2015b). Only results from Dong et al. (2015a) are discussed. Some data from Liu et al. (2009) also are presented in Liu et al. (2011). Specifically, the values for escape time and acquisition on day 6 are identical, although the animal numbers per group differ. Duplicate results are summarized as part of Liu et al. (2009).

end of the acquisition phase in the 22.6-ppm group. No effect was observed in platform crossings or time in target zone in the probe test in any group. Zhu et al. (2012) reported that a 30-day exposure of male and female Sprague Dawley rats (combined for the analysis) to 9.05 ppm resulted in a longer latency to reach the hidden platform across all days of training in a Morris water maze task. Acquisition of the task was not analyzed nor was a memory probe test conducted. No effect was observed at exposure levels of 2.26 or 4.52 ppm.
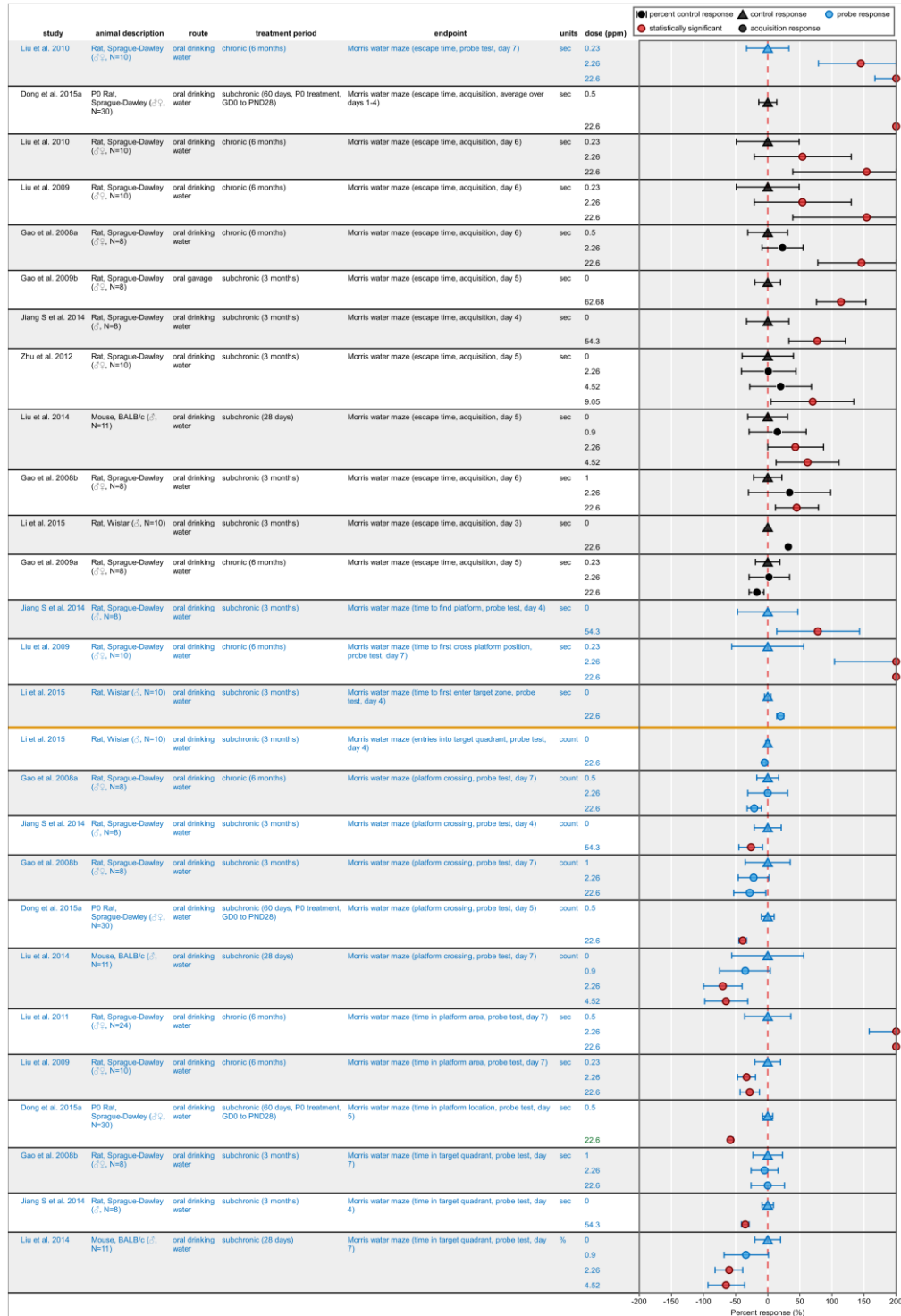
Figure 8. Results from Adult Exposure Studies Using Morris Water Maze

Ordered by direction of expected adverse effect.

Click here for interactive graphic.

Some data from Liu et al. (2009) also are presented in Liu et al. (2011) (escape time, acquisition, day 6), findings are presented under Liu et al. (2009) in figure. Apparently identical data were reported in Dong et al. (2015b) and Dong et al. (2015a); only data from Dong et al. (2015b) are presented in figure. Effects are grouped by a solid orange horizontal line by expected adversity direction (increase from reference/control group, then decrease from reference/control group).
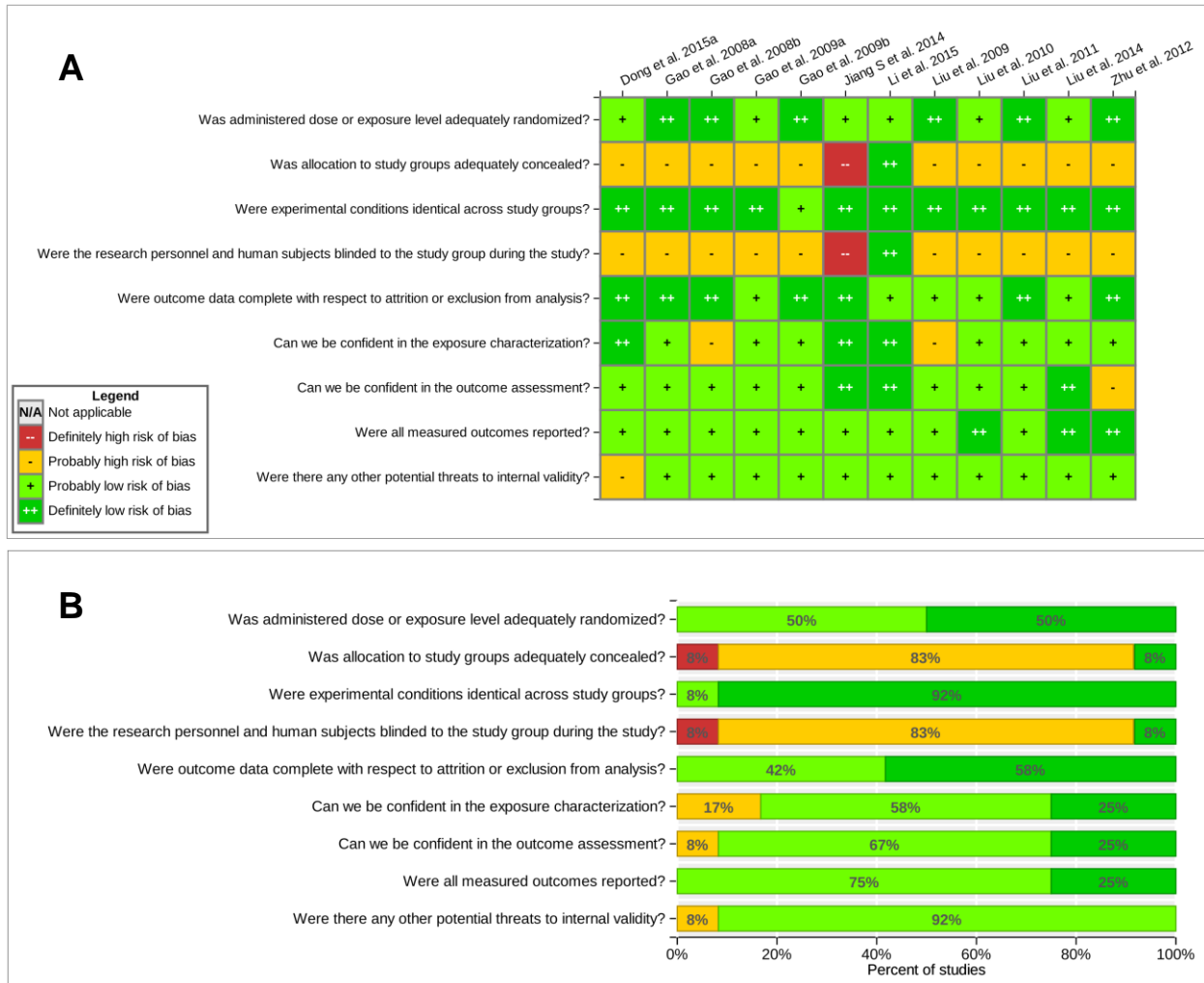
Figure 9. Risk of Bias in Studies Assessing Adult Exposure to Fluoride Using the Morris Water Maze

(A) Risk of bias at the individual study level. (B) Risk of bias across studies.

Extending the duration of exposure in Sprague Dawley rats to 6 months indicated effects on Morris water maze at 2.26 ppm in Liu et al. (2011); Liu et al. (2010); Liu et al. (2009). Acquisition of the task was demonstrated by a progressive decrease in escape latency over the 5 days of training in all groups. A general pattern of longer latencies was reported in fluoride-exposed groups as compared to controls as early as day 2 of training (Liu et al. 2009) (Figure 8). Data were analyzed each day independently and not as a progression over time. In Liu et al. (2010), the probe test suggested a higher latency for the animals to reach the location of the previously hidden platform at both dose levels (Figure 8). **[Some data presented in Liu et al. (2011) appeared identical to those presented in Liu et al. (2009), and results are summarized as part of Liu et al. (2009).]** Using the same dosing paradigm, other studies by the Guiyang Medical College investigators reported no significant effects on Morris water maze latency or probe test at 2.26 ppm (Gao et al. 2009a; Gao et al. 2008a; Gao et al. 2008b). In rats exposed to 22.6 ppm fluoride, escape latency was longer at the end of Morris water maze

training and no effect was observed on the probe test. Data on acquisition over training were not provided. In a study by Li et al. (2015), no statistically significant effects were observed on Morris water maze endpoints in Wister rats exposed to 22.6 ppm fluoride for 3 months.

At higher fluoride concentrations (50–62.68 ppm), longer escape latency at the end of the Morris water maze training session and less preference for the hidden platform quadrant during the probe test have been reported (Dong et al. 2015c; Gao et al. 2008b; Jiang et al. 2014b). **[In these studies, the fluoride-exposed group was used as a control to examine neuroprotective potential for therapeutics. Such data appear identical in Dong et al. (2015a) and Dong et al. (2015b) and, thus, are not considered as independent findings.]** In Jiang et al. (2014b), the effects on escape latency, platform crossing, and time spent in target quadrant also were impaired when assessed 1 week after the acquisition phase, leading the authors to conclude that longer-term memory could be impacted.

Learning is defined as an increase in performance on a task over time and is experimentally measured in animal studies by generating an acquisition curve. Several adult Morris water maze studies did not present acquisition data, instead relying on performance at a single time point at the end of training (Gao et al. 2009a; Gao et al. 2008a; Liu et al. 2011) or averaged over the full training period (Dong et al. 2015c). This approach raises concern for data interpretation. Using terminal performance (latency or distance traveled) as an endpoint of learning—in isolation from previous trials—fails to consider differences in baseline performance that would translate to later evaluations. In the study by Gao et al. (2009a), young adult Sprague Dawley rats (male and female) received four training trials a day for 5 days. Although the four trials per day were reportedly averaged for statistical analysis, latency only on day 6 was evaluated followed by a probe trial on day 7. Escape latency on day 6 showed no difference between controls and the 2.26-ppm dose group and a significant increase was observed in the 22.6-ppm dose group. No differences across groups were observed in the probe test.

Li et al. (2015) treated 5-week-old male Wistar rats with distilled water or 22.6 ppm fluoride in the drinking water for 3 months. Rats were allowed 2 training days of 8 trials per day followed by a probe test on day 3. Escape latency on each day and probe test performance were not statistically significant between groups. In the Morris water maze studies that did present acquisition data over 4 or 5 days, NTP calculated difference scores between the start and end of the acquisition phase to examine the progressive change in the animal's performance on the task. Effects on escape latency were more likely apparent at the end of training and either were not observed on the first day (Liu et al. 2014; Liu et al. 2010; Liu et al. 2009) or were observed but were smaller on the first day than on the last (Gao et al. 2009b; Zhu et al. 2012). With training, the differences in absolute latencies between groups could be magnified. Examining acquisition calculated as a percent change (increased performance) over time revealed that, in approximately half the studies, the change over time was equivalent to controls (Gao et al. 2009b; Jiang et al. 2014b; Liu et al. 2010; Liu et al. 2009) even though actual latency was higher. Other studies indicate impaired learning acquisition. In BALB/c mice, Liu et al. (2014) showed an approximate 45% change over time in latency in controls exposed to 0.9 ppm fluoride for 30 days. Mice exposed to 2.26 ppm showed an approximate 15% change while those exposed to 4.52 ppm showed an approximate 7% change over time. In Sprague Dawley rats exposed to fluoride for 3 months, Zhu et al. (2012) reported no statistically significant change in latency over Morris water maze training sessions. An approximate 50% change in latency was observed

for controls and the 2.26-ppm dose group, 45% for the 4.52-ppm group, and 30% for the 9.05-ppm dose group.

T- Maze and Y-Maze

Learning can be assessed experimentally by examining behavior improvement over training sessions and determining whether a memory of the task develops that enables better performance on subsequent sessions or following an interval of time without a continuation of training trials. T-maze learning is estimated based on spontaneous alternation between arm entries. In the T-maze, adult male Wistar rats exposed to 45.3 ppm fluoride for 30 days showed an arm bias in the spontaneous alternation paradigm and a decrease in exploratory activity (Raghu et al. 2013) (Figure 10). In the same study using a rewarded alternation paradigm, the percentage of correct responses in exposed rats decreased. The influence of the baseline arm bias on the choice of correct arm was not evaluated. In Wistar rats exposed to 22.6 ppm fluoride for 3 months, no differences were observed in alternation rate in a Y-maze (Li et al. 2015).

Using a cued (light)/shock-reinforced Y-maze paradigm to assess learning, the number of choice errors or the number of training trials to reach a predetermined learning criterion was assessed in mice or rats exposed to 0.45–90.5 ppm fluoride (Figure 10). Impaired performance was reported for exposure durations between 30 days and 6 months. Exposure to 2.71 ppm for 8 weeks in mice and 22.6 ppm for 6 months in rats resulted in a performance deficit (Figure 10) measured by number of choice errors or number of training trials to reach learning criteria (Chen and Geng 2011; Niu et al. 2008; Shen et al. 2004; Wang et al. 2006; Xu et al. 2001; Zhang et al. 2013; Zhang et al. 1999).[12]

In a cued Y-maze with shock reinforcement, 30-day-old ICR mice exposed to fluoride for 10 weeks showed a delay in learning (Zhang et al. 1999). At exposure levels of 4.52 ppm fluoride, mice displayed a delay in reaching a criterion of 90% correct choice rate within a session. The mice required approximately 80% more trials to achieve the criterion, as compared to controls. No effect was observed at dose levels of 0.45 and 2.26 ppm. After a 24-hour interval, mice in all groups showed a similar level of performance of 70%. In another study from what appears to be the same research group, Xu et al. (2001) exposed male Kunming mice to 2.71 ppm fluoride for 8 weeks and reported more training runs were required to meet learning criteria, but the percentage of total correct responses was unchanged (Figure 10).

At higher doses, male Wistar rats exposed to 22.6, 45.2, or 67.9 ppm fluoride for 6 months needed more Y-maze training sessions to meet learning criteria; however, the number of correct responses on the last day of training was similar to controls (Chen and Geng 2011). Shen et al. (2004) reported a similar outcome with 5 months of exposure to 67.9 ppm, with a twofold increase in the number of trials to reach criteria and 50% decrease in percentage of correct responses in the memory trial. Niu et al. (2008) examined effects of a 30-day exposure to 67.9 ppm fluoride in male and female (combined) Wistar rats on Y-maze performance. Training consisted of 20 trials per day until rats achieved a criterion of 90% correct choices within a session. Control rats required 3 days of training, while the 67.9-ppm group required 5 days of training. **[The authors state "rats in the three treatment groups spent more days in meeting**

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[12]In several studies (Chen and Geng 2011; Shen et al. 2004; Wang et al. 2006; Xu et al. 2001), whether SD or SEM was reported is unclear. Typically, SD was assumed, based on the magnitude of the values and results of authors' statistical analysis. In Zhang et al. (2013), values were assumed to be SEMs.

the learning standard of the Y-maze test" but the effect is not indicated in the figure as being statistically significant.] Rats in the 67.9-ppm group made significantly more errors at the start of training, but the number of errors showed a progressive decline over test days in both groups and by the last training day, no group differences were evident in error number or total reaction time. **[Variance for error numbers on day 5 was not presented.]**
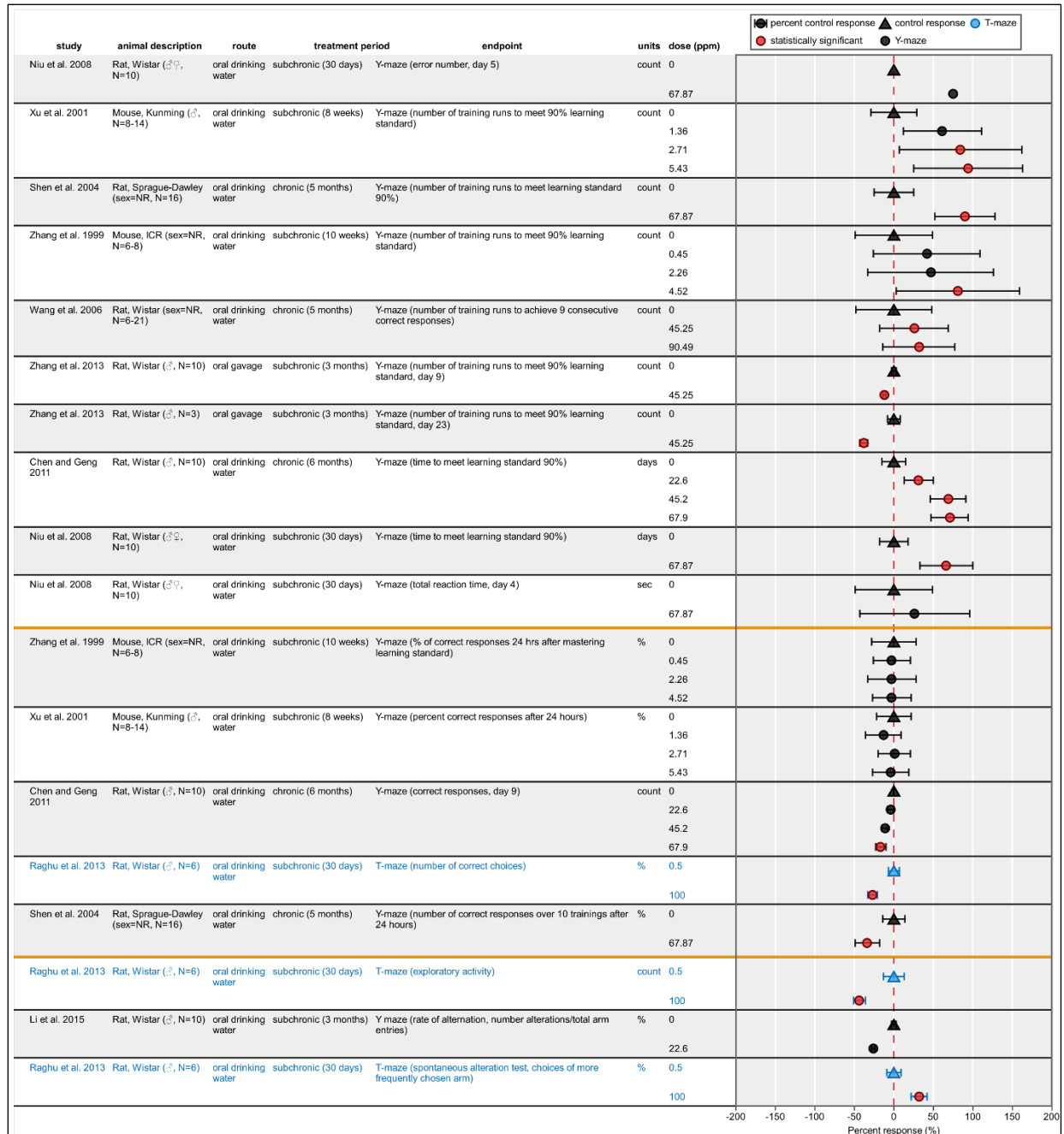
study	animal description	route	treatment period	endpoint	units	dose (ppm)
Niu et al. 2008	Rat, Wistar (♂♀, N=10)	oral drinking water	subchronic (30 days)	Y-maze (error number, day 5)	count	0 67.87
Xu et al. 2001	Mouse, Kunming (♂, N=8-14)	oral drinking water	subchronic (8 weeks)	Y-maze (number of training runs to meet 90% learning standard)	count	0 1.36 2.71 5.43
Shen et al. 2004	Rat, Sprague-Dawley (sex=NR, N=16)	oral drinking water	chronic (5 months)	Y-maze (number of training runs to meet learning standard 90%)	count	0 67.87
Zhang et al. 1999	Mouse, ICR (sex=NR, N=6-8)	oral drinking water	subchronic (10 weeks)	Y-maze (number of training runs to meet 90% learning standard)	count	0 0.45 2.26 4.52
Wang et al. 2006	Rat, Wistar (sex=NR, N=6-21)	oral drinking water	chronic (5 months)	Y-maze (number of training runs to achieve 9 consecutive correct responses)	count	0 45.25 90.49
Zhang et al. 2013	Rat, Wistar (♂, N=10)	oral gavage	subchronic (3 months)	Y-maze (number of training runs to meet 90% learning standard, day 9)	count	0 45.25
Zhang et al. 2013	Rat, Wistar (♂, N=3)	oral gavage	subchronic (3 months)	Y-maze (number of training runs to meet 90% learning standard, day 23)	count	0 45.25
Chen and Geng 2011	Rat, Wistar (♂, N=10)	oral drinking water	chronic (6 months)	Y-maze (time to meet learning standard 90%)	days	0 22.6 45.2 67.9
Niu et al. 2008	Rat, Wistar (♂♀, N=10)	oral drinking water	subchronic (30 days)	Y-maze (time to meet learning standard 90%)	days	0 67.87
Niu et al. 2008	Rat, Wistar (♂♀, N=10)	oral drinking water	subchronic (30 days)	Y-maze (total reaction time, day 4)	sec	0 67.87
Zhang et al. 1999	Mouse, ICR (sex=NR, N=6-8)	oral drinking water	subchronic (10 weeks)	Y-maze (% of correct responses 24 hrs after mastering learning standard)	%	0 0.45 2.26 4.52
Xu et al. 2001	Mouse, Kunming (♂, N=8-14)	oral drinking water	subchronic (8 weeks)	Y-maze (percent correct responses after 24 hours)	%	0 1.36 2.71 5.43
Chen and Geng 2011	Rat, Wistar (♂, N=10)	oral drinking water	chronic (6 months)	Y-maze (correct responses, day 9)	count	0 22.6 45.2 67.9
Raghu et al. 2013	Rat, Wistar (♂, N=6)	oral drinking water	subchronic (30 days)	T-maze (number of correct choices)	%	0.5 100
Shen et al. 2004	Rat, Sprague-Dawley (sex=NR, N=16)	oral drinking water	chronic (5 months)	Y-maze (number of correct responses over 10 trainings after 24 hours)	%	0 67.87
Raghu et al. 2013	Rat, Wistar (♂, N=6)	oral drinking water	subchronic (30 days)	T-maze (exploratory activity)	count	0.5 100
Li et al. 2015	Rat, Wistar (♂, N=10)	oral drinking water	subchronic (3 months)	Y maze (rate of alternation, number alterations/total arm entries)	%	0 22.6
Raghu et al. 2013	Rat, Wistar (♂, N=6)	oral drinking water	subchronic (30 days)	T-maze (spontaneous alteration test, choices of more frequently chosen arm)	%	0.5 100

Legend: percent control response; control response; T-maze; statistically significant; Y-maze

Percent response (%): -200, -150, -100, -50, 0, 50, 100, 150, 200

Figure 10. Results from Adult Exposure Studies Using Y- Maze and T-Maze

Ordered by direction of expected adverse effect.
Click here for interactive graphic.
Effects are grouped by a solid orange horizontal line by expected adversity direction (increase from reference/control group, then decrease from reference/control group, then any change from reference/control group).

Wang et al. (2006) reported a longer latency in the Y-maze in Wistar rats exposed by gavage to 45.2 or 90.49 ppm fluoride for 5 months. Zhang et al. (2013) exposed male Wistar rats to daily gavage delivery of 45.2 ppm fluoride beginning at 6 weeks of age and continuing for 3 months. The number of times the rat entered into the correct arm of the Y maze over a 20-trial session was recorded. After 9 training sessions, the retest was conducted 14 days later. All animals displayed a similar level of acquisition over the training sessions. Although not statistically analyzed by the authors, performance on the initial training day was similar. The number of times the rat entered the correct arm on the last day of training was significantly less in the fluoride-exposed rats. This difference was approximately 15%. Memory of the task assessed 2 weeks later showed an approximately 30% difference, interpreted as lower retention in the fluoride exposed rats.

The following T-maze, Y-maze, or other maze studies were excluded from the primary analysis because of concerns for risk of bias in several critical domains (Basha and Sujitha 2012; Bhatnagar et al. 2002; Elliott 1967; Jetti et al. 2016; Zhang et al. 2001) (see Appendix C for rationale). Except for Zhang et al. (2001), the excluded studies used high concentrations of fluoride (>67 ppm). Across the remaining studies, risk of bias was a "serious" concern based on issues regarding adequate exposure characterization and lack of blinding during outcome assessment (Figure 11). Exclusion of Zhang et al. (2001) did not appreciably alter conclusions because the authors reported findings consistent with those described above for Zhang et al. (1999) and Xu et al. (2001).

Passive and Active Avoidance

In avoidance paradigms, animals are required to learn an association with an adverse event (shock) and to adjust behavior to avoid the event.

Passive Avoidance

In Raghu et al. (2013), male Wistar rats (n = 6) 30 days of age received 100 ppm fluoride in the drinking water for 30 days. No differences were observed during the initial exploration/training phase and in the retention phase; time spent in the unsafe compartment decreased by 60% in controls and 35% in the exposed animals **[details of testing paradigm not described in methods or in the cited textbook]** (Figure 12). Wu et al. (2006) exposed 30-day-old male and female Wistar rats to 45.3 ppm fluoride for up to 90 days. At 30, 60, and 90 days of exposure, rats showed no difference in errors, defined as movement from rubber mat to electrified bar, during the initial training or retention sessions; they did, however, show a shorter latency in making the first error response in the retention trial (Figure 12) shows data from 30 days only).

Two studies assessing passive avoidance were excluded from the analysis due to concerns for risk of bias (Jain et al. 2015; Jetti et al. 2016). Jetti et al. (2016) used the same model system Raghu et al. (2013) used (appear to be the same research group) and found similar results in male Wistar rats exposed to 100 ppm fluoride for 30 days. Jain et al. (2015) reported a longer retention latency in young Wistar rats (sex not reported) treated with 1.36 ppm fluoride for 60 days beginning on PND18 **[details on how or what retention latency refers to were not reported]**. Across the remaining studies, concerns for risk of bias were "serious," based primarily on issues regarding adequate characterization of the exposure and lack of blinding during outcome assessment (Figure 13).
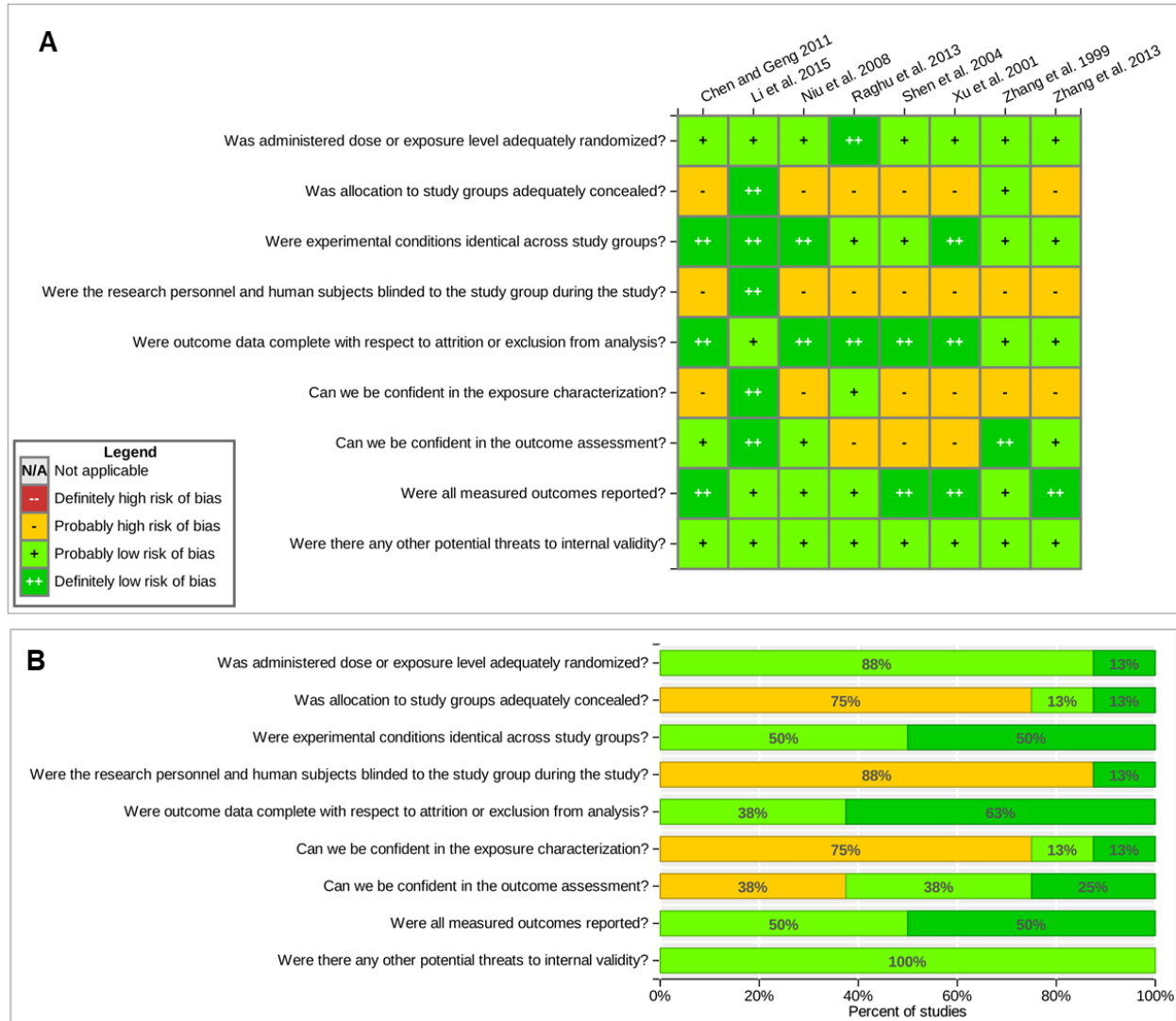
Figure 11. Risk of Bias in Studies Assessing Adult Exposure to Fluoride Using the T- or Y-Maze

(A) Risk of bias at the individual study level. (B) Risk of bias across studies. Studies at high overall risk of bias excluded (Basha and Sujitha 2012; Bhatnagar et al. 2002; Elliott 1967; Jetti et al. 2016; Zhang et al. 2001).
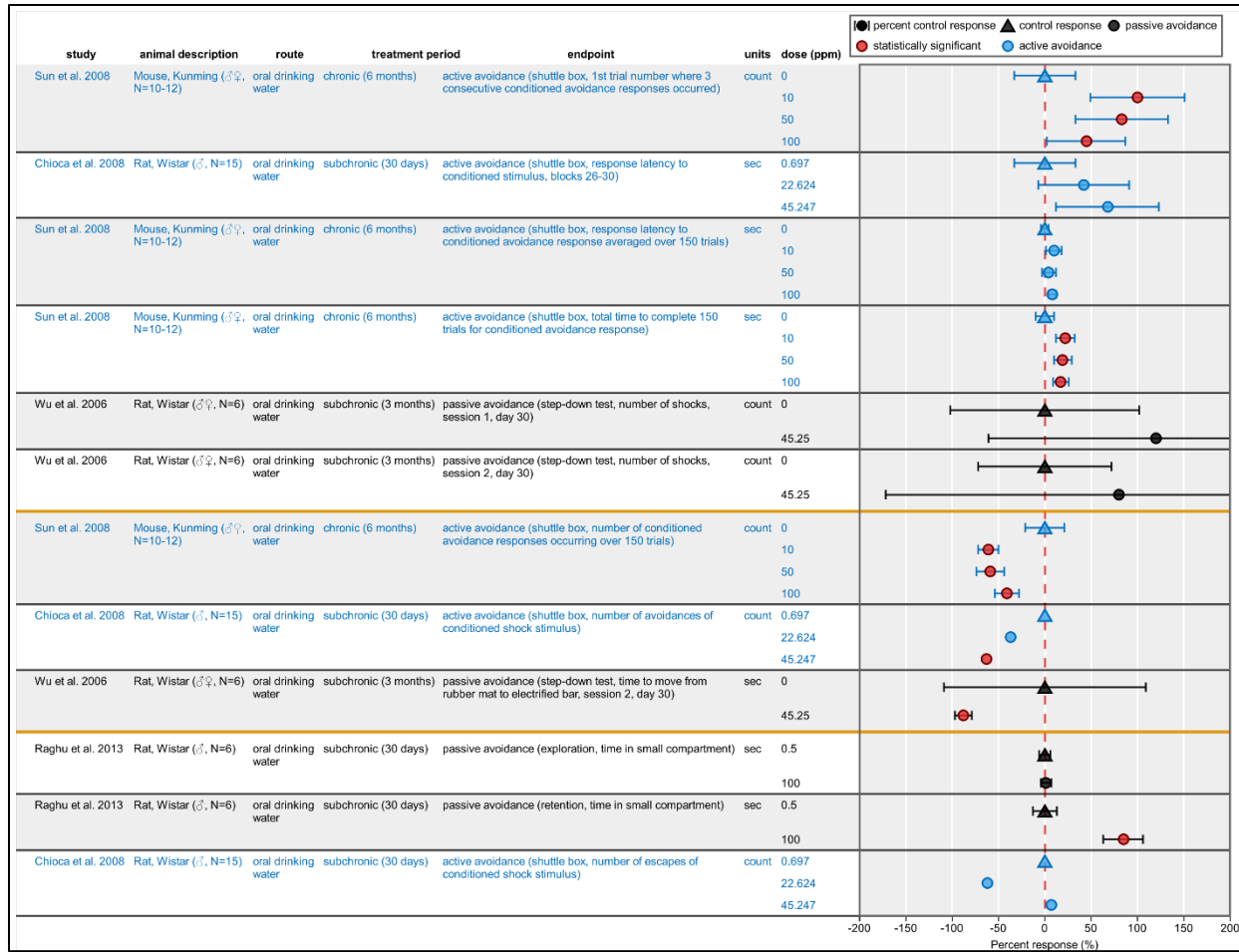
Figure 12. Results from Adult Exposure Studies Using Active and Passive Avoidance Tests

Ordered by direction of expected adverse effect.
Click here for interactive graphic.
Effects are grouped by a solid orange horizontal line by expected adversity direction (increase from reference/control group, then decrease from reference/control group, then any change from reference/control group).
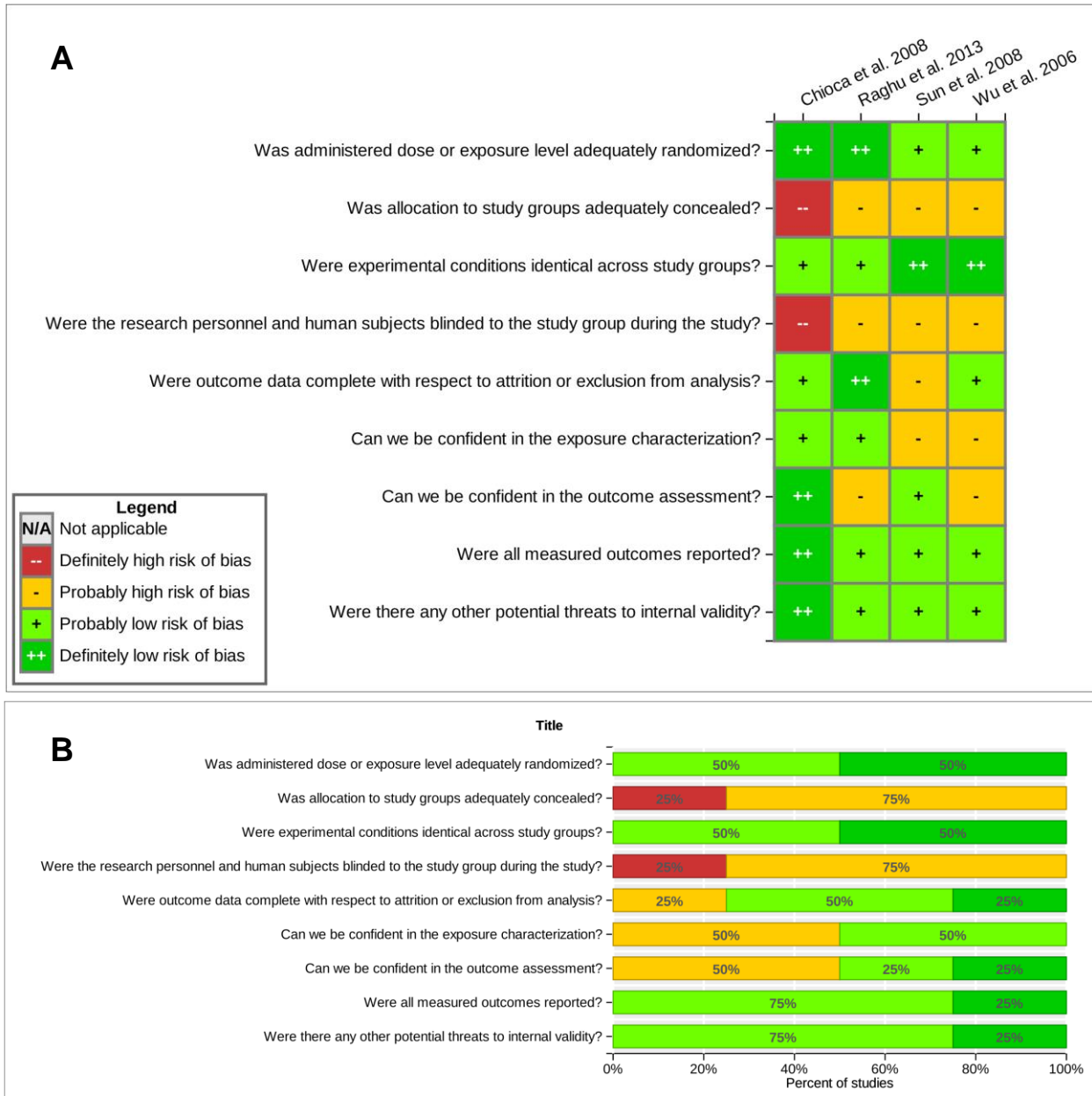
Figure 13. Risk of Bias in Adult Exposure Studies Using Active and Passive Avoidance Tests

(A) Risk of bias at the individual study level. (B) Risk of bias across studies. Studies at high overall risk of bias excluded (Jain et al. 2015; Jetti et al. 2016).

Active Avoidance

The latency to respond to a cued shock decreased over training trials in controls (0.7 ppm fluoride) and in adult male Wistar rats exposed to 22.6 or 45.3 ppm fluoride for 30 days (Chioca et al. 2008). The data showed a high degree of variance. Control animals learned to shift their response pattern to avoid the shock, while a larger proportion of the fluoride-exposed animals displayed an escape, rather than avoidance, response or failed to escape the shock (Figure 12).

In Sun et al. (2008), 60-day-old male and female Kunming mice (n = 10−12) were exposed to 10, 50, or 100 ppm fluoride in drinking water for 6 months. Using avoidance response as a measure of learning, acquisition over five sessions was demonstrated in all exposure groups. Fluoride-exposed mice, however, took longer to complete the 150 conditioned response trials on the last training day, and all fluoride-exposed mice showed a lower level of avoidance response than controls. When data were averaged across the 150 trials, fluoride-exposed mice in all groups required more runs to display the conditioned avoidance response, took longer to perform the task, and showed a lower level of avoidance responses (Figure 12).

Exploration

Exploration in animals can reflect a learning of the environment but also can influence performance on latency or attention-dependent learning tasks. In Liu et al. (2014) 1-month old BALB/c male mice were exposed to fluoride (0.9, 2.26, or 4.52 ppm) via drinking water for 4 weeks. Locomotor activity and rearing were not significantly different across groups, with all groups displaying habituation to the novel arena environment over time. In a test paradigm for novel object recognition, animals showed similar exploratory activity levels and lack of preference for objects during training. When a preference of familiar versus novel was examined, total time spent with novel object was similar across groups. Preference for the novel object, however, was reduced by approximately 20% in the 2.26- and 4.52-ppm groups. Han et al. (2014) exposed adult male Kunming mice to 11, 22, or 45 ppm fluoride for 180 days. No effects were observed on open-field ambulatory or rearing levels, and the time spent in different quadrants of the arena was similar across groups. In a novel object recognition paradigm, the predicted pattern for controls—to show a preference for a novel object over a familiar object— was not observed. This pattern also was not observed in the 11- or 22-ppm fluoride-exposed group, while, in the 45-ppm group, a significant preference for the familiar object was observed. Because of the absence of the predicted control behavior, this study is not included (Figure 14). At a very high concentration of 226 ppm for 60 days, reduced exploratory motor activity was reported in male Wistar rats (Ekambaram and Paul 2003) (Figure 14). Across studies, "no serious" concerns for risk of bias were evident (Figure 15).

Operant Behavior

One study using an appetitive (food) reinforcement task to assess learning is available. Weanling female Sprague Dawley rats were exposed to fluoride in the drinking water at 33.2–155.2 ppm for 8 months. No effect of exposure was observed in rat ability to learn to press a bar to receive food pellets (Whitford et al. 2009) (Figure 14), see days to reach learning criteria and maximum response for acquisition of bar response). Rats were shifted to a fixed ratio (FR) 2 schedule of reinforcement that progressed over 5 days to a final FR-10 schedule. Rats then were placed on a differential reinforcement schedule of low rates of 20 sec (DRL-20) that required them to pause for at least 20 sec between bar presses to earn reinforcement. The total number of bar presses increased with increasing fixed ratios in all dose groups, which was not significantly different across dose groups. All animals displayed the ability to shift to the DRL-20 schedule with a similar level of performance demonstrated over 10 days in all dose groups. (Figure 14, see operant FR-2, FR-10, and DRL-20). Across studies "no serious" concerns for risk of bias were evident (Figure 15).
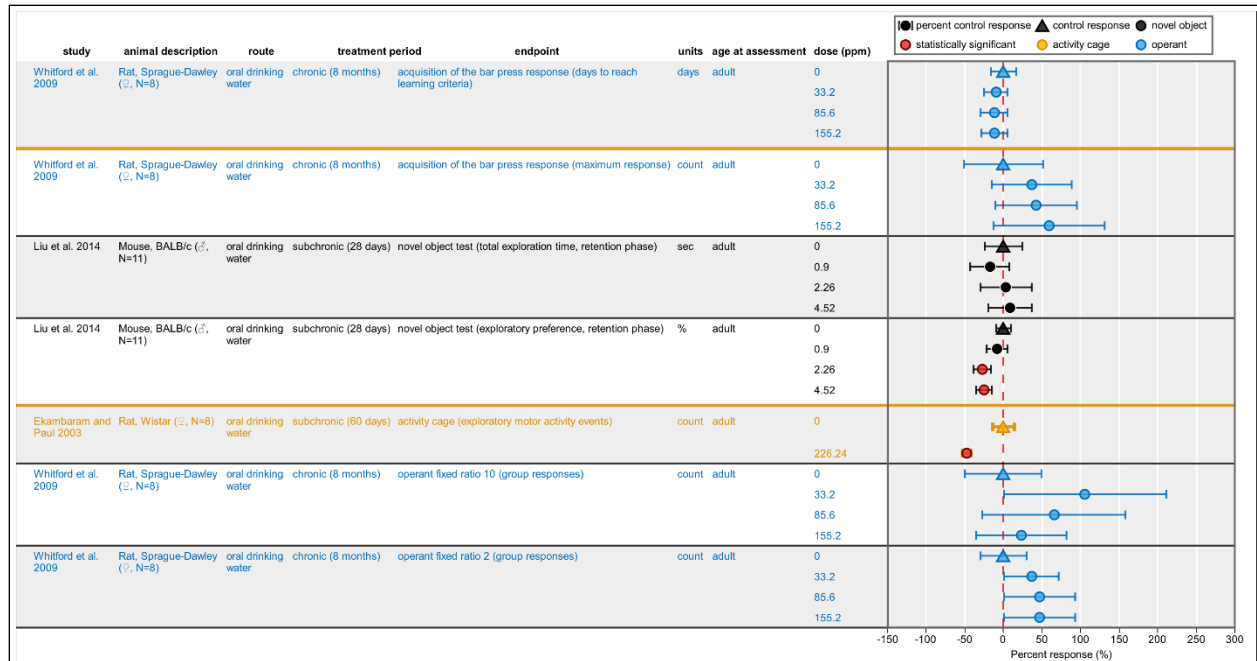
Figure 14. Results from Adult Exposure Studies Using Exploration and Operant Behavior Tests

Ordered by direction of expected adverse effect.
Click here for interactive graphic.
Effects are grouped by a solid orange horizontal line by expected adversity direction (increase from reference/control group, then decrease from reference/control group, then any change from reference/control group).
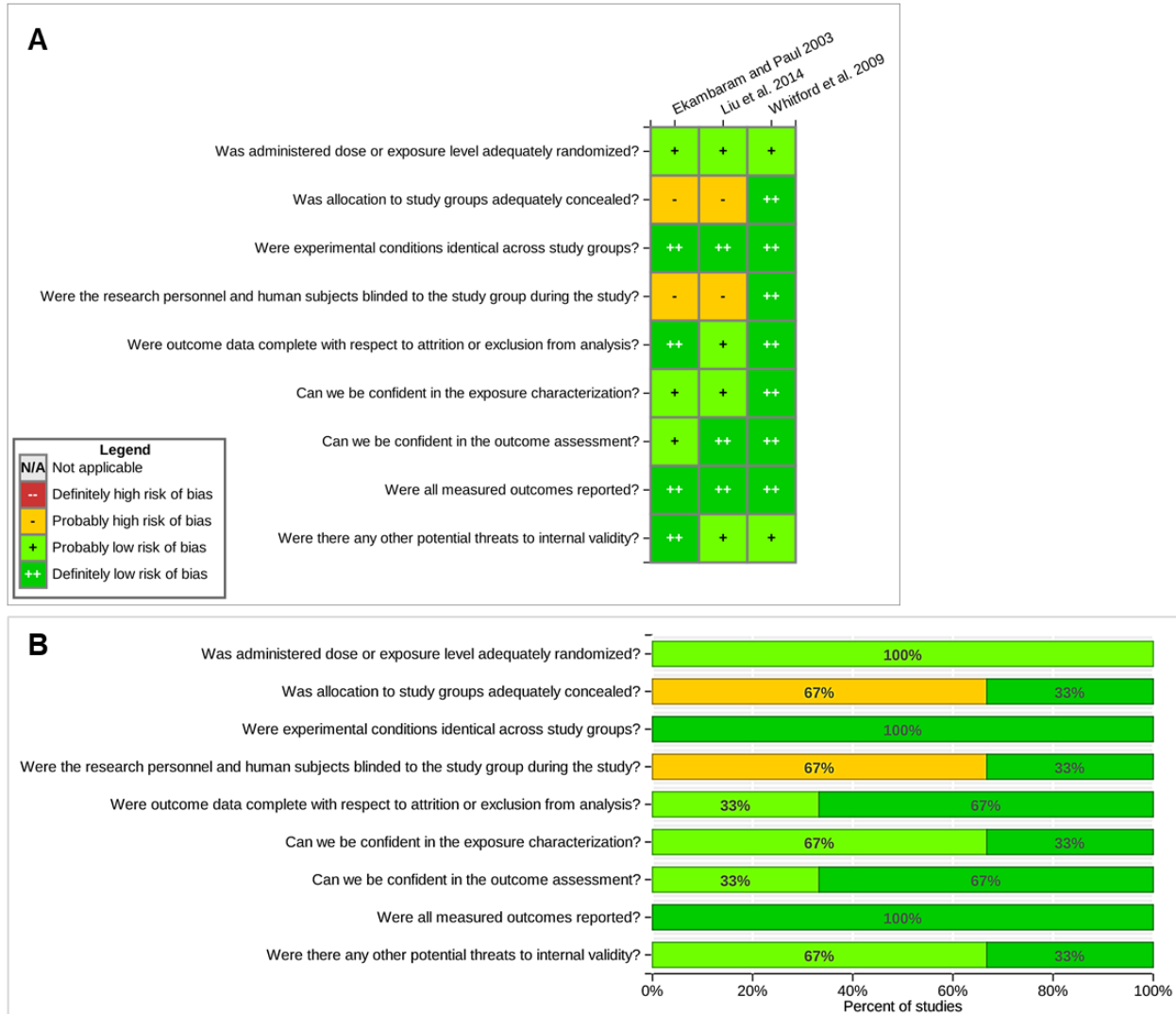
Figure 15. Risk of Bias in Adult Exposure Studies Using Exploration and Operant Behavior Tests

(A) Risk of bias at the individual study level. (B) Risk of bias across studies. Studies at high overall risk of bias excluded (Han et al. 2014).

Findings from High Risk of Bias Studies

Seven adult studies were excluded based on concerns for overall high risk of bias (Table 8). In general, these studies provided evidence to support an effect on learning and memory at high concentrations. Of these, two studies tested concentrations of fluoride at 10 ppm or lower (Elliott 1967; Zhang et al. 2001). In Elliott (1967), no effects were observed in Hooded rats treated for 4 months with 4.24 or 42.4 ppm in an elevated multiple T-maze. In Zhang et al. (2001), male Kunming mice exposed for 8 weeks to 2.26 or 4.52 ppm fluoride required more training runs to reach the learning standard although percentage of correct responses was not affected 24 hours after reaching learning criteria. No effects were observed in the 0.45-ppm group.

Table 8. Learning and Memory Findings from Adult Exposure Studies Excluded Based on Concern for High Overall Risk of Bias

Reference	Treatment	NOEL	LOEL
1. Basha and Sujitha (2012)	Wistar rat (adult, 6 ♂); drinking water for 30 days with 271 ppm (40 mg/kg-d)	–	271 ppm (40 mg/kg-d): T-maze: ↑ time to reach learning standard during retention phase (but no effect on error number during acquisition or retention phase or time to reach standard during acquisition phase)
2. Bhatnagar et al. (2002)	Swiss albino mice (adult, 5 ♀); drinking water for 30 days with 14.7 ppm (54.3 mg/kg-d)	–	14 ppm (54.3 mg/kg-d): "simple maze" test: ↑ latency to complete
3. Elliott (1967)	Hooded rat (adult, 15 ♂); drinking water for 4 months with 4.24 or 42.4 ppm (0.6 or 6 mg/kg-d)	42.4 ppm (6 mg/kg-d) [no effects on error number or time to complete maze test]	–
4. Han et al. (2014)	Kunming mouse (adult, 15 ♂); drinking water for 5 months with 11, 22, or 45 ppm (2.8, 5.6, or 11.5 mg/kg-d)	22 ppm (5.6 mg/kg-d)	45 ppm (11.5 mg/kg-d) novel object test: ↓ discrimination index (no other effects were observed in novel object test)
5. Jain et al. (2015)	Wistar rat (adult, n = not reported, sex not reported) drinking water for 60 days with 11.8 ppm (1.8 mg/kg-d)	–	11.8 ppm (1.8 mg/kg-d): passive avoidance: ↑ latency time during retention phase
6. Jetti et al. (2016)	Wistar rat (adult, 6 ♂); drinking water for 4 months with 100 ppm (14.75 mg/kg-d)	–	100 ppm (14.75 mg/kg-d): T-maze: ↓ number correct choices and exploratory activity, ↑ number of spontaneous alterations. passive avoidance: ↑ retention time in small compartment
7. Zhang et al. (2001)	Kunming mouse (adult, 12-21 ♂); drinking water for 8 weeks with 0.45, 2.26 or 4.52 ppm (0.12, 0.58, or 1.16 mg/kg-d)	0.45 ppm (0.12 mg/kg-d)	2.26 ppm (0.58 mg/kg-d) Y-maze: ↑ number of training runs to reach learning standard (no effect on percentage of correct responses 24 hours after mastering learning standard)

Exposure at Less Than 4 ppm

The 2006 NRC report focused on effects in the 2- to 4-ppm range. Most fluoride neurobehavioral studies identified in the current review used higher fluoride concentrations. Findings at <4 ppm across all behavioral tests (learning and memory, motor function, anxiety, depression, etc.) are summarized in Figure 16 (Bartos et al. 2015; Gao et al. 2009a; Gao et al. 2008a; Gao et al. 2008b; Liu et al. 2014; Liu et al. 2011; Liu et al. 2010; Liu et al. 2009; Xu et al. 2001; Zhang et al. 1999; Zhu et al. 2012). Only one study included developmental exposure (Bartos et al. 2015).

The lowest concentration tested was 0.9 ppm in adult male BALB/c mice for 30 days and no effects were reported (Liu et al. 2014) for locomotor activity, tail suspension immobility time, elevated plus maze, swimming speed, or learning and memory (Morris water maze, novel object recognition). Five studies, four of which were published after the 2006 NRC review, reported learning and memory deficits in mice or rats at 2.26−2.71 ppm, including impaired performance in the Morris water maze (Liu et al. 2014; Liu et al. 2011; Liu et al. 2010; Liu et al. 2009), Y-maze (Xu et al. 2001), and novel object recognition tests (Liu et al. 2014). Liu et al. (2014) showed no alterations in swimming speed prior to Morris water maze testing.

Liu et al. (2014) also reported fewer locomotion events in an activity cage test and a longer tail suspension immobility time (taken as indicative of depression, although it can reflect decreased muscle strength) in BALB/c mice exposed to 2.26 ppm or 4.52 ppm for 30 days. Effects on immobility time in a forced swim test as a measure of depression were observed in the 4.52-ppm dose group. Bartos et al. (2015) did not assess learning and memory but reported delayed eye opening in F1 male and female offspring of Wistar rats treated from GD0 to PND21 with 2.26 ppm fluoride with no differences noted in other neurodevelopmental landmarks. At 90 days of age, these offspring demonstrated lower activity levels (15 min) and an increase in entries and time spent in the open arm of an elevated plus maze. Several studies examined dose levels at 2.26 ppm and reported no statistically significant effects on behavioral assessments (Gao et al. 2009a; Gao et al. 2008b; Zhang et al. 1999; Zhu et al. 2012). The effects on Morris water maze at 2.26 ppm were not consistent. Several of these studies appear to have been conducted by the same research group using a very similar experimental paradigm; the authors found effects in some studies (Liu et al. 2011; Liu et al. 2010; Liu et al. 2009) but not in others (Gao et al. 2009a; Gao et al. 2008a; Gao et al. 2008b).

Risk of bias was of no serious concern among these studies (Figure 17). Reporting quality was an issue, especially regarding a lack of information on concealment during allocation of animals to study groups and blinding of research personnel during the study. Blinding at outcome assessment was not considered consistently, which represents a major concern for any endpoint obtained by observational methods. Endpoints obtained using computer-assisted equipment and measurements were of lesser concern for blinding. One of the two studies considered to have "probably high risk of bias" due to lack of blinding at outcome assessment reported effects at 2.26 ppm (Xu et al. 2001). Information on source and purity of the fluoride was not reported consistently. Several studies that did not report this information, however, measured levels of fluoride in serum, urine, or bone and observed dose-gradients in internal exposure levels (Appendix U).

Systematic Literature Review on the Effects of Fluoride on Learning and Memory in Animal Studies

Figure 16. Results from All Studies Assessing Effects at ≤4 ppm

Ordered by direction of expected adverse effect.
Click here for interactive graphic.
Studies at high overall risk of bias excluded. Data not shown for Morris water maze data on acquisition day 1. Except for the Gao et al. (2009a) study, acquisition day 1 responses were not altered (escape time was significantly longer on acquisition day 1, but not on day 5). Some data from Liu et al. (2009) also are presented in Liu et al. (2011). Specifically, the values for escape time and acquisition on day 6 are identical, although the animal numbers per group differ. Duplicate results are summarized as part of Liu et al. (2009). Effects are grouped by a solid orange horizontal line by expected adversity direction (increase from reference/control group, then decrease from reference/control group, then any change from reference/control group).
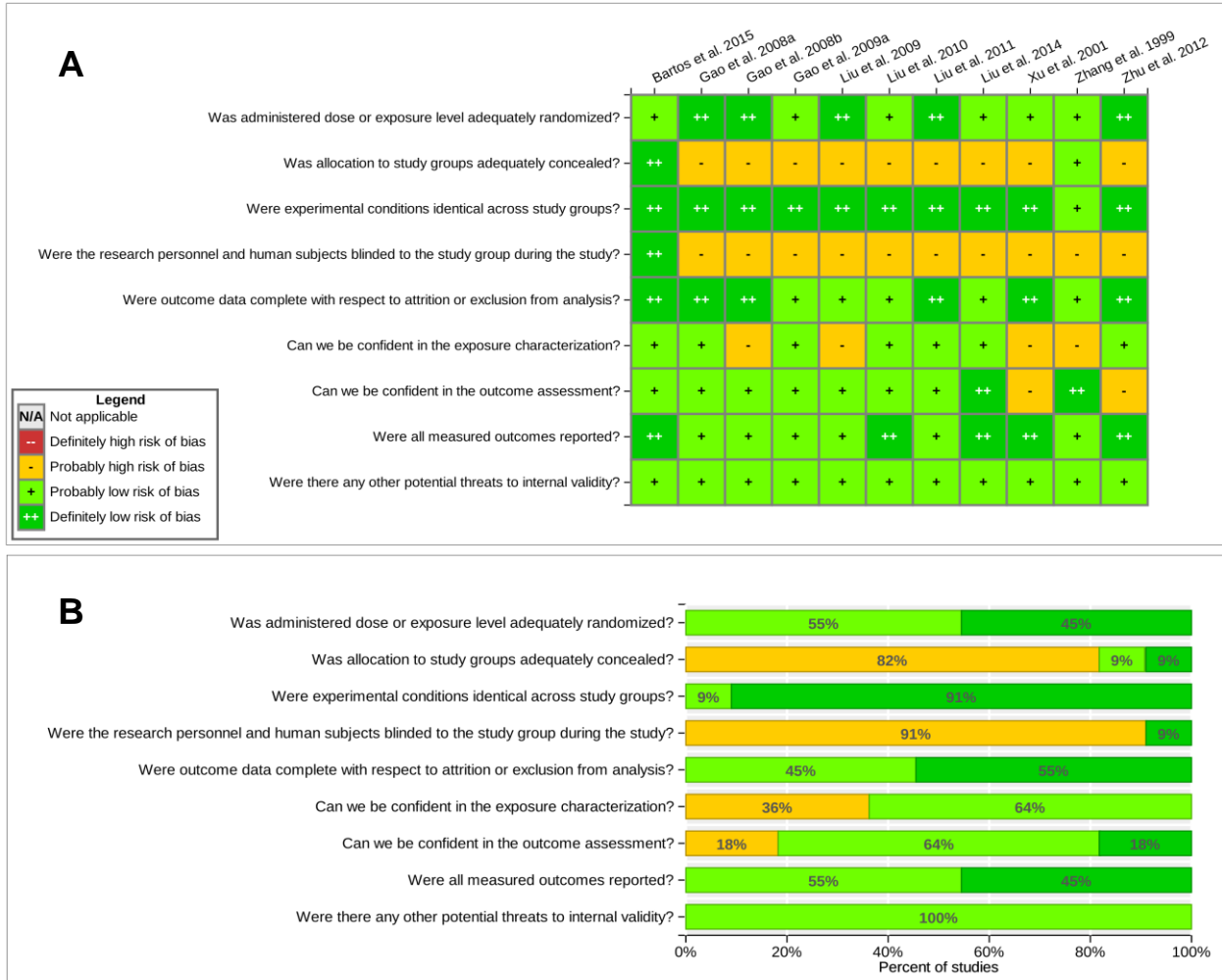
Figure 17. Risk of Bias Ratings for Studies ≤4 ppm

(A) Risk of bias at the individual study level. (B) Risk of bias across studies. Studies with high risk of bias excluded (Elliott 1967; Wu et al. 2008; Zhang et al. 2009; Zhang et al. 2001).

Findings from High Risk of Bias Studies (<5 ppm)

Four studies assessing fluoride levels <5 ppm were excluded based on concerns for overall high risk of bias (Elliott 1967; Wu et al. 2008; Zhang et al. 2009; Zhang et al. 2001); (Table 9). Inclusion of these studies would not have appreciably influenced the conclusions described above. In general, these studies did not provide support for effects at low concentrations. Either no significant effects were observed (Elliott 1967) or effects appeared sporadically. In Zhang et al. (2009), no effects were observed in the Morris water maze at concentrations up to 4.52 ppm in F1 male and female ICR rats of dams treated from GD7 to PND0. This study did report statistically significant decreases in locomotor activity in the open field test: Fewer grid entries occurred at 3 minutes at 2.26 ppm (but not at 1 minute) and fewer rearing events occurred at 1.13 ppm (but not at 2.26 or 4.52 ppm). Wu et al. (2008) found a change in grooming behavior (increased face scrubbing events) at 1 ppm in the open field test in F1 male and female Wistar rats treated from GD0 to PND45 with 1, 5, or 25 ppm fluoride. No other open-field endpoints were altered at 1 ppm, and no effects at this concentration were observed in the Morris water

maze, tests for pain response, reflex and sensory development (surface righting, cliff avoidance, negative taxis), physical development (pinna detachment, incisor eruption,), or motor coordination.

Table 9. Findings from Studies Testing <5 ppm Fluoride Excluded Based on Concern for High Overall Risk of Bias

Reference	Treatment	NOEL	LOEL
1. Elliott (1967)	Hooded rat (adult, 15 ♂); drinking water for 4 months with 4.24 or 42.4 ppm (0.6 or 6 mg/kg-d)	42.4 ppm (6 mg/kg-d) [no effects on error number or time to complete maze test]	–
2. Wu et al. (2008)	Wistar rat (F1, 40 ♂♀); drinking water during development with 1, 5, or 25 ppm (0.2, 1, or 5.3 mg/kg-d) [birth to PND45]	–	5 ppm (1 mg/kg-d): <u>activity</u>: ↑activity time (no effect at 25 ppm), ↓number of squares traveled in open field test most endpoints not altered at 5 ppm; decreased face scrubbing observed at 1 ppm in open field
3. Zhang et al. (2001)	Kunming mouse (adult, 12−21 ♂); drinking water for 8 weeks with 0.45, 2.26, or 4.52 ppm (0.12, 0.58, or 1.16 mg/kg-d)	0.45 ppm (0.12 mg/kg-d)	2.26 ppm (0.58 mg/kg-d) <u>Y-maze</u>: ↑number of training runs to reach learning standard (no effect on percentage of correct responses 24 hours after mastering learning standard)
4. Zhang et al. (2009)	ICR rat (F1, 9−10 ♂♀); drinking water during development with 1.13, 2.26, or 4.52 ppm (0.18, 0.36, or 0.71 mg/kg-d) [GD7 to PND0]	–	1.13 ppm (0.71 mg/kg-d) <u>open field</u>: ↑number of rearing events (no effect at 2.26 or 4.52 ppm) no effects in Morris water maze at any dose level

Quality of Evidence

Results show **low-to-moderate level-of-evidence** in developmental and adult exposure studies for a pattern of findings suggestive of an effect on learning and memory (Table 10). The evidence was strongest for adult exposure studies using the Morris water maze. Level-of-evidence conclusions were rated down due to concern for indirectness and risk of bias.

Table 10. Learning and Memory (Quality of Evidence and Summary of Findings)

Studies	Risk of Bias	Inconsistency	Indirectness	Imprecision	Publication Bias	Magnitude	Dose Response	Quality of Evidence
Impaired Learning and Memory (Developmental Exposure)								
6 Studies (MWM, Passive Avoidance, Exploration)	**Serious**[1]	No serious	**Serious indirectness**[2]	No serious	Not detected[3]	No basis for upgrade	No basis for upgrade[4]	⊕⊕⊖⊖ **Low** risk of bias and indirectness
Impaired Learning and Memory (Adult Exposure)								
14 Studies (MWM, Exploration, Operant Behavior)	No serious	No serious	**Serious indirectness**[2]	No serious	Not detected[3]	No basis for upgrade	No basis for upgrade[4]	⊕⊕⊕⊖ **Moderate** indirectness
12 Studies (T-maze, Y-maze, Passive Avoidance, Active Avoidance)	**Serious**[1]	No serious	**Serious indirectness**[2]	No serious	Not detected[3]	No basis for upgrade	No basis for upgrade[4]	⊕⊕⊖⊖ **Low** risk of bias and indirectness

32 Studies: Quality of evidence: low (⊕⊕⊖⊖) **to moderate** (⊕⊕⊕⊖). Additional studies are required to have higher confidence in the specificity of the responses as learning or memory impairments and in quantitative measures, such as the effect sizes, identification of NOEL or LOEL doses, or parameters for benchmark dose analysis.

Developmental: (Dong et al. 2015c; El-lethey et al. 2010; Gui et al. 2010; Jiang et al. 2014a; Wang et al. 2004; Wei et al. 2014). Duplicate data from Dong et al. (2015b) appear to be reported in another study from this group Dong et al. (2015a).

Adult (MWM, exploration, operant behavior): (Dong et al. 2015c; Ekambaram and Paul 2003; Gao et al. 2009a; Gao et al. 2008b; Gao et al. 2009b; Jiang et al. 2014b; Li et al. 2015; Liu et al. 2014; Liu et al. 2011; Liu et al. 2010; Liu et al. 2009; Whitford et al. 2009; Zhu et al. 2012). Duplicate data from Dong et al. (2015a) for the P0 generation appear to be reported in another study from this group Dong et al. (2015b).

Adult (T-maze, Y-maze, passive avoidance, active avoidance): (Chen and Geng 2011; Chioca et al. 2008; Li et al. 2015; Niu et al. 2008; Raghu et al. 2013; Shen et al. 2004; Sun et al. 2008; Wang et al. 2006; Wu et al. 2006; Xu et al. 2001; Zhang et al. 2013; Zhang et al. 1999).

[1]Only one of the six developmental studies appeared to control for litter effects, a critical risk of bias factor in developmental exposure studies. In the adult studies for T-maze, Y-maze, passive avoidance, or active avoidance, the lack of blinding at outcome assessment and adequate characterization of the fluoride treatment were concerns.

[2]Studies reported impaired learning and memory in fluoride-exposed animals that could reflect a motor activity deficit; however, motor function/activity levels were often not assessed and results from other studies suggest effects on motor impairment. In addition, many studies focused analyses on latency of response rather than on acquisition of learning.

[3]Assessing publication bias through typical approaches was limited, such as through funnel plots, publication of conference abstracts in the peer-review literature, or other means. With respect to conflict of interest, authors report "no conflict of interest," it was not reported, or research was supported by academia or government.

[4]No upgrading was considered for dose response and magnitude of effect because of concern for risk of bias and indirectness (confounding effects of impaired motor function on the learning and memory function tests).

NOTE: OHAT includes consideration of consistency of response across different animal model systems as an upgrade factor when evaluating the quality of the evidence, but consistency of response was not a major consideration in this review because most studies were on rodents, primarily rats.

Discussion

Summary

Results show **low-to-moderate level-of-evidence** in developmental and adult exposure studies for a pattern of findings suggestive of an effect on learning and memory (Table 10). Level-of-evidence conclusions were rated down due to concern for indirectness and risk of bias. The evidence was strongest for adult exposure studies using the Morris water maze. The evidence is strongest (moderate level-of-evidence) in animals exposed as adults tested in the Morris water maze and weaker (low level-of-evidence) in animals exposed during development.

In many cases, across the entire dose range tested, whether the effects were specifically related to learning and memory—versus a possible impact on motor or sensory function that could have impaired the ability of the animal to perform the learning and memory tests as measured—was not possible to discern. Also unclear is the extent to which skeletal fluorosis might have occurred, especially at the highest concentrations tested. Although skeletal fluorosis is associated most often with chronic durations of exposure and longer durations than those used in the neurobehavioral literature. Additional studies are required to achieve higher confidence in the specificity of the responses as learning or memory impairments and in quantitative measures such as effect sizes, points of departure, identification of NOEL or LOEL doses, or parameters for benchmark dose analysis. Based on control values (means and SD/SEs) and the number of animals per group, the studies appear statistically underpowered to detect <10% or <20% change from controls for most behavioral endpoints.

Relevance to Human Exposure Levels

Very few studies assessed learning and memory effects at exposure levels near 0.7 parts per million, the recommended level for community water fluoridation in the United States. No effects were observed at the lowest concentration tested of 0.9 ppm (0.23 mg/kg-d) in adult male BALB/c mice treated with for 30 days (Liu et al. 2014). No effects were observed at this concentration in learning and memory tests (Morris water maze, novel object recognition) or in tests of motor activity, depression, or anxiety. The lowest concentration tested in the included developmental studies was 11.3 ppm (1.63 mg/kg-d) (Jiang et al. 2014a).

Several adult exposure studies, most of which were published after the 2006 NRC review of 2–4 ppm,[13] reported effects at 2.26 ppm (0.33–0.59 mg/kg-d, depending on the study), including impaired performance in the Morris water maze (Dong et al. 2015c; Liu et al. 2014; Liu et al. 2011; Liu et al. 2010; Liu et al. 2009), Y-maze (Xu et al. 2001; Zhang et al. 1999), and novel object recognition tests (Liu et al. 2014). Other studies tested this drinking water concentration but did not report statistically significant effects (Gao et al. 2009a; Gao et al. 2008a; Gao et al. 2008b; Zhu et al. 2012). Several of the Morris water maze studies using 2.26 ppm appear to be conducted by the same research group using a very similar experimental paradigm, finding effects at 2.26 ppm in some studies (Liu et al. 2011; Liu et al. 2010) but not in others (Gao et al. 2009a; Gao et al. 2008b). Some studies appear to combine reporting of the duplicate data with

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[13]At levels below 4.0 mg/L, NRC found no evidence substantial enough to support negative health effects other than severe dental fluorosis (NRC 2006).

new results (Dong et al. 2015b; Dong et al. 2015c; Liu et al. 2011; Liu et al. 2010; Liu et al. 2009).

In the EPA exposure assessment of fluoride intake, the average drinking water concentration for public water systems at a 90% water consumption rate yielded an intake of 0.63–1.23 mg/d for children from 0.5 year to 14 years (US EPA 2010b). In the rodent literature, these daily water intakes were approximated in studies using drinking water concentrations of 7 to 9 ppm.[14] The total oral intake of fluoride from all sources (drinking water and all other sources) was 1.2 to 2.41 mg/d for the same age groups. These total intakes were approximated by animal studies using drinking water concentrations of 9 to 29 ppm.

Assessing effects of fluoride at 0.7-ppm concentration in future studies will be difficult for various reasons. One is the background levels of fluoride in drinking water; the use of reverse osmosis filtration, however, might obviate this issue. When reported, detection level ranges in drinking water were 0.2–1.7 mg/L (Appendix V); the level of quantitation and the margin of error around those levels, however, typically were not reported. More important could be the level of fluoride in normal rodent food. Background levels in diet were less commonly reported, but included 10 ppm (Mullenix et al. 1995) and up to 25.57 mg/kg of diet (Hong et al. 2005).

Several studies in rodents cited in this document measured serum or plasma levels of fluoride following exposures to various elevated levels in drinking water (Appendix U). These studies reported fluoride concentrations in drinking water ranging from approximately 80–8000 times higher than were measured in plasma/serum. This variability is perhaps not surprising because many factors can affect the ratios for substances that are relatively rapidly cleared from blood, including (1) the time between removal from, or the last exposure, to drinking water—which is not reported in studies; (2) the age of the animal and its related capacity of mineralized tissues to take up circulating fluoride; and (3) potential inter strain and species differences in renal clearance rates.

Understanding the relationship between "applied dose," as represented by drinking water concentration, and resulting blood levels is critical in extrapolating adverse health effects from rodent studies to humans. In this respect, the relationship between drinking water fluoride concentrations and resulting blood levels of fluoride in young healthy adult humans has been described as a numerical equivalence between drinking water concentrations expressed as mg/L,

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[14]Using a rat body weight of 0.235 kg and, given that 90th percentile human intake is 0.63 mg/day for a 1-year-old and 1.23 mg/day for a 14-year-old, body weight-adjusted values would be as follows for a 1-year-old and a 14-year-old:
- 0.63 mg/day = 0.07 mg/kg-d for 1-year-old human (assumes body weight of 9 kg)
- 1.23 mg/day = 0.021 mg/kg-d for 14-year-old human (assumes body weight of 60 kg)

Assuming that the same health effects will occur in rats when the intake is adjusted using a $BW^{3/4}$ allometric scaling, the following body weight-adjusted intakes for rats would be:
- 0.07 mg/kg-d $\times$ (9 kg / 0.235 kg)$^{3/4}$ = 1.08 mg/kg-d
- 0.021 mg/kg-d $\times$ (60 kg / 0.235 kg)$^{3/4}$ = 1.31 mg/kg-d

In mg/day:
- 1.08 mg/kg-d $\times$ 0.235 kg = 0.25 mg/day
- 1.31 mg/kg-d $\times$ 0.235 kg = 0.31 mg/day

Converting to ppm would give:
- 0.25 mg/day / 0.034 L/day = 7.4 mg/L = 7 ppm
- 0.31 mg/day / 0.034 L/day = 9.1 mg/L = 9 ppm

and blood concentrations expressed as µmoles/L, or approximately a factor of 1000 (NRC 1993).[15] This value falls within the range of ratios cited above for rodent studies. Dunipace et al. (1995) and National Research Council (NRC 2006), however, postulated that the applied drinking water "dose" to rats to achieve a plasma level equivalent to humans would need to be fivefold higher. Given the myriad variables that could influence these measured relationships in both animal and human studies, assuming approximate equivalence is not unreasonable, nor is assuming that health effects related to drinking water concentrations can be directly compared in rodents and humans from the perspective of kinetics.

Limitations in the Literature Base

- Deficiencies in study design, performance, and reporting limited the utility of several studies. Of the 68 included studies, 19 were considered to have a very severe risk of bias. Duplicate publication of the same results also occurred.

- Methodology descriptions of the behavioral studies often omitted specific information on how behavioral tests were conducted, configuration of the test apparatus, and specifics on test apparatus specifications, for example, for Morris water maze, the relative size of tank and platform and specifications of visual cues; size of activity arena; configuration of avoidance apparatus.

- Information was lacking on alternative sources of fluoride, for example, food or water supply.

- Relatively few developmental studies have focused on levels of fluoride exposure below 25 ppm in drinking water (Table 6). Most of these studies have design issues including lack of reporting of randomization, blinding at outcome assessment, or lack of controlling for litter effects.

- The majority of the learning and memory studies, which indicated an effect of exposure, were maze or avoidance studies. Often, the outcome endpoint was a simple latency measurement in the final training session rather than an evaluation of the acquisition of the task (increase in performance over time) to demonstrate learning. Thus, interpretation of the data is hindered by inability to exclude alterations from baseline levels or differences in motor-related performance over the training session as contributing factors.

Limitations of the Systematic Review

- Evidence synthesis was restricted to potential learning and memory effects of fluoride.

- Additional analyses could be conducted to understand patterns of findings between learning and memory and other behavioral responses, such as motor and sensory function, and general systematic toxicity.

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[15]On page 56–57, the text was changed from "In this respect, the relationship between drinking water fluoride concentrations and resulting blood levels of fluoride in young healthy adult humans has been described as an equivalence between drinking water concentrations expressed as mg/L, and blood concentrations expressed as µg/L, or approximately a factor of 1000 (NRC 1993)" to "In this respect, the relationship between drinking water fluoride concentrations and resulting blood levels of fluoride in young healthy adult humans has been described as a numerical equivalence between drinking water concentrations expressed as mg/L, and blood concentrations expressed as µmoles/L, or approximately a factor of 1000 (NRC 1993)." [August 19, 2016].

- This review focused on selected behavioral measures. Studies examining fluoride exposure on endpoints assessing brain-related cellular, morphometric, or histological endpoints were considered beyond the scope of this analysis. Similarly, effects on thyroid function, which might alter specific neurobehavioral measures, were considered beyond the scope of this review. In the NRC report (NRC 2006), the histological, chemical, and molecular studies were considered to provide evidence that fluorides can interfere with the functions of the brain and the body by direct and indirect means. Potential mechanisms described included reduced brain content of lipids and phospholipids, phosphohydrolases and phospholipase D, and other proteins; inhibition of cholinesterase activity (including acetylcholinesterase); reduction in acetylcholine receptors in the brain; activation of Gp, a protein of the G family, which mediates the release of many central nervous system transmitters; and increased production of free radicals in the brain. Cellular effects of the neocortex, hippocampus, and amygdala also were described in rat studies.

- Dose levels were converted to fluoride equivalents (F) expressed as mg/kg-d and ppm (e.g., 100 ppm sodium fluoride = 45.3 ppm fluoride). Most studies reported sodium fluoride as the form of fluoride administered. In several studies, the specific form of the chemical administered to animals was reported simply as "fluoride" with no details on counter ion, source, or purity, and no conversions to ionic fluoride equivalents were made. Dose levels expressed in fluoride equivalents would overestimate dose levels if, in fact, sodium fluoride were the test compound administered.

- Values for dose conversion (i.e., body weight, food, and water consumption) were based on EPA dosimetry guides (US EPA 1988; 1994). The European Food Safety Authority has developed more recent guidance on default values (EFSA 2012). Use of different default values can influence the dose levels as expressed in mg/kg.

- A meta-analysis was not performed in the current review, but quantitative approaches could be one way to understand sources of heterogeneity more completely and how they might affect confidence ratings, that is, heterogeneity for dose level could lead to greater confidence in dose-response assessment, while heterogeneity due to risk of bias might lead to reduced confidence.

- The ability to address potential publication bias was limited due to (1) challenges of having to select one outcome per study using approaches such as funnel plots when multiple endpoints related to the primary outcome were reported; (2) three or fewer studies being available for certain behavior tests; and (3) a lack of reporting on funding and conflict of interest in papers. In addition, analytical tools, such as funnel plots or trim-and-fill approaches, could be useful to assess publication bias but also have substantial limitations and should be interpreted with caution (Guyatt et al. 2011c). These limitations are especially pertinent for studies having small sample sizes, as is the case in the fluoride literature. In the current analysis, publication bias was described as "undetected." This determination is based on GRADE guidance to use ratings of either "undetected" or "strongly suspected," acknowledging the difficulty in having confidence that publication bias is absent and identifying a threshold to rate down the quality of evidence based on its presence (Guyatt et al. 2011c).

Data Gaps and Research Needs

Additional studies are required to have higher confidence in the specificity of the responses as learning or memory impairments and in quantitative measures such as effect sizes, points of departure, identification of NOEL or LOEL doses, or parameters for benchmark dose analysis. Future research should consider:

- Use of low doses that are more applicable to human exposure levels and include characterization of background levels of fluoride in drinking water and diet. Assessing effects of fluoride at 0.7-ppm concentration will be difficult, given analytical capabilities to quantify low concentrations of fluoride in drinking water and the diet. In addition, use and interpretation of results from studies that use low fluoride rodent diets is likely to be challenging because nutritional components might change as a function of methods to remove fluoride and thus, not adequately support rodent growth, development, and adult health.

- Assessment of potential confounding from effects on motor and sensory function.

- Characterization of differences in response associated with sex, lifestage at exposure, duration of exposure, species, and strain.

- Evaluation and replication of the reported neuropathology associated with exposure.

- Addressing of several common study design and reporting deficiencies in the included studies.

- In particular, additional studies should:
  - Employ a wider range of doses and be appropriately powered with adequate numbers of animals to detect small effect sizes.
  - Be attentive to randomization and blinding during the study and at outcome assessment.
  - Report purity and source of test compound.
  - Control for litter effects in developmental studies.
  - Use repeated measures statistics when appropriate, and perform separate analyses of males and females.

Conclusion

Very few studies assessed learning and memory effects in experimental animals (rats and mice) at exposure levels near 0.7 parts per million, the recommended level for community water fluoridation in the United States. At concentrations higher than 0.7 parts per million, this systematic review found a low to moderate level-of-evidence that suggests adverse effects on learning and memory in animal exposed to fluoride. The evidence is strongest (moderate level-of-evidence) in animals exposed as adults and weaker (low level-of-evidence) in animals exposed during development. Confidence in these findings was reduced primarily based on potential confounding of the learning and memory assessments by deficits in motor function or fear and risk of bias limitations. Additional research is needed, in particular to address potential effects on learning and memory following exposure during development to fluoride at levels nearer to 0.7 parts per million. NTP is conducting laboratory studies in rodents to fill data gaps

identified by this systematic review of the animal studies. The findings from those studies will be included in a future systematic review to evaluate potential neurobehavioral effects from exposure to fluoride during development with consideration of human, experimental animal and mechanistic data.

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Appendix A. Electronic Database Search Strategies

Table of Contents

Tables

A.1. Biosis

Table A-1. Search Strategy for BIOSIS

Search ID	Search Terms	Date: Number of Records
# 4	#1 AND #2 AND #3	2/18/2015: 899 1/14/2016 update: 21
# 1 Animal Studies	**TOPIC:** (("animal model" OR "animal models" OR "invertebrates" OR "chordata" OR "vertebrates" OR "amphibians" OR "birds" OR "fishes" OR "reptiles" OR "mammals" OR "primates" OR "artiodactyla" OR "carnivora" OR "cetacea" OR "chiroptera" OR "elephants" OR "hyraxes" OR "insectivora" OR "lagomorpha" OR "marsupialia" OR "monotremata" OR "perissodactyla" OR "rodentia" OR "scandentia" OR "sirenia" OR "xenarthra" OR "haplorhini" OR "strepsirhini" OR "platyrrhini" OR "tarsii" OR "catarrhini" OR "cercopithecidae" OR "hylobatidae" OR "hominidae" OR "gorilla" OR "pan paniscus" OR "pan troglodytes" OR "pongo pygmaeus" OR animals OR animal OR mice OR mus OR mouse OR murine OR woodmouse OR rats OR rat OR murinae OR muridae OR cottonrat OR cottonrats OR hamster OR hamsters OR cricetinae OR rodentia OR rodent OR rodents OR pigs OR pig OR swine OR swines OR piglets OR piglet OR boar OR boars OR "sus scrofa" OR ferrets OR ferret OR polecat OR polecats OR "mustela putorius" OR "guinea pigs" OR "guinea pig" OR cavia OR callithrix OR marmoset OR marmosets OR cebuella OR hapale OR octodon OR chinchilla OR chinchillas OR gerbillinae OR gerbil OR gerbils OR jird OR jirds OR merione OR meriones OR rabbits OR rabbit OR hares OR hare OR diptera OR flies OR fly OR dipteral OR drosophila OR drosophilidae OR cats OR cat OR carus OR felis OR nematoda OR nematode OR nematodes OR sipunculida OR dogs OR dog OR canine OR canines OR canis OR sheep OR sheeps OR mouflon OR mouflons OR ovis OR goats OR goat OR capra OR capras OR rupicapra OR rupicapras OR chamois OR haplorhini OR monkey OR monkeys OR anthropoidea OR anthropoids OR saguinus OR tamarin OR tamarins OR leontopithecus OR hominidae OR ape OR apes OR "pan paniscus" OR bonobo OR bonobos OR "pan troglodytes" OR gibbon OR gibbons OR siamang OR siamangs OR nomascus OR symphalangus OR chimpanzee OR chimpanzees OR prosimian OR prosimians OR "bush baby" OR bush babies OR galagos OR galago OR pongidae OR gorilla OR gorillas OR "pongo pygmaeus" OR orangutan OR orangutans OR lemur OR lemurs OR lemuridae OR horse OR horses OR equus OR cow OR calf OR bull OR chicken OR chickens OR gallus OR quail OR bird OR birds OR quails OR poultry OR poultries OR fowl OR fowls OR reptile OR reptilia OR reptiles OR snakes OR snake OR lizard OR lizards OR alligator OR alligators OR crocodile OR crocodiles OR turtle OR turtles OR amphibian OR amphibians OR amphibia OR frog OR frogs OR bombina OR salientia OR toad OR toads OR "epidalea calamita" OR salamander OR salamanders OR eel OR eels OR fish OR fishes OR pisces OR catfish OR catfishes OR siluriformes OR arius OR heteropneustes OR sheatfish OR perch OR perches OR percidae OR perca OR trout OR trouts OR char OR chars OR salvelinus OR minnow OR cyprinidae OR carps OR carp OR zebrafish OR zebrafishes OR goldfish OR goldfishes OR guppy OR	2/18/2015: 218,068,453 1/14/2016 update: 150,971

Search ID	Search Terms	Date: Number of Records
# 2 Neurological Outcomes	guppies OR chub OR chubs OR tinca OR barbels OR barbus OR pimephales OR promelas OR "poecilia reticulata" OR mullet OR mullets OR eel OR eels OR seahorse OR seahorses OR mugil curema OR atlantic cod OR shark OR sharks OR catshark OR anguilla OR salmonid OR salmonids OR whitefish OR whitefishes OR salmon OR salmons OR sole OR solea OR lamprey OR lampreys OR pumpkinseed OR sunfish OR sunfishes OR tilapia OR tilapias OR turbot OR turbots OR flatfish OR flatfishes OR sciuridae OR squirrel OR squirrels OR chipmunk OR chipmunks OR suslik OR susliks OR vole OR voles OR lemming OR lemmings OR muskrat OR muskrats OR lemmus OR otter OR otters OR marten OR martens OR martes OR weasel OR badger OR badgers OR ermine OR mink OR minks OR sable OR sables OR gulo OR gulos OR wolverine OR wolverines OR mustela OR llama OR llamas OR alpaca OR alpacas OR camelid OR camelids OR guanaco OR guanacos OR chiroptera OR chiropteras OR bat OR bats OR fox OR foxes OR iguana OR iguanas OR xenopus laevis OR parakeet OR parakeets OR parrot OR parrots OR donkey OR donkeys OR mule OR mules OR zebra OR zebras OR shrew OR shrews OR bison OR bisons OR buffalo OR buffaloes OR deer OR deers OR bear OR bears OR panda OR pandas OR "wild hog" OR "wild boar" OR fitchew OR fitch OR beaver OR beavers OR jerboa OR jerboas OR capybara OR capybaras) NOT (humans OR hominidae)) **TOPIC:** ("nervous system" OR "central nervous" OR brain OR blood- brain OR brain-stem OR cerebral OR limbic OR amygdala OR hippocamp* OR hypothalamus OR parahippocamp* OR perforant OR mesencephalon OR prosencephalon OR rhombencephalon OR meninges OR dura-matter OR spinal-cord OR ganglia OR ganglion OR nerve OR nervous OR neural OR neural near/0 tube* OR afferent OR auditory OR olfactory OR oculomotor OR optic OR retinal OR efferent OR perforant OR neuroglia* OR astrocyte* OR microglia* OR schwann near/0 cell* OR "myelin sheath" OR neuron* OR axon* OR dendrit* OR interneuron* OR lewy near/0 bod* OR neurofibril* OR purkinje-cell* OR pyramidal-cell* OR serotonergic OR neuromuscular OR neuroendocrine OR neurosecret* OR pituitary OR pineal OR synapse* OR synaptic OR presynaptic OR postsynaptic OR plasticity OR hearing OR vision OR taste OR smell OR propriocept* OR neurologic* OR autonomic-nervous OR central near/0 nervous OR demyelinat* OR "multiple sclerosis" OR myasthenia-gravis OR epilep* OR encephalitis OR meningitis OR chronic-fatigue OR dyskinesia* OR dyston* OR parkinson* OR mobility OR motor near/0 control OR motor near/0 abilit* OR motor near/0 skill* OR cranial near/0 nerve OR huntington* OR tourette* OR ataxia OR hydranencephaly OR neurofibra* OR tauopath* OR fibromyalgia OR myopath* OR neurodegenerat* OR neuroimmunomodulat* OR neuromuscular OR neuropath* OR neurosecret* OR neurotox* OR neurobehav* OR dyslexi* OR cognitive OR cognition OR intellect* OR intelligence OR mental-deficienc* OR mental near/0 deficit* OR down-syndrome OR rett-syndrome OR memory near/0 deficit* OR memory near/0 impair* OR memory-loss* OR mental near/0 disorder* OR developmental near/0 delay* OR developmental near/0 disabilit* OR anxiety OR obsessive-compulsive OR phobia* OR phobic OR antisocial OR personality OR schizophrenia OR behavior OR paranoid OR paranoia OR dementia OR senile OR senility OR alzheimer OR learning OR aphasia OR autism OR autistic OR "attention-deficit")	2/18/2015: 5,947,883 1/14/2016 update: 168,166 2/18/2015: 21,679

Search ID	Search Terms	Date: Number of Records
# 3 Fluoride	TITLE=(fluorid* OR flurid* OR fluorin* OR florin* OR fluorosis) NOT TOPIC=((18F OR F-18 OR "fluorine-18" OR 19F OR F-19 OR "fluorine-19" OR (PET AND scan) OR f-labeled OR imaging OR radioligand*))	1/14/2016 update: 450

A.2. EMBASE

Search Note: Animal studies were restricted to the traditional animal models. Any other animal studies would likely be picked up in the other databases.

Table A-2. Search Strategy for EMBASE

Search ID	Search Terms	Date: Number of Records
# 4	#1 AND #2 AND #3	1/7/ 2015: 306 1/14/2016 update: 25
# 1 Animal Studies	'animal model'/exp OR rats OR rat OR mice OR mouse OR rodent* OR murin*	1/7/2015: 3,389,269 1/14/2016 update: 137,941
# 2 Neurological Outcomes	'nervous system'/exp OR 'nervous system' OR 'central-nervous' OR 'brain'/exp OR brain OR 'blood brain' OR 'brain stem'/exp OR 'brain stem' OR cerebral OR cortical OR limbic OR 'amygdala'/exp OR amygdala OR hippocamp* OR 'hypothalamus'/exp OR hypothalamus OR parahippocamp* OR 'mesencephalon'/exp OR mesencephalon OR 'prosencephalon'/exp OR prosencephalon OR 'rhombencephalon'/exp OR rhombencephalon OR 'meninges'/exp OR meninges OR 'dura matter' OR 'spinal cord'/exp OR 'spinal cord' OR 'ganglia'/exp OR ganglia OR 'ganglion'/exp OR ganglion OR 'nerve'/exp OR nerve OR neural OR 'neural tube'/exp OR 'neural tube' OR afferent OR auditory OR olfactory OR oculomotor OR optic OR 'retinal'/exp OR retinal OR efferent OR perforant OR neuroglia* OR astrocyte* OR microglia* OR schwann NEAR/0 cell* OR 'myelin sheath'/exp OR 'myelin sheath' OR neuron* OR axon* OR dendrit* OR interneuron* OR lewy NEXT/0 bod* OR neurofibril* OR purkinje NEXT/0 cell* OR pyramidal NEXT/0 cell* OR serotonergic OR neuromuscular OR neuroendocrine OR neurosecret* OR 'pituitary'/exp OR pituitary OR pineal OR synapse* OR synaptic OR presynaptic OR postsynaptic OR 'plasticity'/exp OR plasticity OR 'hearing'/exp OR hearing OR 'vision'/exp OR vision OR 'taste'/exp OR taste OR 'smell'/exp OR smell OR propriocept* OR nervous AND system AND ('diseases'/exp OR diseases) OR neurologic* OR 'autonomic nervous' OR 'central nervous' OR demyelinat* OR 'multiple sclerosis'/exp OR 'multiple sclerosis' OR 'myasthenia gravis'/exp OR 'myasthenia gravis' OR epilep* OR 'encephalitis'/exp OR encephalitis OR 'meningitis'/exp OR meningitis OR 'chronic fatigue' OR dyskinesia* OR dyston* OR parkinson* OR mobility OR 'motor control'/exp OR 'motor control' OR motor NEXT/0 abilit* OR motor NEXT/0 skill* OR 'cranial nerve'/exp OR 'cranial nerve' OR huntington* OR tourette* OR 'ataxia'/exp OR ataxia OR 'hydranencephaly'/exp OR hydranencephaly OR neurofibra* OR tauopath*	1/7/2015: 4,078,217 1/14/2016 update: 237,733

Search ID	Search Terms	Date: Number of Records
	OR 'fibromyalgia'/exp OR fibromyalgia OR myopath* OR neurodegenerat* OR neuroimmunomodulat* OR neuromuscular OR neuropath* OR neurosecret* OR neurotox* OR neurobehav* OR dyslexi* OR cognitive OR 'cognition'/exp OR cognition OR intellect* OR 'intelligence'/exp OR intelligence OR mental NEAR/0 deficienc* OR mental NEAR/0 deficit* OR 'down syndrome'/exp OR 'down syndrome' OR 'rett syndrome'/exp OR 'rett syndrome' OR memory NEAR/0 deficit* OR memory NEAR/0 impair* OR memory NEAR/0 loss* OR mental AND disorders OR developmental AND disabilities OR developmental NEAR/0 delay* OR developmental NEAR/0 disabilit* OR 'anxiety'/exp OR anxiety OR 'obsessive compulsive' OR phobia* OR phobic OR antisocial OR 'personality'/exp OR personality OR 'schizophrenia'/exp OR schizophrenia OR 'behavior'/exp OR behavior OR 'paranoid'/exp OR paranoid OR 'paranoia'/exp OR paranoia OR 'dementia'/exp OR dementia OR senile OR 'senility'/exp OR senility OR alzheimer OR 'learning'/exp OR learning OR 'aphasia'/exp OR aphasia OR 'autism'/exp OR autism OR autistic OR 'attention deficit'/exp OR 'attention deficit'	
# 3 Fluoride	2/18/2015: fluoride:de OR fluorid* NOT ('f labeled' OR 'fluorine 18'/exp OR 'fluorine 18' OR 'imaging'/exp OR imaging OR radioligand*) 1/14/2016 update: fluoride:de OR fluorid* OR flurid* OR fluorinated OR 'fluorosis'/exp OR fluorosis NOT ('18f'/exp OR 18f OR 'f 18'/exp OR 'f 18' OR 19f OR 'f 19' OR 'fluorine-19'/exp OR 'fluorine-19' OR (pet AND scan) OR 'f labeled' OR 'fluorine 18'/exp OR 'fluorine 18' OR 'imaging' OR 'imaging'/exp OR imaging OR radioligand*) AND [2015-2016]/py	1/7/2015: 59,675 1/14/2016 update: 1,635

A.3. PsycINFO

Search Note: Given how studies fluoride studies were included in this database, the search was not restricted to neurological outcomes in animals.

Table A-3. Search Strategy for PsycINFO

Search ID	Search Terms	Date: Number of Records
# 1	1/7/2015: Title: fluorid* OR flurid* OR fluorin* OR florin* 1/14/2016 update: Title: fluorid* OR flurid* OR fluorin* OR florin* OR fluorosis *OR* Abstract: fluorid* OR flurid* OR fluorin* OR florin* OR fluorosis *AND* Year: 2015 *To* 2016	1/7/2015: 74 1/14/2016 update: 36

A.4. PubMed

Table A-4. Search Strategy for PubMed

Search ID	Search Terms	Date: Number of Records
# 4	#1 AND #2 AND #3	1/7/2015: 2417 1/14/2016 update: 53
# 1 Animal Studies [from Hooijmans et al. (2010)]	("animal experimentation"[MeSH Terms] OR "models, animal"[MeSH Terms] OR "invertebrates"[MeSH Terms] OR "Animals"[Mesh:noexp] OR "animal population groups"[MeSH Terms] OR "chordata"[MeSH Terms:noexp] OR "chordata, nonvertebrate"[MeSH Terms] OR "vertebrates"[MeSH Terms:noexp] OR "amphibians"[MeSH Terms] OR "birds"[MeSH Terms] OR "fishes"[MeSH Terms] OR "reptiles"[MeSH Terms] OR "mammals"[MeSH Terms:noexp] OR "primates"[MeSH Terms:noexp] OR "artiodactyla"[MeSH Terms] OR "carnivora"[MeSH Terms] OR "cetacea"[MeSH Terms] OR "chiroptera"[MeSH Terms] OR "elephants"[MeSH Terms] OR "hyraxes"[MeSH Terms] OR "insectivora"[MeSH Terms] OR "lagomorpha"[MeSH Terms] OR "marsupialia"[MeSH Terms] OR "monotremata"[MeSH Terms] OR "perissodactyla"[MeSH Terms] OR "rodentia"[MeSH Terms] OR "scandentia"[MeSH Terms] OR "sirenia"[MeSH Terms] OR "xenarthra"[MeSH Terms] OR "haplorhini"[MeSH Terms:noexp] OR "strepsirhini"[MeSH Terms] OR "platyrrhini"[MeSH Terms] OR "tarsii"[MeSH Terms] OR "catarrhini"[MeSH Terms:noexp] OR "cercopithecidae"[MeSH Terms] OR "hylobatidae"[MeSH Terms] OR "hominidae"[MeSH Terms:noexp] OR "gorilla gorilla"[MeSH Terms] OR "pan paniscus"[MeSH Terms] OR "pan troglodytes"[MeSH Terms] OR "pongo pygmaeus"[MeSH Terms]) OR ((animals[tiab] OR animal[tiab] OR mice[Tiab] OR mus[Tiab] OR mouse[Tiab] OR murine[Tiab] OR woodmouse[tiab] OR rats[Tiab] OR rat[Tiab] OR murinae[Tiab] OR muridae[Tiab] OR cottonrat[tiab] OR cottonrats[tiab] OR hamster[tiab] OR hamsters[tiab] OR cricetinae[tiab] OR rodentia[Tiab] OR rodent[Tiab] OR rodents[Tiab] OR pigs[Tiab] OR pig[Tiab] OR swine[tiab] OR swines[tiab] OR piglets[tiab] OR piglet[tiab] OR boar[tiab] OR boars[tiab] OR "sus scrofa"[tiab] OR ferrets[tiab] OR ferret[tiab] OR polecat[tiab] OR polecats[tiab] OR "mustela putorius"[tiab] OR "guinea pigs"[Tiab] OR "guinea pig"[Tiab] OR cavia[Tiab] OR callithrix[Tiab] OR marmoset[Tiab] OR marmosets[Tiab] OR cebuella[Tiab] OR hapale[Tiab] OR octodon[Tiab] OR chinchilla[Tiab] OR chinchillas[Tiab] OR gerbillinae[Tiab] OR gerbil[Tiab] OR gerbils[Tiab] OR jird[Tiab] OR jirds[Tiab] OR merione[Tiab] OR meriones[Tiab] OR rabbits[Tiab] OR rabbit[Tiab] OR hares[Tiab] OR hare[Tiab] OR diptera[Tiab] OR flies[Tiab] OR fly[Tiab] OR dipteral[Tiab] OR drosophila[Tiab] OR drosophilidae[Tiab] OR cats[Tiab] OR cat[Tiab] OR carus[Tiab] OR felis[Tiab] OR nematoda[Tiab] OR nematode[Tiab] OR nematodes[Tiab] OR sipunculida[Tiab] OR dogs[Tiab] OR dog[Tiab] OR canine[Tiab] OR canines[Tiab] OR canis[Tiab] OR sheep[Tiab] OR sheeps[Tiab] OR mouflon[Tiab] OR mouflons[Tiab] OR ovis[Tiab] OR goats[Tiab] OR goat[Tiab] OR capra[Tiab] OR capras[Tiab] OR rupicapra[Tiab] OR rupicapras[Tiab] OR chamois[Tiab] OR haplorhini[Tiab] OR monkey[Tiab] OR monkeys[Tiab] OR anthropoidea[Tiab] OR anthropoids[Tiab] OR saguinus[Tiab] OR	1/7/2015: 5,723,242 1/14/2016 update: 209,076

Search ID	Search Terms	Date: Number of Records
	tamarin[Tiab] OR tamarins[Tiab] OR leontopithecus[Tiab] OR hominidae[Tiab] OR ape[Tiab] OR apes[Tiab] OR "pan paniscus"[Tiab] OR bonobo[Tiab] OR bonobos[Tiab] OR "pan troglodytes"[Tiab] OR gibbon[Tiab] OR gibbons[Tiab] OR siamang[Tiab] OR siamangs[Tiab] OR nomascus[Tiab] OR symphalangus[Tiab] OR chimpanzee[Tiab] OR chimpanzees[Tiab] OR prosimian[Tiab] OR prosimians[Tiab] OR "bush baby"[Tiab] OR bush babies[Tiab] OR galagos[Tiab] OR galago[Tiab] OR pongidae[Tiab] OR gorilla[Tiab] OR gorillas[Tiab] OR "pongo pygmaeus"[Tiab] OR orangutan[Tiab] OR orangutans[Tiab] OR lemur[Tiab] OR lemurs[Tiab] OR lemuridae[Tiab] OR horse[Tiab] OR horses[Tiab] OR equus[Tiab] OR cow[Tiab] OR calf[Tiab] OR bull[Tiab] OR chicken[Tiab] OR chickens[Tiab] OR gallus[Tiab] OR quail[Tiab] OR bird[Tiab] OR birds[Tiab] OR quails[Tiab] OR poultry[Tiab] OR poultries[Tiab] OR fowl[Tiab] OR fowls[Tiab] OR reptile[Tiab] OR reptilia[Tiab] OR reptiles[Tiab] OR snakes[Tiab] OR snake[Tiab] OR lizard[Tiab] OR lizards[Tiab] OR alligator[Tiab] OR alligators[Tiab] OR crocodile[Tiab] OR crocodiles[Tiab] OR turtle[Tiab] OR turtles[Tiab] OR amphibian[Tiab] OR amphibians[Tiab] OR amphibia[Tiab] OR frog[Tiab] OR frogs[Tiab] OR bombina[Tiab] OR salientia[Tiab] OR toad[Tiab] OR toads[Tiab] OR "epidalea calamita"[Tiab] OR salamander[Tiab] OR salamanders[Tiab] OR eel[Tiab] OR eels[Tiab] OR fish[Tiab] OR fishes[Tiab] OR pisces[Tiab] OR catfish[Tiab] OR catfishes[Tiab] OR siluriformes[Tiab] OR arius[Tiab] OR heteropneustes[Tiab] OR sheatfish[Tiab] OR perch[Tiab] OR perches[Tiab] OR percidae[Tiab] OR perca[Tiab] OR trout[Tiab] OR trouts[Tiab] OR char[Tiab] OR chars[Tiab] OR salvelinus[Tiab] OR minnow[Tiab] OR cyprinidae[Tiab] OR carps[Tiab] OR carp[Tiab] OR zebrafish[Tiab] OR zebrafishes[Tiab] OR goldfish[Tiab] OR goldfishes[Tiab] OR guppy[Tiab] OR guppies[Tiab] OR chub[Tiab] OR chubs[Tiab] OR tinca[Tiab] OR barbels[Tiab] OR barbus[Tiab] OR pimephales[Tiab] OR promelas[Tiab] OR "poecilia reticulata"[Tiab] OR mullet[Tiab] OR mullets[Tiab] OR eel[Tiab] OR eels[Tiab] OR seahorse[Tiab] OR seahorses[Tiab] OR mugil curema[Tiab] OR atlantic cod[Tiab] OR shark[Tiab] OR sharks[Tiab] OR catshark[Tiab] OR anguilla[Tiab] OR salmonid[Tiab] OR salmonids[Tiab] OR whitefish[Tiab] OR whitefishes[Tiab] OR salmon[Tiab] OR salmons[Tiab] OR sole[Tiab] OR solea[Tiab] OR lamprey[Tiab] OR lampreys[Tiab] OR pumpkinseed[Tiab] OR sunfish[Tiab] OR sunfishes[Tiab] OR tilapia[Tiab] OR tilapias[Tiab] OR turbot[Tiab] OR turbots[Tiab] OR flatfish[Tiab] OR flatfishes[Tiab] OR sciuridae[Tiab] OR squirrel[Tiab] OR squirrels[Tiab] OR chipmunk[Tiab] OR chipmunks[Tiab] OR suslik[Tiab] OR susliks[Tiab] OR vole[Tiab] OR voles[Tiab] OR lemming[Tiab] OR lemmings[Tiab] OR muskrat[Tiab] OR muskrats[Tiab] OR lemmus[Tiab] OR otter[Tiab] OR otters[Tiab] OR marten[Tiab] OR martens[Tiab] OR martes[Tiab] OR weasel[Tiab] OR badger[Tiab] OR badgers[Tiab] OR ermine[Tiab] OR mink[Tiab] OR minks[Tiab] OR sable[Tiab] OR sables[Tiab] OR gulo[Tiab] OR gulos[Tiab] OR wolverine[Tiab] OR wolverines[Tiab] OR mustela[Tiab] OR llama[Tiab] OR llamas[Tiab] OR alpaca[Tiab] OR alpacas[Tiab] OR camelid[Tiab] OR camelids[Tiab] OR guanaco[Tiab] OR guanacos[Tiab] OR chiroptera[Tiab] OR chiropteras[Tiab] OR bat[Tiab] OR bats[Tiab] OR fox[Tiab] OR foxes[Tiab] OR iguana[Tiab] OR iguanas[Tiab] OR xenopus laevis[Tiab] OR parakeet[Tiab] OR parakeets[Tiab] OR parrot[Tiab] OR parrots[Tiab] OR donkey[Tiab] OR donkeys[Tiab] OR mule[Tiab] OR mules[Tiab] OR	

Search ID	Search Terms	Date: Number of Records
	zebra[Tiab] OR zebras[Tiab] OR shrew[Tiab] OR shrews[Tiab] OR bison[Tiab] OR bisons[Tiab] OR buffalo[Tiab] OR buffaloes[Tiab] OR deer[Tiab] OR deers[Tiab] OR bear[Tiab] OR bears[Tiab] OR panda[Tiab] OR pandas[Tiab] OR "wild hog"[Tiab] OR "wild boar"[Tiab] OR fitchew[Tiab] OR fitch[Tiab] OR beaver[Tiab] OR beavers[Tiab] OR jerboa[Tiab] OR jerboas[Tiab] OR capybara[Tiab] OR capybaras[Tiab]) NOT medline[sb])	
# 2 Neurologica l Outcomes	Nervous system[mh] OR Nervous system physiological processes[mh] OR nervous-system[tiab] OR central-nervous[tiab] OR brain[tiab] OR blood-brain[tiab] OR brain-stem[tiab] OR cerebral[tiab] OR cortical[tiab] OR limbic OR amygdala OR hippocamp*[tiab] OR hypothalamus[tiab] OR parahippocamp*[tiab] OR perforant[tiab] OR mesencephalon OR prosencephalon OR rhombencephalon OR meninges OR dura-matter[tiab] OR spinal- cord[tiab] OR ganglia[tiab] OR ganglion[tiab] OR nerve[tiab] OR nervous[tiab] OR neural[tiab] OR neural-tube[tiab] OR afferent[tiab] OR auditory[tiab] OR olfactory[tiab] OR oculomotor [tiab] OR optic[tiab] OR retinal[tiab] OR efferent[tiab] OR perforant[tiab] OR neuroglia*[tiab] OR astrocyte*[tiab] OR microglia*[tiab] OR schwann-cell*[tiab] OR "myelin sheath"[tiab] OR neuron*[tiab] OR axon*[tiab] OR dendrit*[tiab] OR interneuron*[tiab] OR lewy- bod*[tiab] OR neurofibril*[tiab] OR purkinje-cell*[tiab] OR pyramidal-cell*[tiab] OR serotonergic[tiab] OR neuromuscular[tiab] OR neuroendocrine[tiab] OR neurosecret*[tiab] OR pituitary[tiab] OR pineal[tiab] OR synapse*[tiab] OR synaptic[tiab] OR presynaptic[tiab] OR postsynaptic[tiab] OR plasticity[tiab] OR hearing[tiab] OR vision[tiab] OR taste[tiab] OR smell[tiab] OR propriocept*[tiab] OR Nervous system diseases[mh] OR neurologic*[tiab] OR autonomic-nervous[tiab] OR central- nervous[tiab] OR demyelinat*[tiab] OR multiple-sclerosis[tiab] OR myasthenia-gravis[tiab] OR epilep*[tiab] OR encephalitis[tiab] OR meningitis[tiab] OR chronic-fatigue[tiab] OR dyskinesia*[tiab] OR dyston*[tiab] OR parkinson*[tiab] OR mobility[tiab] OR motor-control[tiab] OR motor-abilit*[tiab] OR motor-skill*[tiab] OR cranial- nerve[tiab] OR huntington*[tiab] OR tourette*[tiab] OR ataxia[tiab] OR hydranencephaly[tiab] OR neurofibra*[tiab] OR tauopath*[tiab] OR fibromyalgia[tiab] OR myopath*[tiab] OR neurodegenerat*[tiab] OR neuroimmunomodulat*[tiab] OR neuromuscular[tiab] OR neuropath*[tiab] OR neurosecret*[tiab] OR neurotox*[tiab] OR neurobehav*[tiab] OR dyslexi*[tiab] OR cognitive[tiab] OR cognition[tiab] OR intellect*[tiab] OR intelligence[tiab] OR mental- deficienc*[tiab] OR mental-deficit*[tiab] OR down-syndrome[tiab] OR rett-syndrome[tiab] OR memory-deficit*[tiab] OR memory- impair*[tiab] OR memory-loss*[tiab] OR Mental disorders[mh] OR Developmental disabilities[mh] OR developmental-delay*[tiab] OR developmental-disabilit*[tiab] OR anxiety[tiab] OR obsessive-compulsive[tiab] OR phobia*[tiab] OR phobic[tiab] OR antisocial[tiab] OR personality[tiab] OR schizophrenia[tiab] OR behavior[tiab] OR paranoid[tiab] OR paranoia[tiab] OR dementia[tiab] OR senile[tiab] OR senility[tiab] OR alzheimer[tiab] OR learning[tiab] OR aphasia[tiab] OR autism[tiab] OR autistic[tiab] OR attention-deficit[tiab]	1/7/2015: 5,326,936 1/14/2016 update: 276,401
# 3 Fluoride	1/7/2015: (Fluorides[mh:noexp] OR fluorides, topical[mh] OR sodium fluoride[mh] OR fluorid*[tiab] OR flurid*[tiab] OR fluorin*[tiab] OR florin*[tiab]) NOT (f-labeled[tiab] OR "fluorine- 18"[tiab] OR imaging[tiab] OR radioligand*[tiab]) 1/14/2016 update: (Fluorides[mh:noexp] OR	1/7/2015: 58,732 1/14/2016 update: 2813

Search ID	Search Terms	Date: Number of Records
	fluorides, topical[mh] OR sodium fluoride[mh] OR fluorid*[tiab] OR flurid*[tiab] OR fluorin*[tiab] OR florin*[tiab]) NOT (18F[tiab] OR f-18[tiab] OR 19F[tiab] OR f-19[tiab] OR f-labeled[tiab] OR "fluorine-18"[tiab] OR "fluorine-19"[tiab] OR pet-scan[tiab] OR imaging[tiab] OR radioligand*[tiab]) Sort by: PublicationDate Filters: Publication date from 2015/01/01 to 2016/12/31	

A.5. SCOPUS

Table A-5. Search Strategy for SCOPUS

Search ID	Search Terms	Date: Number of Records
# 4	#1 AND #2 AND #3	1/7/2015: 1759 1/14/2016 update: 89
# 1 Animal Studies	TITLE-ABS (animal* OR mice OR mus OR mouse OR murine OR rats OR rat OR murinae OR muridae OR "cotton rat" OR "cotton rats" OR hamster* OR criceticae OR rodent* OR pigs OR pig OR swine* OR piglet* OR "guinea pigs" OR "guinea pig" OR cavia OR callithrix OR marmoset* OR cebuella OR hapale OR octodon OR chinchilla* OR gerbillinae OR gerbil* OR rabbit* OR hares OR hare OR cats OR cat OR carus OR felis OR dogs OR dog OR canine* OR canis OR sheep* OR mouflon* OR ovis OR goat* OR capra OR haplorhini OR monkey* OR anthropoid* OR saguinus OR tamarin* OR leontopithecus OR hominidae OR ape OR apes OR "pan paniscus" OR bonobo* OR "pan troglodytes" OR gibbon* OR nomascus OR symphalangus OR chimpanzee* OR prosimian* OR pongidae OR gorilla* OR "pongo pygmaeus" OR orangutan* OR lemur* OR lemuridae OR horse* OR equus OR cow OR calf OR bull OR chicken* OR gallus OR quail* OR bird OR birds OR poultry OR fowl OR fowls OR reptil* OR snake* OR lizard* OR turtle* OR amphibia* OR frog* OR xenopus OR bombina OR salientia OR toad* OR "epidalea calamita" OR salamander* OR fish OR fishes OR pisces OR catfish OR perch OR percidae OR perca OR trout OR char OR salmon OR salvelinus OR minnow* OR cyprinidae OR carp OR zebrafish OR "zebra fish" OR nematode* OR elegans OR diptera OR flies OR dipteral OR drosophil* OR squirrel* OR chipmunk* OR bear OR bears OR ursidae OR dolphin* OR porpoise* OR whale* OR cetacea)	1/7/2015: 5,159,982 1/14/2016 update: 214,886
# 2 Neurological Outcomes	TITLE-ABS ("nervous system" OR "central-nervous" OR brain OR blood-brain OR brain-stem OR cerebral OR cortical OR limbic OR amygdala OR hippocamp* OR hypothalamus OR parahippocamp* OR perforant OR mesencephalon OR prosencephalon OR rhombencephalon OR meninges OR dura-matter OR spinal-cord OR ganglia OR ganglion OR nerve OR nervous OR neural OR neural-tube OR afferent OR auditory OR olfactory OR oculomotor OR optic OR retinal OR efferent OR perforant OR neuroglia* OR astrocyte* OR microglia* OR schwann-cell* OR "myelin sheath" OR neuron* OR axon* OR dendrit* OR interneuron* OR lewy-bod* OR neurofibril* OR purkinje-cell* OR pyramidal-cell* OR serotonergic OR neuromuscular OR neuroendocrine	1/7/2015: 7,558,830 1/14/2016 update: 453,341

Search ID	Search Terms	Date: Number of Records
	OR neurosecret* OR pituitary OR pineal OR synapse* OR synaptic OR presynaptic OR postsynaptic OR plasticity OR hearing OR vision OR taste OR smell OR propriocept* OR Nervous system diseases OR neurologic* OR autonomic-nervous OR central- nervous OR demyelinat* OR multiple-sclerosis OR myasthenia-gravis OR epilep* OR encephalitis OR meningitis OR chronic-fatigue OR dyskinesia* OR dyston* OR parkinson* OR mobility OR motor-control OR motor-abilit* OR motor-skill* OR cranial-nerve OR huntington* OR tourette* OR ataxia OR hydranencephaly OR neurofibra* OR tauopath* OR fibromyalgia OR myopath* OR neurodegenerat* OR neuroimmunomodulat* OR neuromuscular OR neuropath* OR neurosecret* OR neurotox* OR neurobehav* OR dyslexi* OR cognitive OR cognition OR intellect* OR intelligence OR mental- deficienc* OR mental-deficit* OR down-syndrome OR rett-syndrome OR memory-deficit* OR memory-impair* OR memory-loss* OR Mental disorders OR Developmental disabilities OR developmental- delay* OR developmental-disabilit* OR anxiety OR obsessive- compulsive OR phobia* OR phobic OR antisocial OR personality OR schizophrenia OR behavior OR paranoid OR paranoia OR dementia OR senile OR senility OR alzheimer OR learning OR aphasia OR autism OR autistic OR attention-deficit)	
# 3 Fluoride	1/7/2015: TITLE-ABS ((fluorid* OR flurid* OR fluorin* OR florin*) AND NOT (f-labeled OR fluorine-18 OR imaging OR radioligand*))1/14/2016 update: TITLE-ABS(fluorid* OR flurid* OR fluorin* OR florin* OR fluorosis) AND NOT (18F OR F-18 OR "fluorine-18" OR 19F OR F-19 OR "fluorine-19" OR (PET AND scan) OR f-labeled OR imaging OR radioligand*) AND PUBYEAR>2014	1/7/2015: 153,560 1/14/2016 update: 7895

A.6. Web of Science

Search Note: Searched 1/7/2015.

Table A-6. Search Strategy for Web of Science

Search ID	Search Terms	Date: Number of Records
# 4	#1 AND #2 AND #3 Indexes=SCI-EXPANDED, SSCI, CPCI-S, CPCI-SSH, BKCI-S, BKCI-SSH, CCR-EXPANDED,	1/7/2015: 1,836 1/14/2016 update: 59
# 1 Animal Studies	**TOPIC:** ("animal model" OR "animal models" OR "invertebrates" OR "chordata" OR "vertebrates" OR "amphibians" OR "birds" OR "fishes" OR "reptiles" OR "mammals" OR "primates" OR "artiodactyla" OR "carnivora" OR "cetacea" OR "chiroptera" OR "elephants" OR "hyraxes" OR "insectivora" OR "lagomorpha" OR "marsupialia" OR "monotremata" OR "perissodactyla" OR "rodentia" OR "scandentia" OR "sirenia" OR "xenarthra" OR "haplorhini" OR "strepsirhini" OR "platyrrhini" OR "tarsii" OR "catarrhini" OR "cercopithecidae" OR "hylobatidae" OR "hominidae" OR "gorilla" OR "pan paniscus" OR "pan troglodytes" OR "pongo pygmaeus" OR animals OR animal OR mice OR mus OR mouse OR murine OR woodmouse OR rats OR rat OR murinae OR muridae OR cottonrat OR cottonrats OR hamster OR hamsters OR	1/7/2015: 5,945,667 1/14/2016 update: 248,464

Search ID	Search Terms	Date: Number of Records
	cricetinae OR rodentia OR rodent OR rodents OR pigs OR pig OR swine OR swines OR piglets OR piglet OR boar OR boars OR "sus scrofa" OR ferrets OR ferret OR polecat OR polecats OR "mustela putorius" OR "guinea pigs" OR "guinea pig" OR cavia OR callithrix OR marmoset OR marmosets OR cebuella OR hapale OR octodon OR chinchilla OR chinchillas OR gerbillinae OR gerbil OR gerbils OR jird OR jirds OR merione OR meriones OR rabbits OR rabbit OR hares OR hare OR diptera OR flies OR fly OR dipteral OR drosophila OR drosophilidae OR cats OR cat OR carus OR felis OR nematoda OR nematode OR nematodes OR sipunculida OR dogs OR dog OR canine OR canines OR canis OR sheep OR sheeps OR mouflon OR mouflons OR ovis OR goats OR goat OR capra OR capras OR rupicapra OR rupicapras OR chamois OR haplorhini OR monkey OR monkeys OR anthropoidea OR anthropoids OR saguinus OR tamarin OR tamarins OR leontopithecus OR hominidae OR ape OR apes OR "pan paniscus" OR bonobo OR bonobos OR "pan troglodytes" OR gibbon OR gibbons OR siamang OR siamangs OR nomascus OR symphalangus OR chimpanzee OR chimpanzees OR prosimian OR prosimians OR "bush baby" OR bush babies OR galagos OR galago OR pongidae OR gorilla OR gorillas OR "pongo pygmaeus" OR orangutan OR orangutans OR lemur OR lemurs OR lemuridae OR horse OR horses OR equus OR cow OR calf OR bull OR chicken OR chickens OR gallus OR quail OR bird OR birds OR quails OR poultry OR poultries OR fowl OR fowls OR reptile OR reptilia OR reptiles OR snakes OR snake OR lizard OR lizards OR alligator OR alligators OR crocodile OR crocodiles OR turtle OR turtles OR amphibian OR amphibians OR amphibia OR frog OR frogs OR bombina OR salientia OR toad OR toads OR "epidalea calamita" OR salamander OR salamanders OR eel OR eels OR fish OR fishes OR pisces OR catfish OR catfishes OR siluriformes OR arius OR heteropneustes OR sheatfish OR perch OR perches OR percidae OR perca OR trout OR trouts OR char OR chars OR salvelinus OR minnow OR cyprinidae OR carps OR carp OR zebrafish OR zebrafishes OR goldfish OR goldfishes OR guppy OR guppies OR chub OR chubs OR tinca OR barbels OR barbus OR pimephales OR promelas OR "poecilia reticulata" OR mullet OR mullets OR eel OR eels OR seahorse OR seahorses OR mugil curema OR atlantic cod OR shark OR sharks OR catshark OR anguilla OR salmonid OR salmonids OR whitefish OR whitefishes OR salmon OR salmons OR sole OR solea OR lamprey OR lampreys OR pumpkinseed OR sunfish OR sunfishes OR tilapia OR tilapias OR turbot OR turbots OR flatfish OR flatfishes OR sciuridae OR squirrel OR squirrels OR chipmunk OR chipmunks OR suslik OR susliks OR vole OR voles OR lemming OR lemmings OR muskrat OR muskrats OR lemmus OR otter OR otters OR marten OR martens OR martes OR weasel OR badger OR badgers OR ermine OR mink OR minks OR sable OR sables OR gulo OR gulos OR wolverine OR wolverines OR mustela OR llama OR llamas OR alpaca OR alpacas OR camelid OR camelids OR guanaco OR guanacos OR chiroptera OR chiropteras OR bat OR bats OR fox OR foxes OR iguana OR iguanas OR xenopus laevis OR parakeet OR parakeets OR parrot OR parrots OR donkey OR donkeys OR mule OR mules OR zebra OR zebras OR shrew OR shrews OR bison OR bisons OR buffalo OR buffaloes OR deer OR deers OR bear OR bears OR panda OR pandas OR "wild hog" OR "wild boar" OR fitchew OR fitch OR beaver OR beavers OR jerboa OR jerboas OR capybara OR capybaras) Indexes=SCI-EXPANDED, SSCI, CPCI-S, CPCI-SSH, BKCI-S, BKCI-SSH, CCR-EXPANDED,	

Systematic Literature Review on the Effects of Fluoride on Learning and Memory in Animal Studies

Search ID	Search Terms	Date: Number of Records
# 2 Neurolog-ical outcomes	**TOPIC:** ("nervous system" OR "central-nervous" OR brain OR blood- brain OR brain-stem OR cerebral OR cortical OR limbic OR amygdala OR hippocamp* OR hypothalamus OR parahippocamp* OR perforant OR mesencephalon OR prosencephalon OR rhombencephalon OR meninges OR dura-matter OR spinal-cord OR ganglia OR ganglion OR nerve OR nervous OR neural OR neural-tube OR afferent OR auditory OR olfactory OR oculomotor OR optic OR retinal OR efferent OR perforant OR neuroglia* OR astrocyte* OR microglia* OR schwann- cell* OR "myelin sheath" OR neuron* OR axon* OR dendrit* OR interneuron* OR lewy-bod* OR neurofibril* OR purkinje-cell* OR pyramidal-cell* OR serotonergic OR neuromuscular OR neuroendocrine OR neurosecret* OR pituitary OR pineal OR synapse* OR synaptic OR presynaptic OR postsynaptic OR plasticity OR hearing OR vision OR taste OR smell OR propriocept* OR Nervous system diseases OR neurologic* OR autonomic-nervous OR central- nervous OR demyelinat* OR multiple-sclerosis OR myasthenia-gravis OR epilep* OR encephalitis OR meningitis OR chronic-fatigue OR dyskinesia* OR dyston* OR parkinson* OR mobility OR motor-control OR motor-abilit* OR motor-skill* OR cranial-nerve OR huntington* OR tourette* OR ataxia OR hydranencephaly OR neurofibra* OR tauopath* OR fibromyalgia OR myopath* OR neurodegenerat* OR neuroimmunomodulat* OR neuromuscular OR neuropath* OR neurosecret* OR neurotox* OR neurobehav* OR dyslexi* OR cognitive OR cognition OR intellect* OR intelligence OR mental- deficienc* OR mental-deficit* OR down-syndrome OR rett-syndrome OR memory-deficit* OR memory-impair* OR memory-loss* OR Mental disorders OR Developmental disabilities OR developmental- delay* OR developmental-disabilit* OR anxiety OR obsessive-compulsive OR phobia* OR phobic OR antisocial OR personality OR schizophrenia OR behavior OR paranoid OR paranoia OR dementia OR senile OR senility OR alzheimer OR learning OR aphasia OR autism OR autistic OR attention-deficit) Indexes=SCI-EXPANDED, SSCI, CPCI-S, CPCI-SSH, BKCI-S, BKCI-SSH, CCR-EXPANDED,	1/7/2015: 7,065,701 1/14/2016 update: 419,568
# 3 Fluoride	1/7/2015: **TOPIC:** ((fluorid* OR flurid* OR fluorin* OR florin*) NOT (f-labeled OR "fluorine-18" OR imaging OR radioligand*)) Indexes=SCI-EXPANDED, SSCI, CPCI-S, CPCI-SSH, BKCI-S, BKCI-SSH, CCR-EXPANDED1/14/2016 update: TI=(fluorid* OR flurid* OR fluorin* OR florin* OR fluorosis) NOT TS=((18F OR F-18 OR "fluorine-18" OR 19F OR F-19 OR "fluorine-19" OR (PET AND scan) OR f-labeled OR imaging OR radioligand*)) Indexes=SCI-EXPANDED, SSCI, CPCI-S, CPCI-SSH, BKCI-S, BKCI-SSH, CCR-	1/7/2015: 147,851 1/14/2016 update: 2551

Appendix B. Data Extraction Elements for Animal Studies

Table B-1. Data Extraction Elements for Animal Studies

Data Type	Element
Funding	Funding source(s)
	Reporting of COI by authors and/or translators (*reporting bias)
Animal Model	Sex
	Species
	Strain
Treatment	Chemical name and CAS number
	Source of chemical
	Purity of chemical (*information bias)
	Dose levels or concentration (as presented and converted to mg/kg bw/d when possible)
	Other dose-related details, such as whether administered dose level was verified by measurement, information on internal dosimetry (*information bias)
	Vehicle used for exposed animals
	Route of administration (e.g., oral, inhalation, dermal, injection)
	Age or lifestage at start of dosing and at health outcome assessment
	Duration and frequency of dosing (e.g., hours, days, weeks when administration was ended, days per week)
Methods	Study design (e.g., single treatment, acute, subchronic (e.g., 90 days in a rodent), chronic, multigenerational, developmental, other)
	Guideline compliance (i.e., use of EPA, OECD, NTP or another guideline for study design, conducted under GLP guideline conditions, non-GLP but consistent with guideline study, non-guideline peer-reviewed publication)
	Number of animals per group (and dams per group in developmental studies) (*missing data bias)
	Randomization procedure, allocation concealment, blinding during outcome assessment (*selection bias)
	Method to control for litter effects in developmental studies (*information bias)
	Use of negative controls and whether controls were untreated, vehicle-treated, or both
	Endpoint health category (e.g., reproductive)
	Endpoint (e.g., infertility)
	Diagnostic or method to measure endpoint (*information bias)
	Statistical methods (*information bias)

Data Type	Element
Results	Measures of effect at each dose or concentration level (e.g., mean, median, frequency, measures of precision or variance) or description of qualitative results. When possible, OHAT will convert measures of effect to a common metric with associated 95% confidence intervals (CI). Most often, measures of effect for continuous data will be expressed as percent control response, mean difference, or standardized mean difference. Categorical data will be expressed as relative risk (RR, also called risk ratio).
	No observed effect level (NOEL), lowest observed effect level (LOEL), benchmark dose (BMD) analysis, statistical significance of other dose levels, or other estimates of effect presented in paper. **Note:** The NOEL and LOEL are highly influenced by study design, give no quantitative information about the relationship between dose and response, and can be subject to author's interpretation (e.g., a statistically significant effect might not be considered biologically important). Also, a NOEL does not necessarily mean zero response. Ideally, the response rate or effect size at specific dose levels is used as the primary measure to characterize the response.
	If not presented in the study, statistical power can be assessed during data extraction using an approach that assesses the ability to detect a 10% to 20% change from control group's response for continuous data, or a relative risk or odds ratio of 1.5–2 for categorical data, using the outcome frequency in the control group to determine sample size. Recommended sample sizes to achieve 80% power for a given effect size, i.e., 10% or 20% change from control, will be compared to sample sizes used in the study to categorize statistical power. Studies will be considered adequately powered when sample size for 80% power is met.
	Observations on dose response (e.g., trend analysis, description of whether dose-response shape appears to be monotonic, nonmonotonic)
	Data on internal concentration, toxicokinetics, or toxicodynamics (when reported)
Other	Documentation of author queries, use of digital rulers to estimate data values from figures, exposure unit, statistical result conversions, etc.

Appendix C. Studies Excluded from the Analysis for Having "Probably High" or "Definitely High" Risk of Bias in Multiple Critical Domains

Table C-1. Studies Excluded from Analysis for Risk of Bias

Click to See Risk of Bias Rationale	Study	No Randomization[1]	Concern or Lack of Information on Exposure Characterization	Concern for Lack of Blinding Outcome Assessment[1]	Lack of Control for Litter Effects[1]	Other Factors
1	Băran-Poesina et al. (2013)	X	X	X	X	*Were outcome data incomplete due to attrition or exclusion from analysis?* Not reported. 16 animals per group were treated but sample size was not reported for any results. Stated that 2 pairs of each F1 group were mated to create the F2 generation, but no information provided on how many animals were tested.
2	Basha and Sujitha (2012)	X	X	X		
3	Basha et al. (2011)	X		X	X	*Were outcome data incomplete due to attrition or exclusion from analysis?* Not reported. Stated that 8 dams in the F0 generation treated, but no indication of how many F1, F2, or F3 animals were tested. A few results tables for non-behavior outcomes state n = 6.
4	Bera et al. (2007)	X	X	X		
5	Bhatnagar et al. (2002)					*Were experimental conditions identical across study groups?* Controls drank deionized, defluorinated water and were untreated and maintained "isolated in a corner of animal room for two days…kept undisturbed, and the room was locked for 18 hrs. Just after opening the animal room, all mice were sacrificed." Similar housing conditions were not reported for treatment groups, which were described as receiving "…(NaF) orally, dissolved in water…
6	Elliott (1967)	X	X	X		

Click to See Risk of Bias Rationale	Study	No Randomization[1]	Concern or Lack of Information on Exposure Characterization	Concern for Lack of Blinding Outcome Assessment[1]	Lack of Control for Litter Effects[1]	Other Factors
7	Gopal et al. (2006)	X	X			*Were experimental conditions identical across study groups?* Controls did not receive treatment, and experimental conditions stated only to be "standard laboratory condition."
8	Han et al. (2014)		X	X		*Were there any other potential threats to internal validity?* Absence of expected response in control animals in exploratory behavior test.
9	Hong et al. (2005)		X	X	X	*Were outcome data incomplete due to attrition or exclusion from analysis?* Not reported. 6 dams treated in each group and offspring tested at PND30, 60, and 90, but number of offspring assessed not reported.
10	Jain et al. (2015)		X	X		*Were outcome data incomplete due to attrition or exclusion from analysis?* Not reported and cannot be inferred, as neither number of treated animals nor animals assessed for behavioral effects reported. *Were there any other potential threats to internal validity?* Statistical analyses were reasonable (one-way ANOVA followed by Bonferroni post-test), but consideration of homogeneity of variance not reported. Sex of animals used or if equal numbers of each sex were used not reported. If both sexes were used, results were reported for the sexes together.
11	Jetti et al. (2016)	X	X	X		
12	Liu (1989)		X	X	X	*Were outcome data incomplete due to attrition or exclusion from analysis?* Unclear. 150 offspring generated from 70 dams were divided into 3 groups (unclear how many dams became pregnant in each group). Also unclear how many offspring were tested; results for 22 offspring reported in Table 1 (controls = 8; low fluoride = 7; high fluoride = 7).

Click to See Risk of Bias Rationale	Study	No Randomization[1]	Concern or Lack of Information on Exposure Characterization	Concern for Lack of Blinding Outcome Assessment[1]	Lack of Control for Litter Effects[1]	Other Factors
13	Mullenix et al. (1995)		X		X	*Were outcome data incomplete due to attrition or exclusion from analysis?* Death occurred in about half the weanlings exposed to 175 ppm. Unclear how much attrition in other treatments. Not all numbers reported and in some cases they do not sum to numbers of controls or treated animals. *Were all measured outcomes reported?* Unclear. Results appear most complete for animals whose treatment started at weaning or adulthood. Data presentation for RS statistic and K functions difficult to follow. K scores appear presented for only certain behavior clusters.
14	Niu et al. (2009)	X		X	X	
15	Niu et al. (2014)	X	X		X	
16	Rumiantsev et al. (1988)	X		X		*Were experimental conditions identical across study groups?* Very few details on experimental conditions provided and whether control group was based on inhalation or oral administration unclear, as chemicals administered by different routes of administration but presented in a table with a single control group. *Were outcome data incomplete due to attrition or exclusion from analysis?* Not reported and cannot be inferred, as neither number of treated animals nor animals assessed for behavioral effects reported. *Were all measured outcomes reported?* Very little description in methods of procedure, sample sizes not reported, unclear whether SD or SE is reported in the results table. Were there any other potential threats to internal validity? Statistical methods not reported.
17	Wu et al. (2008)	X	X	X	X	*Were outcome data incomplete due to attrition or exclusion from analysis?* Number of dams treated not clearly reported for all groups. Authors report that 6 of 18 litters in high dose

Click to See Risk of Bias Rationale	Study	No Randomization[1]	Concern or Lack of Information on Exposure Characterization	Concern for Lack of Blinding Outcome Assessment[1]	Lack of Control for Litter Effects[1]	Other Factors
						group lost all pups due to no or low milk production. Table 2 reports results for 9−11 litters in each group. Considered to pose potential risk of bias because number of litters varies across groups and number of treated dams not reported in all groups and loss of pups in the high dose group. Results appear to be obtained on 40 pups in each group, but unclear for all outcomes. *Were all measured outcomes reported?* Unclear. Description of methods too incomplete to evaluate "Behavioral teratology testing of the offspring rats was conducted according to procedures outlined in the literature." *Were there any other potential threats to internal validity?* Statistical methods not reported.
18	Zhang et al. (2009)		X	X	X	*Were outcome data incomplete due to attrition or exclusion from analysis?* Unclear. One dam treated per group, resulting in 37 F1 offspring (numbers range from 9−10 per group). Sample size in results not presented but no loss of animals reported.
19	Zhang et al. (2001)		X	X		*Were outcome data incomplete due to attrition or exclusion from analysis?* Unclear. Authors did not report in the methods section number of mice treated and inferring is difficult because the number of mice among treatment and control groups differed and number of mice reported under same treatment group differed between Table 1 and Table 2.

[1]Information often is not reported and is assumed not conducted, resulting in an assessment of "probably high" risk of bias. Authors were queried for missing information and responses received were used to update risk of bias assessments.

Appendix D. Learning and Memory: Morris Water Maze (All Studies)

Studies: n = 19 (Gao et al. 2008b, a; Wu et al. 2008; Q Gao et al. 2009; YL Gao et al. 2009; Liu et al. 2009; Zhang et al. 2009; Gui et al. 2010; Liu et al. 2010; Liu et al. 2011; Zhu et al. 2012; C Jiang et al. 2014; S Jiang et al. 2014; Liu et al. 2014; Wei et al. 2014; Y Dong et al. 2015b, a; YT Dong et al. 2015; Li et al. 2015)

A

(See note in A, below, for the link to this figure.)

Excluded due to RoB concerns (n = 2): (Wu et al. 2008; Zhang et al. 2009)

Animal models: rats (n = 18: ICR, Sprague-Dawley, Wistar); mice (n = 1: BALB/c)

Life stage during exposure: developmental [n = 7 (Wu et al. 2008; Zhang et al. 2009; Gui et al. 2010; C Jiang et al. 2014; Wei et al. 2014; Y Dong et al. 2015a; YT Dong et al. 2015)]; adult [n = 13 (Gao et al. 2008b, a; Q Gao et al. 2009; YL Gao et al. 2009; Liu et al. 2009; Liu et al. 2010; Liu et al. 2011; Zhu et al. 2012; S Jiang et al. 2014; Liu et al. 2014; Y Dong et al. 2015b; YT Dong et al. 2015; Li et al. 2015)]

Exposure and Route: sodium fluoride (NaF) or "fluoride"; drinking water (n = 17), gavage (n = 1), diet (n = 1)

Outcome assessment: Morris water maze

Setting where research conducted: China (n = 19; 10 studies were non-English)

Figure D-1. Learning and Memory: Morris Water Maze (All Studies)

(A) Descriptive features and results of studies expressed as percent change from control response. Click to see interactive graphic sorted by study. *Note:* Some data from Liu et al. (2009) are also presented in Liu et al. (2011) (escape time, acquisition, day 6). Findings are presented under Liu et al. (2009) in the figure. Apparently, identical data were reported in Dong et al. (2015a); Dong et al. (2015b) and Dong et al. (2015c). Only data from Dong et al. (2015a) is presented in figure. **(B)** Risk of bias across studies. **(C)** Risk of bias assessments for individual studies (*study excluded from primary analysis due to concerns for risk of bias).

Appendix E. Learning and Memory: T-Maze

Studies: n = 5 (Elliott 1967; Basha et al. 2011; Basha and Sujitha 2012; Jetti et al. 2016; Raghu et al. 2013) **Excluded due to RoB concerns (n = 4):** (Elliott 1967; Basha et al. 2011; Basha and Sujitha 2012; Jetti et al. 2016) **Animal models:** rats (n = 5: Wistar, Hooded)

Life stage during exposure: developmental [n = 1 (Basha et al. 2011)]; adult [n = 4 (Elliott 1967; Basha and Sujitha 2012; Jetti et al. 2016; Raghu et al. 2013)]

Exposure and Route: sodium fluoride (NaF) or "fluoride"; drinking water (n = 4), diet (n = 1)

Outcome assessment: T-maze (n = 4), multiple T-maze (Biel maze; n = 1)

Setting where research conducted: Canada (n = 1), India (n = 4)

Figure E-1. Learning and Memory: T-Maze

(A) Descriptive features and results of studies expressed as percent change from control response. Click to see interactive graphic sorted by study. **(B)** Risk of bias across studies. **(C)** Risk of bias assessments for individual studies (*study excluded from primary analysis due to concerns for risk of bias).

Appendix F. Learning and Memory: Y-Maze

Studies: n = 10 (Zhang et al. 1999; Xu and Shen 2001; Zhang et al. 2001; Shen and Xu 2004; Wang et al. 2006; Niu et al. 2008; Niu et al. 2009; Chen and Geng 2011; Zhang et al. 2013; Li et al. 2015)

Excluded due to RoB concerns (n = 2): (Zhang et al. 2001; Niu et al. 2009)

Animal models: rats (n = 7: Sprague-Dawley, Wistar), mice (n = 3: ICR, Kunming)

Life stage during exposure: developmental [n = 1 (Niu et al. 2009)]; adult [n = 9 (Zhang et al. 1999; Xu and Shen 2001; Zhang et al. 2001; Shen and Xu 2004; Wang et al. 2006; Niu et al. 2008; Chen and Geng 2011; Zhang et al. 2013; Li et al. 2015)]

Exposure and Route: sodium fluoride (NaF); drinking water (n = 9) or gavage (n = 1)

Outcome assessment: Y-maze

Setting where research conducted: China (n = 10; 5 studies were non-English)

Figure F-1. Learning and Memory: Y-Maze

(A) Descriptive features and results of studies expressed as percent change from control response. Click to see interactive graphic sorted by study. **(B)** Risk of bias across studies. **(C)** Risk of bias assessments for individual studies (*study excluded from primary analysis due to concerns for risk of bias).

Appendix G. Learning and Memory: Other Maze Tests

Studies: n = 3 (Bhatnagar et al. 2002; El-lethey et al. 2010; Baran-Poesina et al. 2013)
Excluded due to RoB concerns (n = 2): (Bhatnagar et al. 2002; Baran-Poesina et al. 2013)
Animal models: rats (n = 1: Wistar), mice (n = 2: NMRI, Swiss albino)

Life stage during exposure: developmental [n = 2 (El-lethey et al. 2010; Baran-Poesina et al. 2013)]; adult [n = 1 (Bhatnagar et al. 2002)]

Exposure and Route: sodium fluoride (NaF), drinking water (n = 3)

Outcome assessment: "classic maze" or unspecified maze tests

Setting where research conducted: Egypt (n = 1), India (n = 1), Romania (n = 1)

Figure G-1. Learning and Memory: Other Maze Tests

(A) Descriptive features and results of studies expressed as percent change from control response. Click to see interactive graphic sorted by study. *Note:* For Bhatnagar et al. (2002), data presented only for control and high dose groups (0-, 3.68-, 7.37-, and 14.72-ppm dose groups treated). **(B)** Risk of bias across studies. **(C)** Risk of bias assessments for individual studies (*study excluded from primary analysis due to concerns for risk of bias).

Appendix H. Learning and Memory: Exploratory Behavior

Studies: n = 6 (Ekambaram and Paul 2003; Bera et al. 2007; El-lethey et al. 2010; Han et al. 2014; Liu et al. 2014; Niu et al. 2014)

Excluded due to RoB concerns (n = 3): (Bera et al. 2007; Han et al. 2014; Niu et al. 2014)

Animal models: rats (n = 3: Wistar), mice (n = 3: BALB/c, Kunming)

[n = 3 (Bera et al. 2007; El-lethey et al. 2010; Niu et al. 2014)]; adult [n = 3 (Ekambaram and Paul 2003; Han et al. 2014; Liu et al. 2014)]

Exposure and Route: sodium fluoride (NaF); drinking water (n = 6) or gavage (n = 1)

Outcome assessment: novel object test, mini-holeboard, activity cage

Setting where research conducted: China (n = 3), Egypt (n = 1), India (n = 1), Italy (n = 1)

Figure H-1. Learning and Memory: Exploratory Behavior

(A) Descriptive features and results of studies expressed as percent change from control response. Click to see interactive graphic sorted by study. **(B)** Risk of bias across studies. **(C)** Risk of bias assessments for individual studies (*study excluded from primary analysis due to concerns for risk of bias).

Appendix I. Learning and Memory: Passive Avoidance

Studies: n = 7 (Wang et al. 2004; Hong et al. 2005; Wu et al. 2006; Bera et al. 2007; Jetti et al. 2016; Raghu et al. 2013; Jain et al. 2015)

Excluded due to RoB concerns (n = 4): (Hong et al. 2005; Bera et al. 2007; Jetti et al. 2016; Jain et al. 2015)

Animal models: rats (n = 7: Wistar)

Life stage during exposure: developmental [n = 3 (Wang et al. 2004; Hong et al. 2005; Bera et al. 2007)]; adult [n = 4 (Wu et al. 2006; Jetti et al. 2016; Raghu et al. 2013; Jain et al. 2015)]

Exposure and Route: sodium fluoride (NaF); drinking water (n = 5) or gavage (n = 1)

Outcome assessment: step-down test or unspecified

Setting where research conducted: China (n = 3; 1 study was non-English), India (n = 3), Italy (n = 1)

Figure I-1. Learning and Memory: Passive Avoidance

(A) Descriptive features and results of studies expressed as percent change from control response. Click to see interactive graphic sorted by study. *Note:* For Wang et al. (2004), percent control response value for passive avoidance (step-down test, number of shocks, session 2, day 30) could not be calculated due to a zero response (count) for the control. **(B)** Risk of bias across studies. **(C)** Risk of bias assessments for individual studies (*study excluded from primary analysis due to concerns for risk of bias).
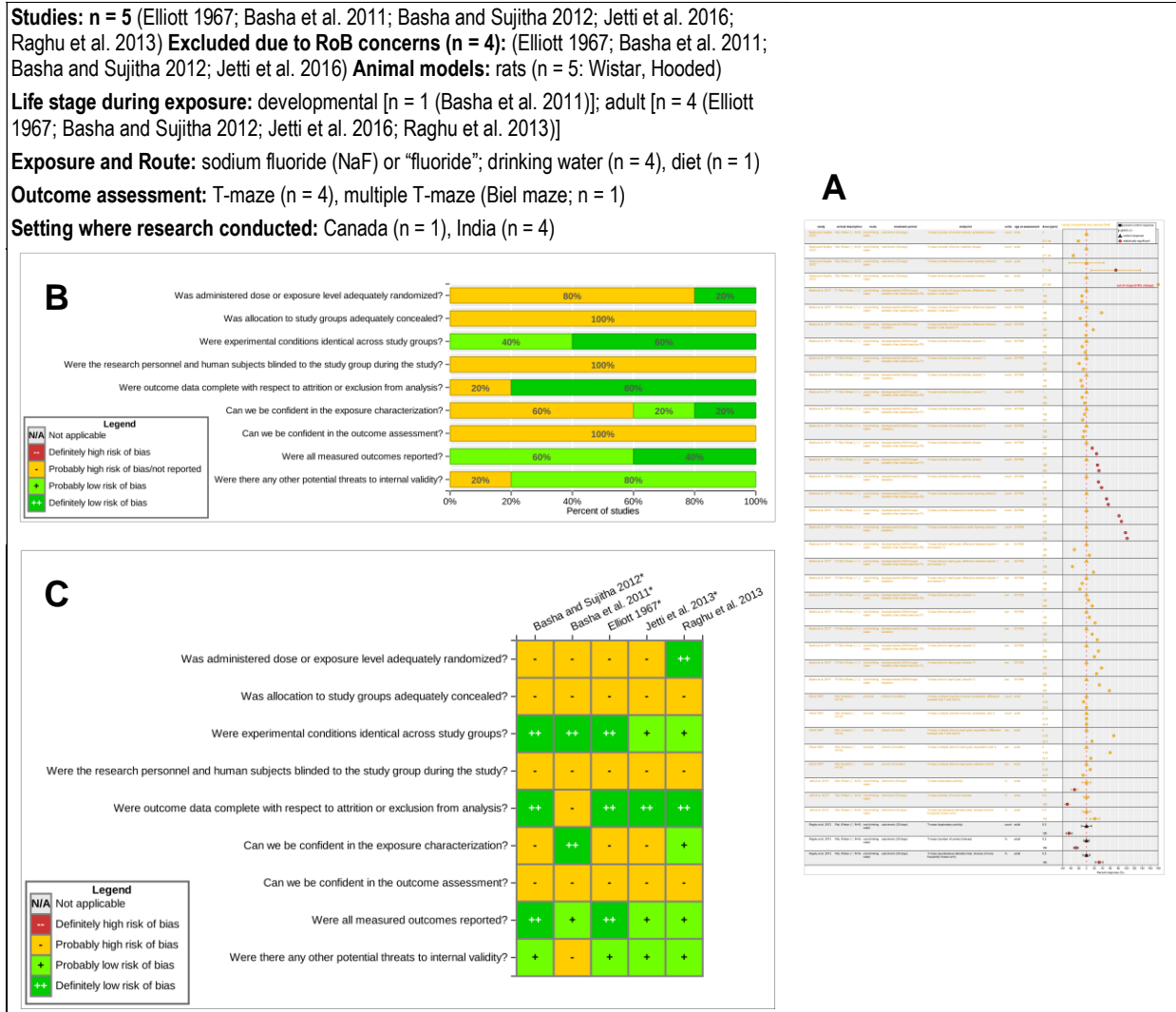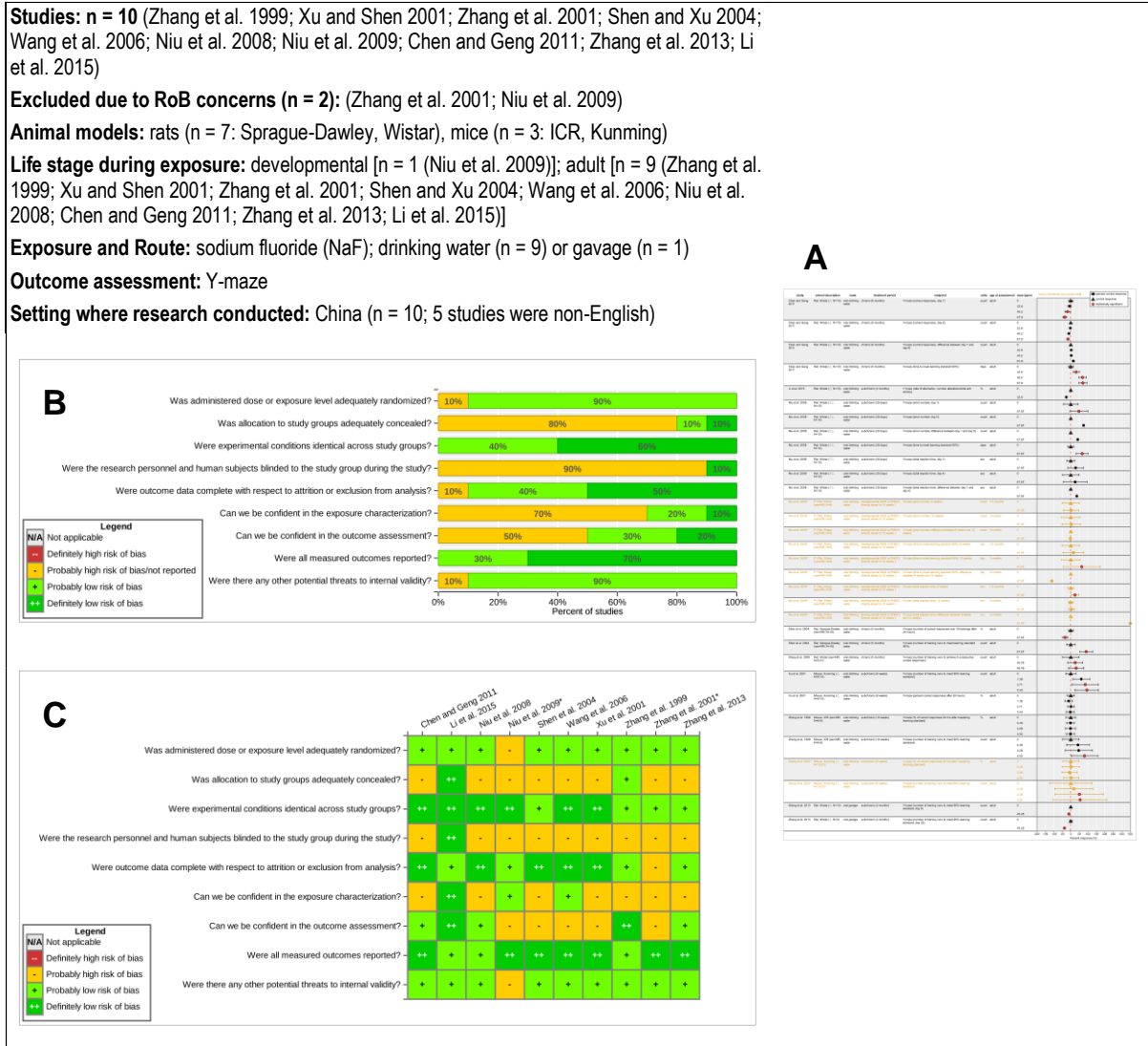
Appendix J. Learning and Memory: Active Avoidance
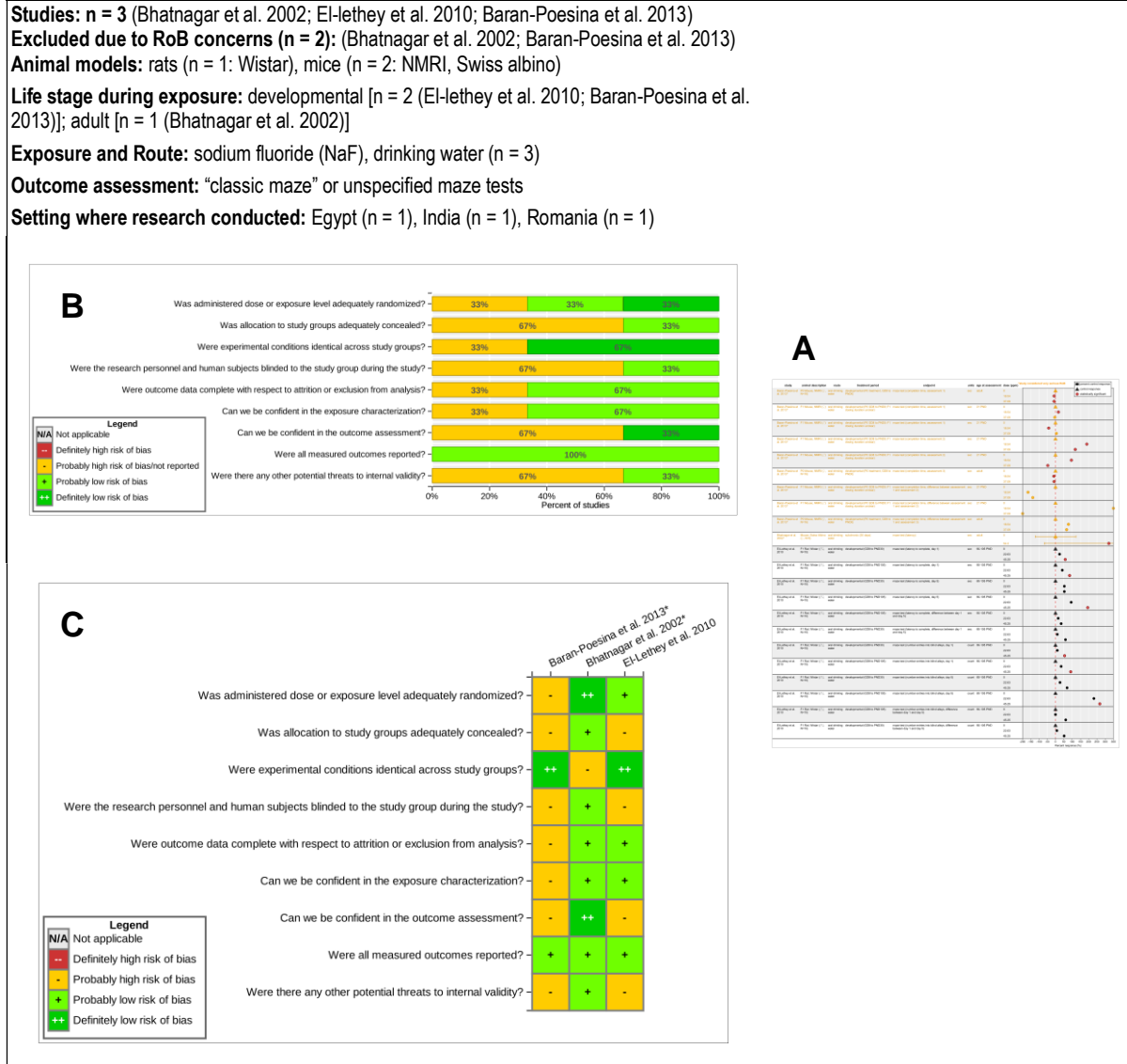
Studies: n = 4 (Liu 1989; Bera et al. 2007; Chioca et al. 2008; Sun et al. 2008)

Excluded due to RoB concerns (n = 2): (Liu 1989; Bera et al. 2007)

Animal models: rats (n = 6: Wistar), mice (n = 1: Kunming)

Life stage during exposure: developmental [n = 2 (Liu 1989; Bera et al. 2007)]; adult [n = 3 (Chioca et al. 2008; Sun et al. 2008)]

Exposure and Route: sodium fluoride (NaF) or "fluoride"; drinking water (n = 3), gavage (n = 1)

Outcome assessment: shuttle box

Setting where research conducted: Brazil (n = 1), China (n = 2; 1 was non-English), Italy (n = 1)
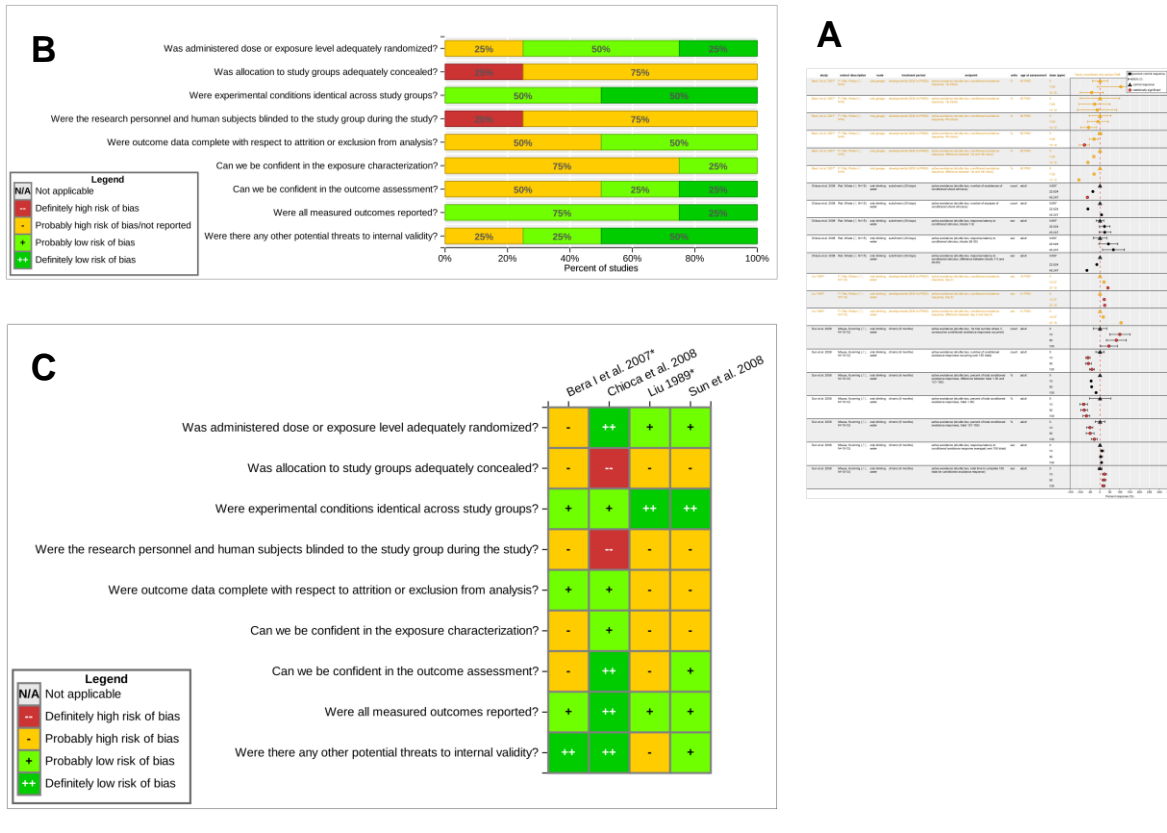
Figure J-1. Learning and Memory: Active Avoidance

(A) Descriptive features and results of studies expressed as percent change from control response. Click to see interactive graphic sorted by study. *Note:* Data from Wang et al. (2004) for active avoidance (step-down test, number of shocks, session 2, day 30) could not be plotted as percent change from control because the response and SD were 0 in the control group. A significant increase in the number of shocks was observed in the 45-ppm treatment group. **(B)** Risk of bias across studies. **(C)** Risk of bias assessments for individual studies (*study excluded from primary analysis due to concerns for risk of bias).

Appendix K. Motor and Sensory Function: Locomotor Activity
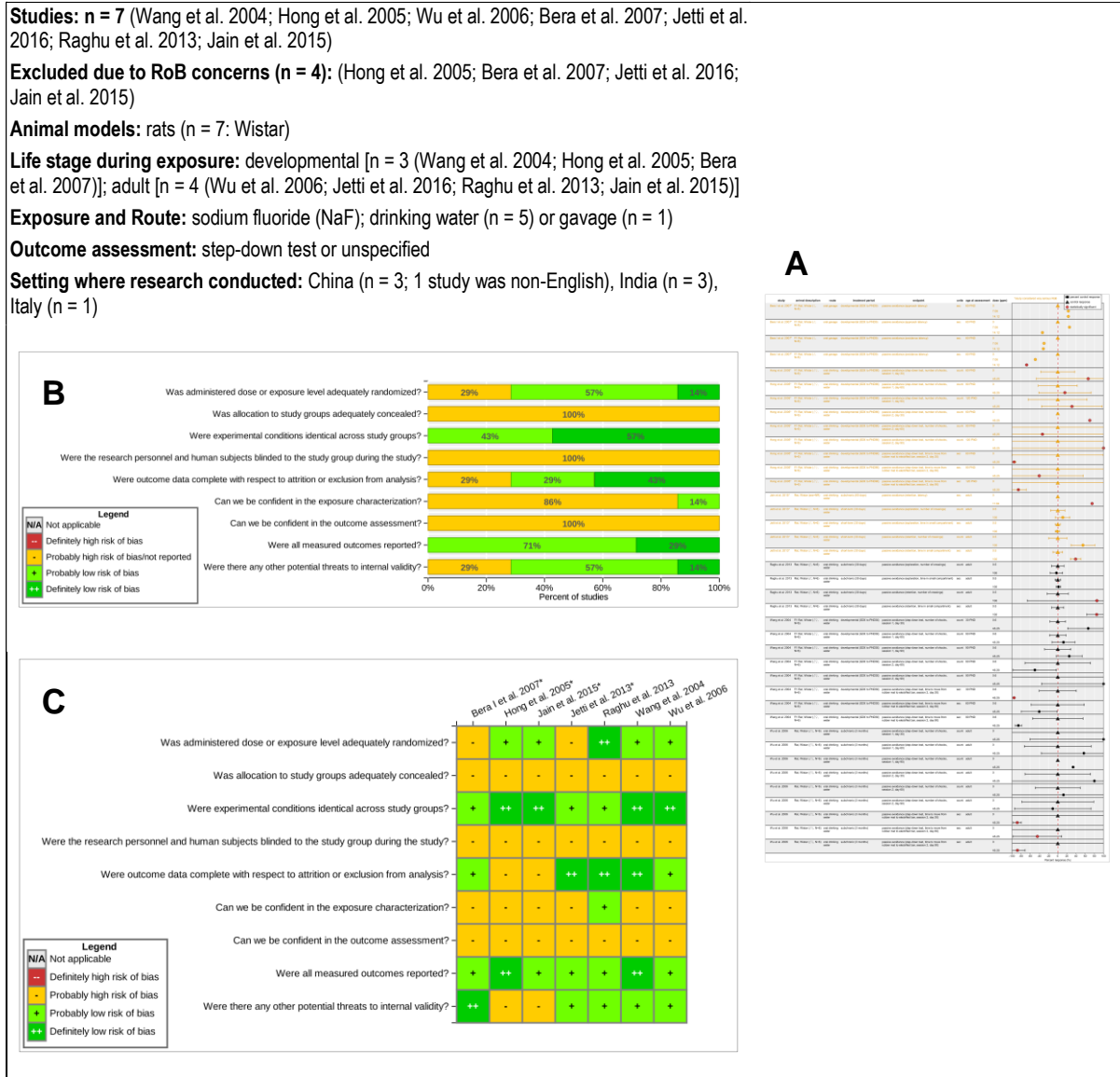
Studies: n = 25 (Rumiantsev et al. 1988; Mullenix et al. 1995; Paul et al. 1998; Ekambaram and Paul 2001; Xu and Shen 2001; Ekambaram and Paul 2002; Gopal et al. 2006; Bera et al. 2007; Chioca et al. 2008; Niu et al. 2008; Wu et al. 2008; Zhang et al. 2009; El-lethey et al. 2010; Chen and Geng 2011; El-lethey and Kamel 2011; Pereira et al. 2011; Kivrak 2012; Banji et al. 2013; Han et al. 2014; Liu et al. 2014; Niu et al. 2014; Sarkozi et al. 2014; Balaji et al. 2015; Bartos et al. 2015; Ma et al. 2015)

A

(See note in A, below, for the link to this figure.)

Excluded due to RoB concerns (n = 8): (Rumiantsev et al. 1988; Mullenix et al. 1995; Gopal et al. 2006; Bera et al. 2007; Wu et al. 2008; Zhang et al. 2009; Han et al. 2014; Niu et al. 2014)

Animal models: rats (n = 19: albino, ICR, Sprague-Dawley, Wistar, other − not reported), mice (n = 8: BALB/c, C57/Bl, Kunming, Swiss, Swiss albino OF1)

Life stage during exposure: developmental [n = 9 (Mullenix et al. 1995; Bera et al. 2007; Wu et al. 2008; Zhang et al. 2009; El-lethey et al. 2010; Banji et al. 2013; Niu et al. 2014; Bartos et al. 2015)]; adult [n = 19 (Rumiantsev et al. 1988; Mullenix et al. 1995; Paul et al. 1998; Ekambaram and Paul 2001; Xu and Shen 2001; Ekambaram and Paul 2002; Gopal et al. 2006; Chioca et al. 2008; Niu et al. 2008; Chen and Geng 2011; El-lethey and Kamel 2011; Pereira et al. 2011; Kivrak 2012; Han et al. 2014; Liu et al. 2014; Sarkozi et al. 2014; Balaji et al. 2015; Ma et al. 2015)]

Exposure and Route: sodium fluoride (NaF) or "fluoride"; drinking water (n = 20), gavage (n = 5), subcutaneous injection (n = 1)

Outcome assessment: open field or activity cage

Setting where research conducted: Argentina (n = 1); Brazil (n = 2), China (n = 9; 4 were non-English), Egypt (n = 1), Hungary (n = 1), India (n = 7), Italy (n = 1), Russia (n = 1), Turkey (n = 1), United States (n = 1)
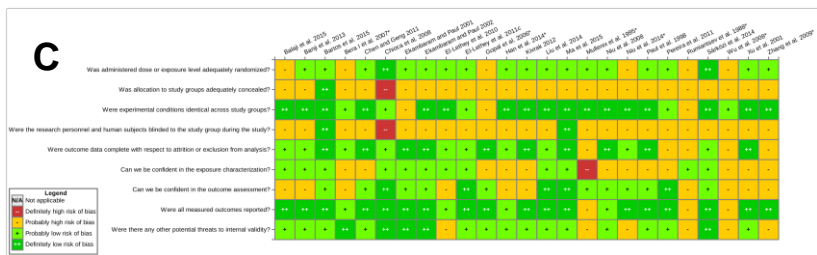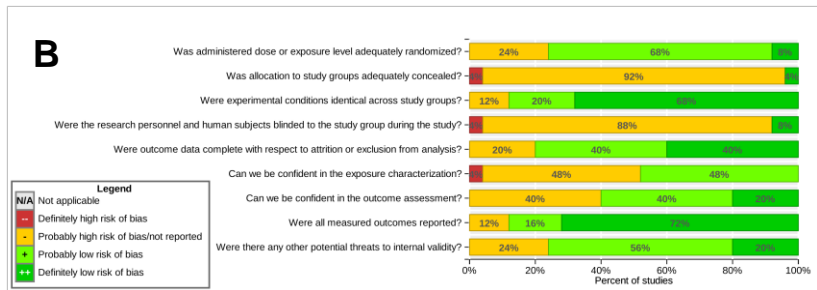
Figure K-1. Motor and Sensory Function: Locomotor Activity

(A) Descriptive features and results of studies expressed as percent change from control response. Click the following link to see interactive graphic sorted by study. *Note:* Findings for Bera et al. (2007) are not shown because a single conclusion on "locomotor activity" was reported (no significant effects, data not shown). **(B)** Risk of bias across studies. **(C)** Risk of bias assessments for individual studies (*study excluded from primary analysis due to concerns for risk of bias).

Appendix L. Motor and Sensory Function: Movement Coordination

Studies: n = 12 (Paul et al. 1998; Ekambaram and Paul 2001; Bhatnagar et al. 2002; Ekambaram and Paul 2002, 2003; Gopal et al. 2006; Bera et al. 2007; Wu et al. 2008; El-lethey and Kamel 2011; Banji et al. 2013; Balaji et al. 2015; Reddy and Karnati 2015)

Excluded due to RoB concerns (n = 4): (Bhatnagar et al. 2002; Gopal et al. 2006; Bera et al. 2007; Wu et al. 2008)

Animal models: rats (n = 10: albino, Wistar), mice (n = 2: Swiss albino, Swiss albino OF1)

Life stage during exposure: developmental [(n = 3) (Bera et al. 2007; Wu et al. 2008; Banji et al. 2013)[, adult [(n = 9) (Paul et al. 1998; Ekambaram and Paul 2001; Bhatnagar et al. 2002; Ekambaram and Paul 2003; Gopal et al. 2006; El-lethey and Kamel 2011; Balaji et al. 2015; Reddy and Karnati 2015)]

Exposure and Route: sodium fluoride (NaF) or "fluoride"; drinking water (n = 8), gavage (n = 3)

Outcome assessment: akinesia/catalepsy, plank walking, rotarod, slanted surface, swim test
Setting where research conducted: China (n = 1), Egypt (n = 1), India (n = 9), Italy (n = 1)
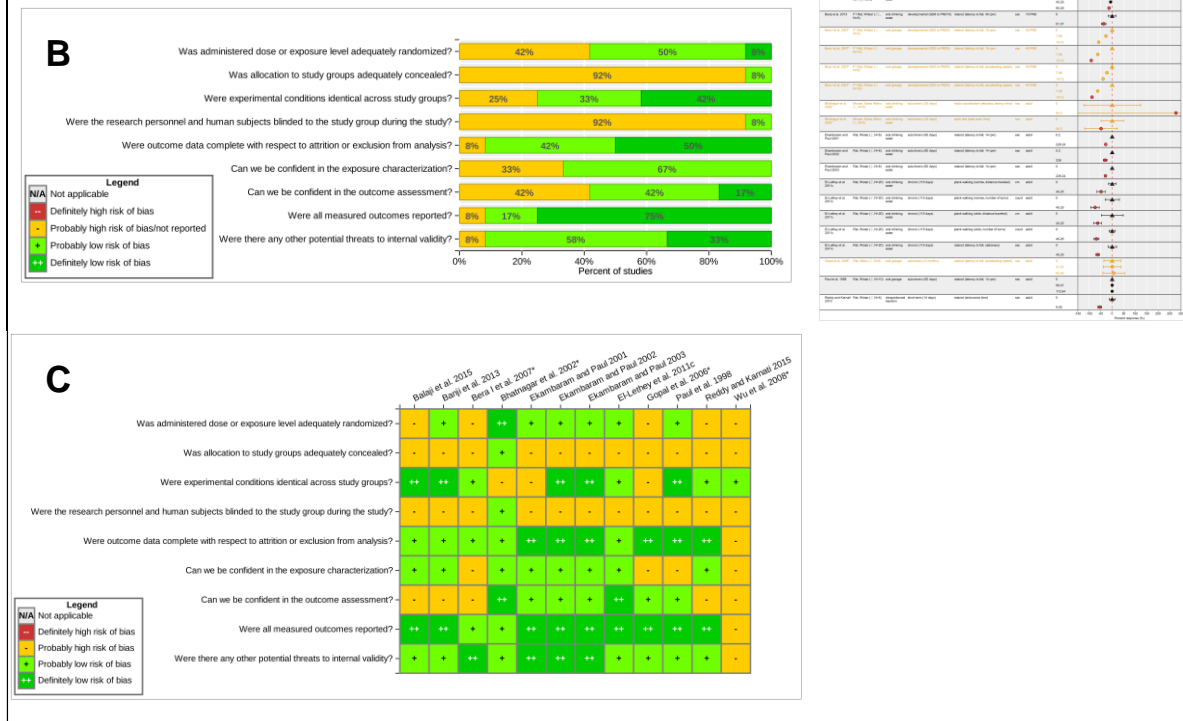
Figure L-1. Motor and Sensory Function: Movement Coordination

(A) Descriptive features and results of studies expressed as percent change from control response. Click to see interactive graphic sorted by study. *Note:* For Bhatnagar et al. (2002), data presented in paper only for control and high dose groups (0-, 3.68-, 7.37-, and 14.72-ppm dose groups were tested) and data for (catalepsy, latency time) are not included in figure because control values were not presented in paper (findings described as "a significant high value was observed in treated animals (7.0 +/- 3.3)." The following endpoints from Wu et al. (2008) were described as not affected (data not shown): swim test, slanted surface, forelimb suspension. **(B)** Risk of bias across studies. **(C)** Risk of bias assessments for individual studies (*study excluded from primary analysis due to concerns for risk of bias).

Appendix M. Motor and Sensory Function: Reflex and Motor Sensory Development

Studies: n = 8 (Rumiantsev et al. 1988; Liu 1989; Balayssac et al. 2002; Wu et al. 2008; Flace et al. 2010; Wei et al. 2014; Bartos et al. 2015; Ma et al. 2015)

Excluded due to RoB concerns (n = 3): (Rumiantsev et al. 1988; Liu 1989; Wu et al. 2008)

Animal models: rats (n = 7: Lou-C, Sprague-Dawley, Wistar, other – not reported), mice (n = 1: C57/Bl)

Life stage during exposure: developmental [n = 5 (Liu 1989; Wu et al. 2008; Flace et al. 2010; Wei et al. 2014; Bartos et al. 2015)], adult [n = 2 (Rumiantsev et al. 1988; Balayssac et al. 2002; Ma et al. 2015)]

Exposure and Route: sodium fluoride (NaF) or "fluoride"; drinking water (n = 6), gavage (n = 2)

Outcome assessment: auditory startle, cliff avoidance, surface righting, negative taxis, pain response, pivoting/orienting reflex, tail immersion, Von Frey hair test

Setting where research conducted: Argentina (n = 1), China (n = 4; 2 studies were non-English), France (n = 1), Italy (n = 1), Russian (n = 1)
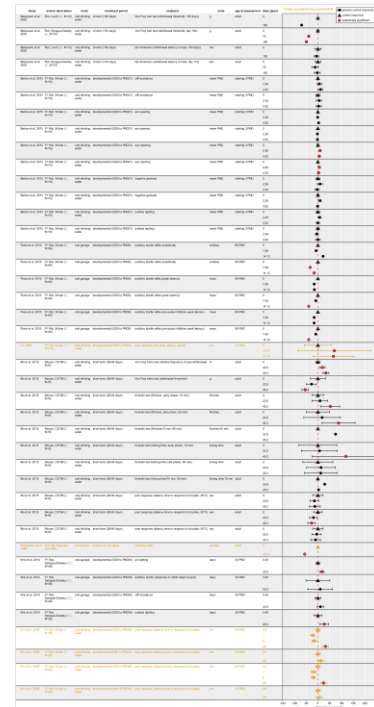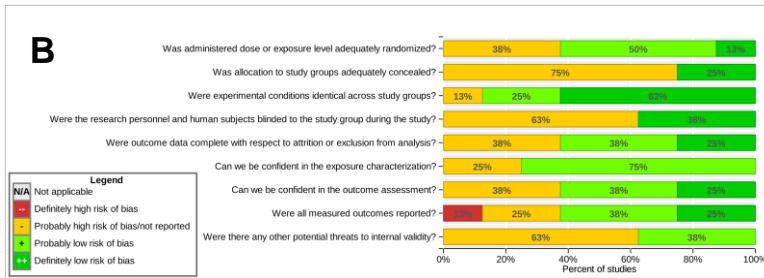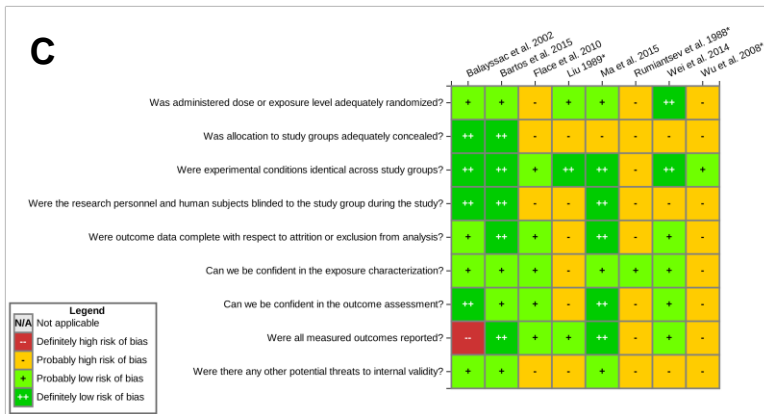
Figure M-1. Motor and Sensory Function: Reflex and Motor Sensory Development

(A) Descriptive features and results of studies expressed as percent change from control response. Click to see interactive graphic sorted by study. *Note:* The following endpoints from Wu et al. (2008) were described as not affected (data not shown): auditory startle, cliff avoidance, negative taxis, pivoting task, surface righting. **(B)** Risk of bias across studies. **(C)** Risk of bias assessments for individual studies (*study excluded from primary analysis due to concerns for risk of bias).

Appendix N. Anxiety

Studies: n = 4 (Banji et al. 2013; Liu et al. 2014; Balaji et al. 2015; Bartos et al. 2015)

Excluded due to RoB concerns: none

Animal models: rats (n = 2: Wistar), mice (n = 2:BALB/c, Swiss albino OF1)

Life stage during exposure: developmental [n = 2 (Banji et al. 2013; Bartos et al. 2015)]; adult [n = 2 (Liu et al. 2014; Balaji et al. 2015)]

Exposure and Route: sodium fluoride (NaF); drinking water (n = 4)

Outcome assessment: elevated plus maze

Setting where research conducted: Argentina (n = 1), China (n = 1), India (n = 2)

Figure N-1. Anxiety

(A) Descriptive features and results of studies expressed as percent change from control response. Click to see interactive graphic sorted by study. **(B)** Risk of bias across studies. **(C)** Risk of bias assessments for individual studies (*study excluded from primary analysis due to concerns for risk of bias).

Appendix O. Grooming, Defecation, Urination

Studies: n = 5 (Mullenix et al. 1995; Wu et al. 2008; Kivrak 2012; Balaji et al. 2015; Bartos et al. 2015)

Excluded due to RoB concerns (n = 2): (Mullenix et al. 1995; Wu et al. 2008)

Animal models: rats (n = 3: Sprague-Dawley, Wistar), mice (n = 2:Swiss, Swiss albino OF1)

Life stage during exposure: developmental [n = 3 (Mullenix et al. 1995; Wu et al. 2008; Bartos et al. 2015)]; adult [n = 3 (Mullenix et al. 1995; Kivrak 2012; Balaji et al. 2015)]

Exposure and Route: sodium fluoride (NaF) or "fluoride"; drinking water (n = 5)

Outcome assessment: activity box, open field

Setting where research conducted: Argentina (n = 1), China (n = 1), India (n = 1), Turkey (n = 1), United States (n = 1)

Figure O-1. Grooming, Defecation, Urination

(A) Descriptive features and results of studies expressed as percent change from control response. Click to see interactive graphic sorted by study. *Note*: The following endpoint from Wu et al. (2008) was described as not affected (data not shown): defecation and urination in open field test. **(B)** Risk of bias across studies. **(C)** Risk of bias assessments for individual studies (*study excluded from primary analysis due to concerns for risk of bias).

Appendix P. Sexual Behavior

Studies: n = 4 (Bataineh and Nusier 2006; Bera et al. 2007; El-Lethey et al. 2011; El-lethey and Shaheed 2011)

Excluded due to RoB concerns (n = 1): (Bera et al. 2007)

Animal models: rats (n = 1: Wistar) , mice (n = 3: BALB/c, Swiss albino OF1)

Life stage during exposure: developmental [n = 2 (Bera et al. 2007; El-Lethey et al. 2011)]; adult [n = 2 (Bataineh and Nusier 2006; El-lethey and Shaheed 2011)]

Exposure and Route: sodium fluoride (NaF) or "fluoride"; drinking water (n = 4)

Outcome assessment: sexual behavior tests

Setting where research conducted: Egypt (n = 2), Italy (n = 1), Jordan (n = 1)
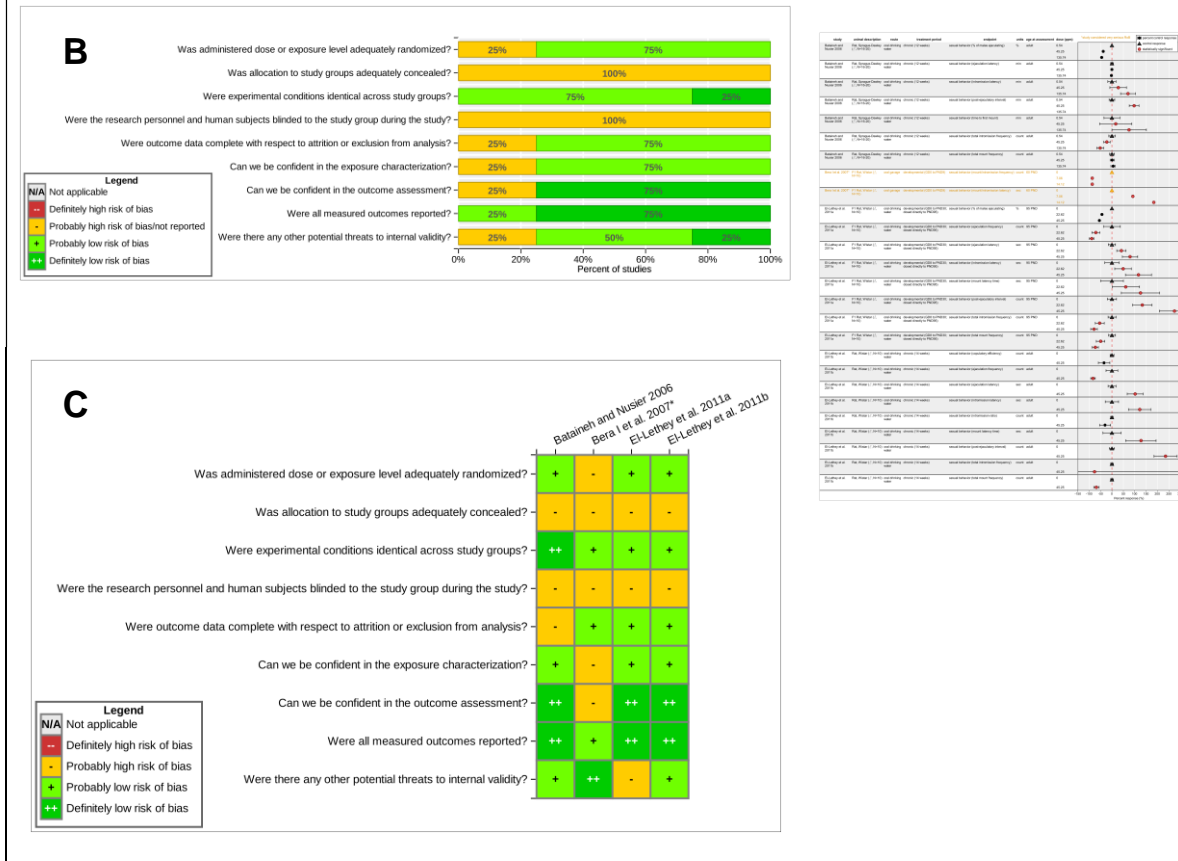
Figure P-1. Sexual Behavior

(A) Descriptive features and results of studies expressed as percent change from control response. Click to see interactive graphic sorted by study. **(B)** Risk of bias across studies. **(C)** Risk of bias assessments for individual studies (*study excluded from primary analysis due to concerns for risk of bias).

Appendix Q. Territorial Aggression

Studies: n = 3 (Bataineh and Nusier 2006; El-Lethey et al. 2011; El-lethey and Shaheed 2011)

Excluded due to RoB concerns: none

Animal models: rats (n = 3: Sprague-Dawley, Wistar)

Life stage during exposure: developmental [n = 1 (El-Lethey et al. 2011)], adult [n = 2 (El-Lethey et al. 2011)]

Exposure and Route: sodium fluoride (NaF); drinking water (n = 3)

Outcome assessment: territorial aggression tests

Setting where research conducted: Egypt (n = 2), Jordan (n = 1)

Figure Q-1. Territorial Aggression

(A) Descriptive features and results of studies expressed as percent change from control response. Click to see interactive graphic sorted by study. **(B)** Risk of bias across studies. **(C)** Risk of bias assessments for individual studies (*study excluded from primary analysis due to concerns for risk of bias).

Appendix R. Other

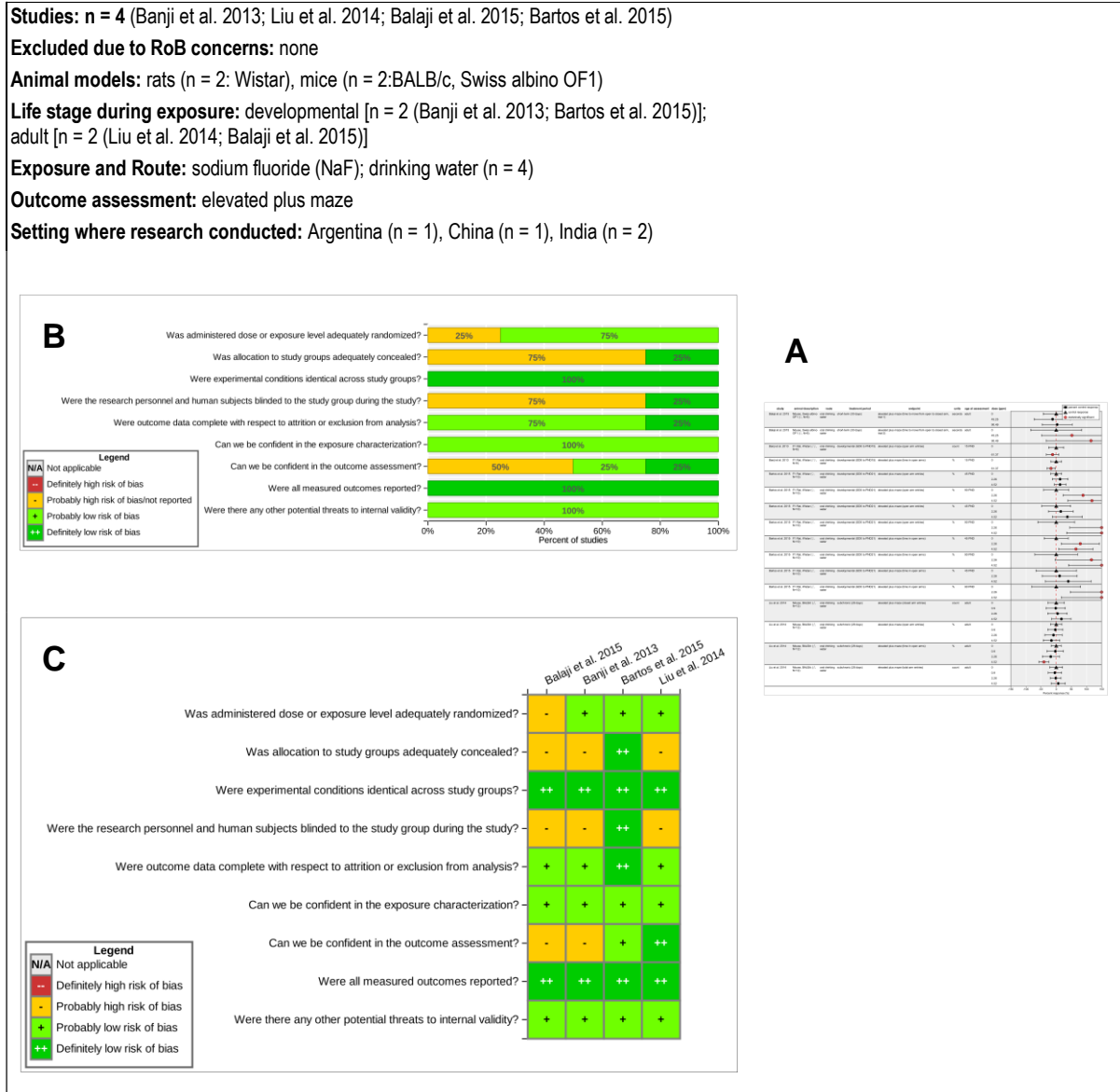**Studies: n = 2** (Flace et al. 2010; Banji et al. 2013)

Excluded due to RoB concerns: none

Animal models: rats (n = 2: Wistar)

Life stage during exposure: developmental [n = 2 (Flace et al. 2010; Banji et al. 2013)]

Exposure and Route: sodium fluoride (NaF); drinking water (n = 1), gavage (n = 1)

Outcome assessment: motor development, vocalizations

Setting where research conducted: India (n = 1), Italy (n = 1)

A

B
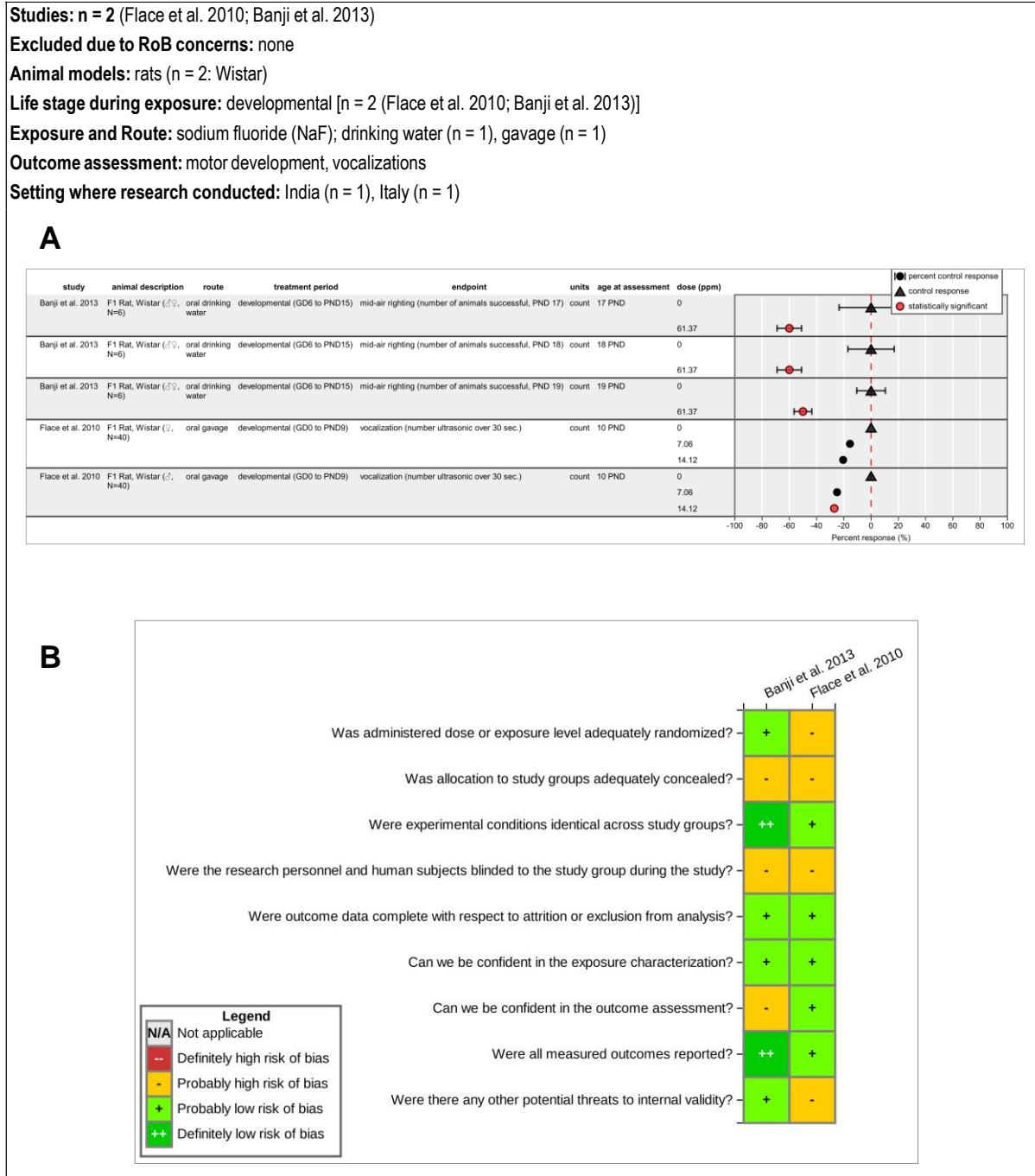
Figure R-1. Other

(A) Descriptive features and results of studies expressed as percent change from control response. Click to see interactive graphic sorted by study. (B) Risk of bias assessments for individual studies (*study excluded from primary analysis due to concerns for risk of bias).

Appendix S. Fluoride Administered and Converted Doses (Individual Studies)
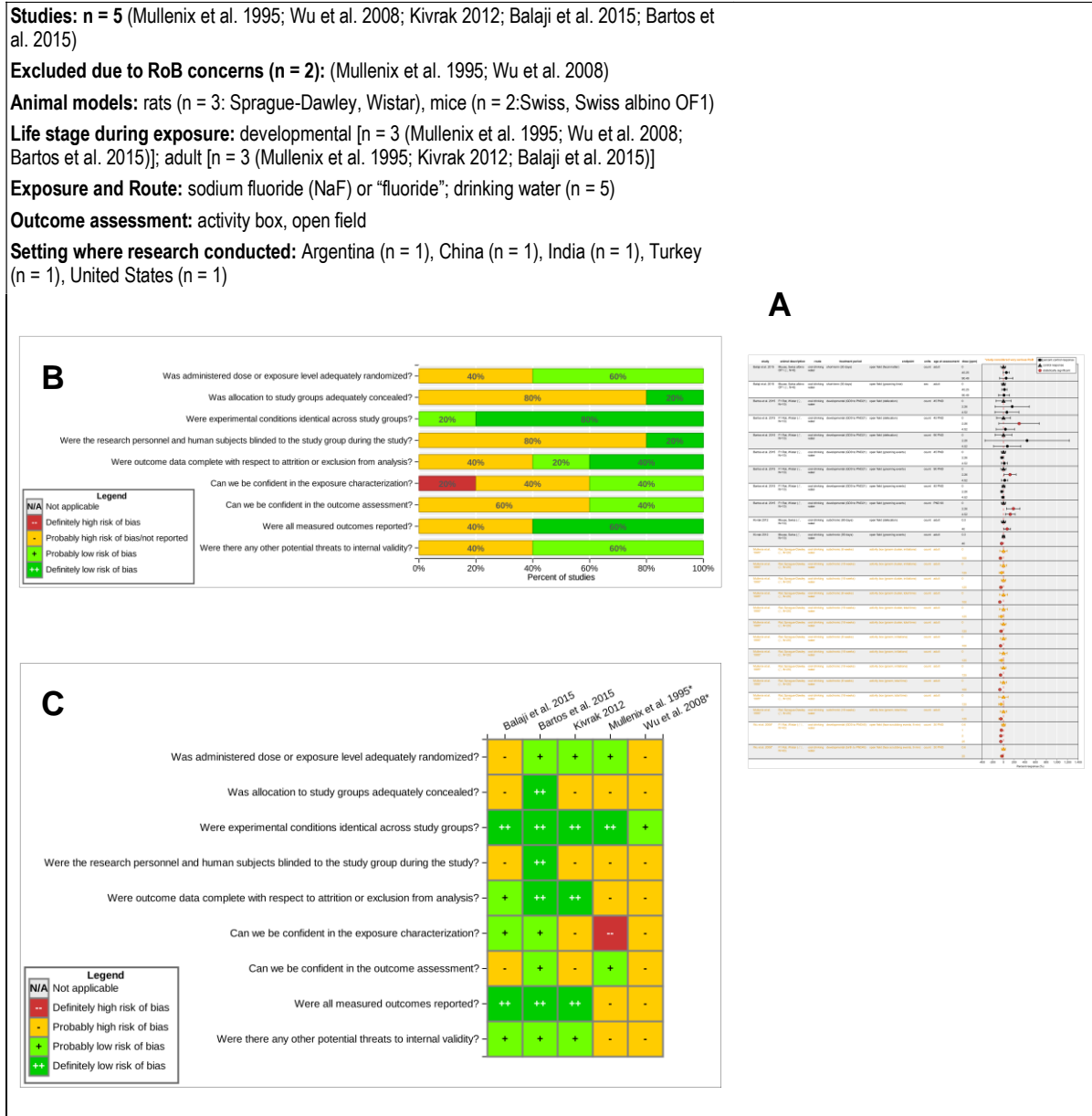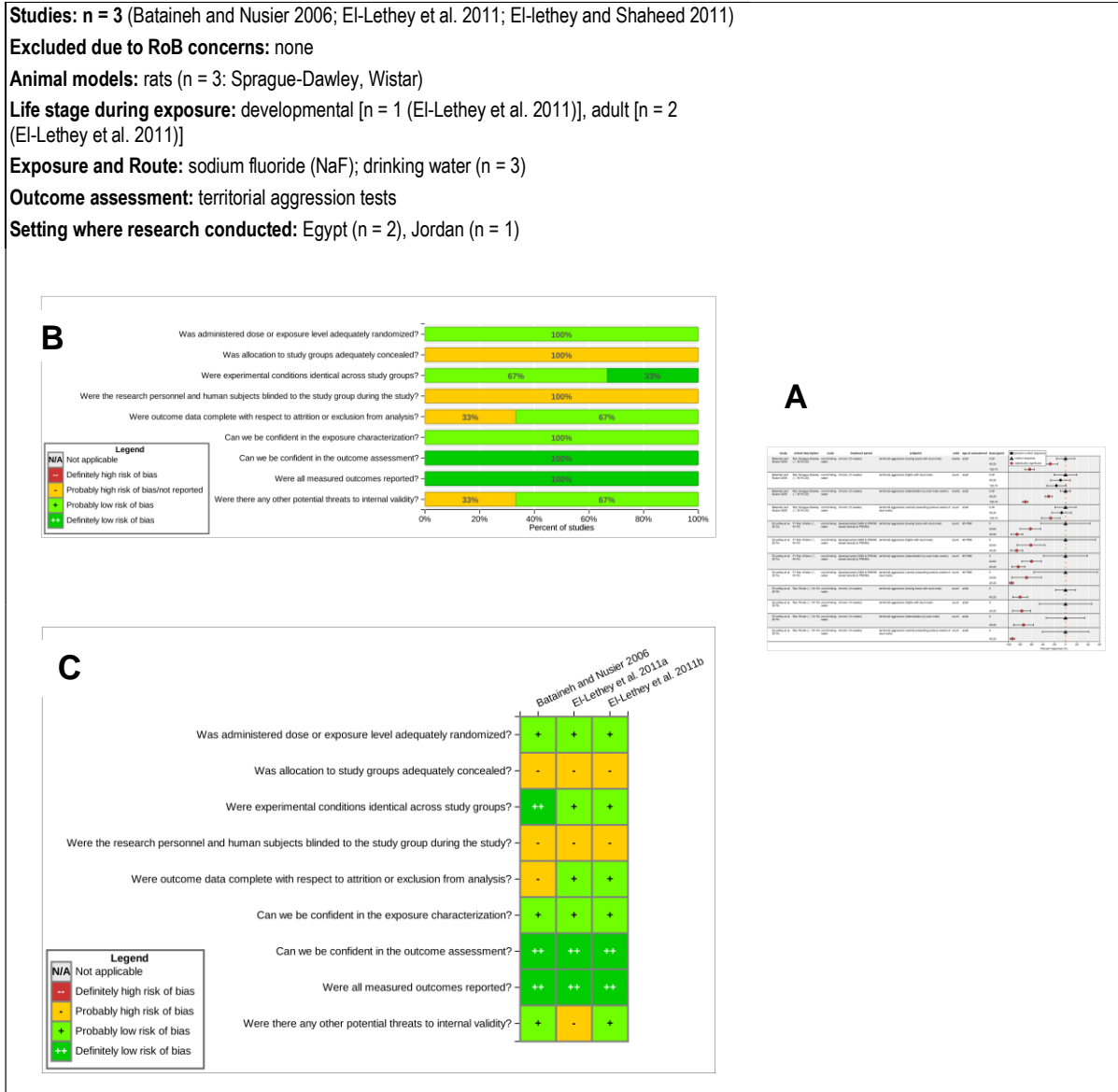
Table S-1. Summary of Fluoride Administered and Converted Doses (Individual Studies)

Study	Fluoride Concentration[1] ppm	Fluoride Concentration[1] mg/kg-d
Oral Drinking Water		
Balaji et al. (2015)	0, 45.3, 90.5	0, 12.3, 24.5
Balayssac et al. (2002)	0, 150 (Lou/C rat) 0, 75, 150 (SD rat)	0, 20.8 (Lou/C rat) 0, 10.4, 20.8 (SD rat)
Banji et al. (2013)	0, 61.4 (F1)	0, 9.1 (F1)
Băran-Poesina et al. (2013)	0, 18.5, 37.1 (P0, F1, F2)	0, 5, 10 (P0, F1, F2)
Bartos et al. (2015)	0, 2.3, 4.5 (F1)	0, 0.27, 0.54 (F1)
Basha and Sujitha (2012)	0, 271.5	0, 40
Basha et al. (2011)	1, 100, 200 (F1, F2, F3)	0.16, 16.03, 32.1 (F1, F2, F3)
Bataineh and Nusier (2006)	0.54, 45.3, 135.7	0.08, 6.3, 18.8
Bhatnagar et al. (2002)	0, 54.3	0, 14.7
Chen and Geng (2011)	0, 22.6, 45.2, 67.9	0, 3.3, 6.7, 10
Chioca et al. (2008)	0.7, 22.6, 45.2	0.045, 2.33, 4.87
Dong et al. (2015a); Dong et al. (2015b); Dong et al. (2015c)	0.5, 22.6 (F1)	0.07, 3.3 (F1)
Ekambaram and Paul (2003)	0.2, 226	0.03, 36.3
Ekambaram and Paul (2002)	0.2, 226	0.04, 40.4
Ekambaram and Paul (2003)	0, 226	0, 110.7
El-lethey et al. (2010)	0, 22.6, 45.3 (F1)	0, 2.3, 4.9 (F1)
El-lethey et al. (2011)	0, 22.6, 45.3 (F1)	0, 3.1, 6.3 (F1)
El-lethey and Shaheed (2011)	0, 45.3	0, 6.7
El-lethey and Kamel (2011)	0, 45.3	0, 6.7
Gao et al. (2008a)	1, 2.3, 22.6	0.14, 0.33, 3.3
Gao et al. (2008b)	0.5, 2.3, 22.6	0.07, 0.33, 3.3
Gao et al. (2009a)	0.23, 2.7, 22.6	0.03, 0.33, 3.3
Han et al. (2014)	0, 11, 22, 45	0, 2.8, 5.6, 11.5
Hong et al. (2005)	0, 45.3 (F1)	0, 6.9 (F1)
Jain et al. (2015)	0, 11.8	0, 1.8
Jetti et al. (2016)	0.5, 100	0.07, 14.8
Jiang et al. (2014a)	0.3, 11.3, 22.6, 45.3 (F1)	0.05, 1.6, 3.3, 6.5 (F1)
Jiang et al. (2014b)	0, 54.3	0, 7.5
Kivrak (2012)	0.3, 40	0.07, 9.6

Systematic Literature Review on the Effects of Fluoride on Learning and Memory in Animal Studies

Study	Fluoride Concentration[1] ppm	Fluoride Concentration[1] mg/kg-d
Li et al. (2015)	0, 22.6	0, 3.3
Liu (1989)	0, 13.6, 27.2 (F1)	0, 2.2, 4.4 (F1)
Liu et al. (2009)	0.23, 2.3, 22.6	0.03, 0.3, 3.3
Liu et al. (2010)	0.23, 2.3, 22.6	0.03, 0.3, 3.3
Liu et al. (2011)	0.5, 2.3, 22.6	0.07, 0.3, 3.3
Liu et al. (2014)	0, 0.9, 2.3, 4.5	0, 0.23, 0.58, 1.2
Ma et al. (2015)	0, 22.6, 45.2	0, 5.79, 11.6
Mullenix et al. (1995) (6-wk exposure)	0, 100	0, 21.46
Mullenix et al. (1995) (16-wk exposure)	0, 125	0, 19
Niu et al. (2008)	0, 67.9	0, 10.4
Niu et al. (2009)	0, 67.9 (F1)	0, 10.9 (F1)
Niu et al. (2014)	0, 67.9 (F1)	0, 18.4 (F1)
Pereira et al. (2011)	0.7, 45.2	0.1, 6.7
Raghu et al. (2013)	0.5, 100	0.07, 14.8
Shen et al. (2004)	0, 67.9	0, 9.8
Sun et al. (2008)	0, 10, 50, 100	0, 2.6, 13.1, 26.3
Wang et al. (2004)	0.6, 45.3 (F1)	0.01, 6.9 (F1)
Wang et al. (2006)	0, 45.3, 90.5	0, 6.9, 13.8
Wei et al. (2014)		
Whitford et al. (2009)	0, 33.2, 85.6, 155.2	0, 3, 6, 12
Wu et al. (2006)	0, 45.3	0, 5.8
Wu et al. (2008)	0.6, 25 (F1 postnatal) 0.6, 1, 5, 25 (F1 prenatal and postnatal)	0.1, 5.3 (F1 postnatal) 0.1, 0.2, 1.1, 5.3 (F1 prenatal and postnatal)
Xu et al. (2001)	0, 1.4, 2.7, 5.4	0, 0.4, 0.7, 1.4
Zhang et al. (1999)	0, 0.5, 2.3, 4.5	0, 0.1, 0.6, 1.2
Zhang et al. (2001)	0, 0.5, 2.3, 4.5	0, 0.1, 0.6, 1.2
Zhang et al. (2009)	0, 1.1, 2.3, 4.5 (F1)	0, 0.18, 0.36, 0.71 (F1)
Zhu et al. (2012)	0, 2.3, 4.5, 9.05	0, 0.34, 0.68, 1.4
Oral diet		
Elliott (1967)	0, 4.2, 42.4	0, 0.61, 6.2
Gui et al. (2010)	1.8, 29.8 (F1)	0.26, 4.3 (F1)
Gavage		
Bera et al. (2007)	0, 7.1, 14.1 (F1)	0, 1.1, 2.3 (F1)
Flace et al. (2010)	0, 7.1, 14.1 (F1)	0, 1.1, 2.3 (F1)
Gao et al. (2009a)	0, 62.7	0, 9.05

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Study	Fluoride Concentration[1] ppm	Fluoride Concentration[1] mg/kg-d
Gopal et al. (2006)	0, 31.3, 62.6	0, 4.52, 9.05
Paul et al. (1998)	0, 56.5, 112.9	0, 9.05, 18.1
Rumiantsev et al. (1988)	0, 33.3	0, 5
Sarkozi et al. (2015)	0, 14.8	0, 2.3
Wei et al. (2014)	0.5, 22.6 (F1)	0.07, 3.44 (F1)
Zhang et al. (2013)	0, 45.3	0, 6.7
Subcutaneous or Intraperitoneal Injection		
Mullenix et al. (1995)	–	0, 0.06 mg/kg (P0) (9 prenatal injections)
Reddy and Karnati (2015)	–	0, 9.05 mg/kg bw (single injection)

[1]In general, the administered chemical in studies is sodium fluoride (NaF), and not all studies provide F equivalents. Dose levels are presented here as average daily doses of fluoride (mg/kg-d) and equivalent water concentrations of fluoride (ppm). As needed, dose conversions were made using US EPA (1988; 1994) default food or water consumption rates and body weights (using subchronic age and experiment duration) for the species/strain and sex of the animal of interest. Dose levels in mg/kg-d can vary for a given ppm across different studies if the studies use different species/strains and sex of animals that are assumed to have different water consumption rates.

Appendix T. Fluoride Administered and Converted Doses (Range across Studies for a Given ppm Level)

Table T-1. Summary of Fluoride Administered and Converted Doses (Across Studies)

Fluoride Concentrations[1] ppm	Fluoride Concentrations[1] mg/kg-d	References
Oral Drinking Water		
0.20	0.03–0.04	Ekambaram and Paul (2001; 2002)
0.23	0.03	Gao et al. (2009a); Liu et al. (2010); Liu et al. (2009)
0.30	0.07	Kivrak (2012)
0.34	0.05	Jiang et al. (2014a)
0.45	0.12	Zhang et al. (2001); Zhang et al. (1999)
0.50	0.07	Dong et al. (2015a); Dong et al. (2015b); Dong et al. (2015c); Gao et al. (2008a); Jetti et al. (2016); Liu et al. (2011); Raghu et al. (2013)
0.54	0.08	Bataineh and Nusier (2006)
0.60	0.01–0.13	Wang et al. (2004); Wu et al. (2008)
0.70	0.05–0.10	Chioca et al. (2008); Pereira et al. (2011)
0.90	0.23	Liu et al. (2014)
1.0	0.14–0.21	Basha et al. (2011); Gao et al. (2008b); Wu et al. (2008)
1.1	0.18	Zhang et al. (2009)
1.4	0.35	Xu et al. (2001)
2.3	0.27–0.59	Bartos et al. (2015); Gao et al. (2009a); Gao et al. (2008a); Gao et al. (2008b); Liu et al. (2014); Liu et al. (2011); Liu et al. (2010); Liu et al. (2009); Zhang et al. (2009); Zhang et al. (2001); Zhang et al. (1999); Zhu et al. (2012)
2.7	0.7	Xu et al. (2001)
4.5	0.54–1.19	Bartos et al. (2015); Liu et al. (2014); Zhang et al. (2009); Zhang et al. (2001); Zhang et al. (1999); Zhu et al. (2012)
5.0	1.1	Wu et al. (2008)
5.4	1.4	Xu et al. (2001)
9.1	1.34	Zhu et al. (2012)
10.0	2.6	Sun et al. (2008)
11.0	2.8	Han et al. (2014)
11.3	1.6	Jiang et al. (2014a)
11.8	1.8	Jain et al. (2015)
13.6	2.2	Liu (1989)
18.5	5.0	Băran-Poesina et al. (2013)
22.0	5.6	Han et al. (2014)

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Fluoride Concentrations[1] ppm	Fluoride Concentrations[1] mg/kg-d	References
22.6	2.3–5.8	Chen and Geng (2011); Chioca et al. (2008); Dong et al. (2015b); Dong et al. (2015c); El-lethey et al. (2011); El-lethey et al. (2010); Gao et al. (2009a); Gao et al. (2008a); Gao et al. (2008b); Jiang et al. (2014a); Li et al. (2015); Liu et al. (2011); Liu et al. (2010); Liu et al. (2009) Ma et al. (2015)
25.0	5.3	Wu et al. (2008)
27.2	4.4	Liu (1989)
33.2	3	Whitford et al. (2009)
37.1	10	Băran-Poesina et al. (2013)
40.0	9.6	Kivrak (2012)
45.0	11.5	Han et al. (2014)
45.2–45.3	4.9–12.3	Balaji et al. (2015); Chen and Geng (2011); Chioca et al. (2008); El-lethey et al. (2011); El-lethey and Kamel (2011); El-lethey et al. (2010); El-lethey and Shaheed (2011); Hong et al. (2005); Jiang et al. (2014a); Wang et al. (2006); Wang et al. (2004); Wu et al. (2006) Bataineh and Nusier (2006); Ma et al. (2015); Pereira et al. (2011)
50.0	13.1	Sun et al. (2008)
54.3	7.5–14.7	Bhatnagar et al. (2002); Jiang et al. (2014b)
61.4	9.1	Banji et al. (2013)
67.9	9.8–18.4	Chen and Geng (2011); Niu et al. (2014); Niu et al. (2009); Niu et al. (2008); Shen et al. (2004)
75.0	10.4	Balayssac et al. (2002)
85.6	6	Whitford et al. (2009)
90.5	13.8–24.5	Balaji et al. (2015); Wang et al. (2006)
100	14.8–26.3	Basha et al. (2011); Jetti et al. (2016); Mullenix et al. (1995); Raghu et al. (2013); Sun et al. (2008) (6-wk exposure)
125	19	Mullenix et al. (1995) (16-wk exposure)
135.7	18.8	Bataineh and Nusier (2006)
150	20.8	Balayssac et al. (2002)
155.2	12	Whitford et al. (2009)
200	32	Basha et al. (2011)
226	36.3–110.7	Ekambaram and Paul (2001; 2002); Ekambaram and Paul (2003)
271.5	40.0	Basha and Sujitha (2012)
Oral Diet		
1.8	0.3	Gui et al. (2010)
4.2	0.6	Elliott (1967)
29.8	4.3	Gui et al. (2010)
42.4	6.1	Elliott (1967)

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Fluoride Concentrations[1] ppm	Fluoride Concentrations[1] mg/kg-d	References
Gavage		
0.45	0.07	Wei et al. (2014)
7.1	1.1	Bera et al. (2007); Flace et al. (2010)
14.1	2.3	Bera et al. (2007); Flace et al. (2010)
14.8	2.3	Sarkozi et al. (2015)
22.6	3.4	Wei et al. (2014)
31.3	4.5	Gopal et al. (2006)
33.3	5.0	Rumiantsev et al. (1988)
45.3	6.7	Zhang et al. (2013)
56.5	9.1	Paul et al. (1998)
62.6	9.1	Gopal et al. (2006)
62.7	9.1	Gao et al. (2009b)
112.9	18.1	Paul et al. (1998)
Subcutaneous or Intraperitoneal Injection		
–	0.06 (9 prenatal injections)	Mullenix et al. (1995)
–	9.1 (single injection)	Reddy and Karnati (2015)

In general, the administered chemical in studies is sodium fluoride (NaF), and not all studies provide F equivalents. Dose levels are presented here as average daily doses of fluoride (mg/kg-d) and equivalent water concentrations of fluoride (ppm). As needed, dose conversions were made using US EPA (1988; 1994) default food or water consumption rates and body weights (using subchronic age and experiment duration) for the species/strain and sex of the animal of interest. Dose levels in mg/kg-d can vary for a given ppm across different studies if the studies use different species/strains and sex of animals that are assumed to have different water consumption rates.

Appendix U. Pharmacokinetics and Concentrations of Fluoride Measured in Tissue, Blood, Urine, or Bone

The predominant route and source of human exposure to fluoride is by oral ingestion of drinking water (either with naturally occurring or added fluoride), food, and dental products containing fluorides (US DHHS 2015; US EPA 2010b). Fluoride from water and dental products is in the form of fluoride ion, released in solution from water-soluble fluorides such as sodium fluoride, hydrogen fluoride, fluorosilicic acid, or sodium monofluorophosphate (Ekstrand et al. 1978; Spak et al. 1982). Fluoride compounds with low water solubility such as calcium fluoride, magnesium fluoride, or aluminum fluoride are poorly absorbed (IPCS 2002). The pharmacokinetics of fluoride are driven primarily by pH and storage in bone (NRC 2006). The acidic environment of the stomach promotes the conversion of fluoride ion to hydrogen fluoride, which is rapidly absorbed by passive diffusion from the stomach and small intestine (Whitford and Pashley 1984). Approximately 70–90% of ingested fluoride is absorbed in the alimentary tract and, for very soluble forms such as sodium fluoride, absorption is almost 100% (NRC 2006). Peak plasma concentrations are reached within 30 min (IPCS 2002). Calcified tissues such as bone and teeth readily incorporate fluoride (NRC 2006).

When taken with food, the rate of fluoride absorption from the gut and overall bioavailability are both lowered, thought due to binding of fluoride ions to calcium or other food constituents (Ekstrand and Ehrnebo 1979; Shulman and Vallejo 1990; Trautner and Einwag 1989). In healthy young adults, blood levels of fluoride, when expressed as micrograms per liter, approximate the consumed water concentration of fluoride expressed as mg/L (NRC 1993). Fluoride is cleared from plasma primarily through uptake by bone and excretion in urine, and clearance by these two routes is approximately equal in healthy adult humans (Whitford 1996). Clearance of fluoride from plasma occurs with a half-life estimated between 3 and 10 hours, depends on dose, and is affected by renal function and ongoing bone accretion and dissolution (Waterhouse et al. 1980). A significant amount of fluoride is cleared from blood by deposition into mineralized tissues. In animals and humans, approximately 99% of the body burden of fluoride is in bones and teeth (Hamilton 1992; Kaminsky et al. 1990). Fluoride is incorporated into the crystal lattice structure of mineralized tissues in the form of partially fluoridated hydroxyapatite (WHO 1994).

In infants and children, fluoride intake varies with age and diet, and relative clearance from plasma into mineralized tissues is extensive; clearance in children is larger compared to adults because of their growing skeletal system (Ekstrand et al. 1994; Ophaug et al. 1980). The features of developing versus mature bone provide more surface area for reaction with fluoride (Whitford 1996). In the elderly, blood fluoride levels can be elevated because of reduced capacity of bone to absorb fluoride, diminishing bone mass, and impaired renal function (IPCS 2002). Fluoride freely crosses the placenta, although concentrations in breast milk are lower than in plasma (Shen and Taves 1974). Whether consumption of fluoridated versus nonfluoridated water by the mother materially affects breast-milk fluoride concentrations is not yet settled (Dabeka et al. 1986; Ekstrand et al. 1981). Formula-fed infants are exposed to higher levels of fluoride than breast-fed infants, particularly when formula is reconstituted with fluoridated water (IPCS 2002). The blood-brain barrier is thought to reduce fluoride transfer (Whitford 1996).

Rats appear to require higher chronic exposure than humans to achieve the same plasma and bone fluoride concentrations, with one estimate of five times higher water concentrations

required to achieve the same plasma concentration (Dunipace et al. 1995). With respect to bone, one study estimated that "humans incorporate fluoride ~18 times more readily than rats when the rats are on a normal calcium diet" (Turner et al. 1995). Several studies in rodents cited in this document have measured serum or plasma levels of fluoride following exposures to various elevated levels in drinking water (Table U-1). These studies have reported relationships between fluoride concentrations in drinking water and plasma/serum ranging from approximately 70–8000 times higher levels in water than were measured in plasma/serum (Table U-2). This variability is perhaps not surprising because the ratio for a substance that is relatively rapidly cleared from blood could be affected by many factors including (1) the time between removal from or the last exposure to drinking water, which is not reported in studies; (2) the age of the animal and the related capacity of its mineralized tissues to take up circulating fluoride; and (3) potential interstrain and species differences in renal clearance rates.

Table U-1. Concentrations of Fluoride Measured in Tissue, Blood, Urine, or Bone

Study	Treatment[1]	Fluoride Concentration ppm (mg/kg-d)	Internal Doses[2]			
			Brain or Other Tissue	Serum/ Plasma	Urine	Bone
Balaji et al. (2015)	Swiss albino mouse (adult, 6 ♀); drinking water for 30 days	0 (0), 45.25 (12.27), 90.49 (24.53)	0.083, 0.24, 0.42	–	–	–
Balayssac et al. (2002)	Sprague Dawley rat (adult, 10 ♂); drinking water for 104 days	0 (0), 75 (10.393), 150 (20.787)	–	1, 5.5, 10.9 µM	–	–
	Lou/C rat (adult, 12 ♂); drinking water for 195 days	0 (0), 150 (20.787)		1.1, 12.3 µM		
Banji et al. (2013)	Wistar rat (adult P0 generation, 6 ♀); drinking water from GD6-PND15	0 (0), 61.37 (9.05)	–	*P0* 0.12, 0.32 (day 15) 0.14, 0.42 (day 30)	–	–
Basha et al. (2011)	Wistar rat (adult P0 generation, 8 ♀); drinking water from GD0 through lactation	0 (0), 100 (16.025), 200 (32.051)	*F1* 0.318, 1.83, 2.32 CB 0.672, 2.28, 3.43 CC 0.258, 0.993, 1.93 HC 0.358, 2.12, 3.09 MO *F2* 0.32, 2.07, 2.61 CB 0.675, 2.89, 3.66 CC 0.256, 1.24, 2.34 HC 0.362, 2.78, 3.53 MO *F3* 0.319, 2.51, 2.87 CB 0.673, 3.11, 3.92 CC 0.26, 1.65, 2.73 HC 0.359, 3.03, 3.84 MO	–	–	–

Study	Treatment[1]	Fluoride Concentration ppm (mg/kg-d)	Internal Doses[2]			
			Brain or Other Tissue	Serum/ Plasma	Urine	Bone
Dong et al. (2015c)	Sprague Dawley rat (adult P0 generation, 30 ♂♀); drinking water from GD0-PND28	0.23 (0.03), 22.6 (3.27)	–	–	*P0* 1.7, 2.16	*P0* 34.67, 211.07
Dong et al. (2015b)	Sprague Dawley rat (adult, 30 ♂♀); drinking water for 10 months	0.23 (0.03), 22.6 (3.27)	–	–	1.7, 2.16	1.52, 2.31
Ekambaram and Paul (2001) V 2001	Wistar rat (adult, 8 ♀); drinking water for 60 days	0 (0), 226.24 (36.26)	–	0.22, 1.94	–	–
Ekambaram and Paul (2002)	Wistar rat (adult, 8 ♀); drinking water for 60 days	0.2 (0.04), 226 (40.4)	–	0.22, 1.94	–	–
Gao et al. (2009a)	Sprague Dawley rat (adult, 8 ♂♀); drinking water for 6 months	0.23 (0.03), 2.26 (0.33), 22.6 (3.27)	–	–	1.02, 2.59, 5.96	669.4, 1135.2, 1304.3
Gao et al. (2009b)	Sprague Dawley rat (adult, 8 ♂♀); gavage for 3 months	0 (0), 62.68 (9.05)	–	0.018, 7.135	1.462, 64.966	
Gui et al. (2010)	Sprague Dawley rat (adult P0 generation, 9 ♂♀); diet for 6 months prior to mating, through lactation F1 (10 ♂♀); diet through PND30	*F1* 1.77 (0.26), 29.81 (4.3)	*F1* 0.36, 0.74	–	*F1* 0.98, 8.52	*F1* 1124, 1873
Han et al. (2014)	Kunming mouse (adult, 15 ♂); drinking water for 6 months	0 (0), 11 (2.82) 22 (5.63), 45 (11.52)	23.5, 34.2, 38.8, 39.6 HC	–	–	–
Jiang et al. (2014b)	Sprague Dawley rat (adult, 8 ♂); drinking water for 3 months	0 (0), 54.3 (7.52)	0.25, 1.19 cortex 0.24, 1.14 HC	0.09, 0.4	–	–
Li et al. (2015)	Wistar rat (juvenile-adult, 10 ♂); drinking water for 3 months	0 (0), 22.6 (3.34)	–	–	6.67, 22.5	0.0646, 0.189
Mullenix et al. (1995)	Sprague Dawley rat (adult, 20 ♀); drinking water for 6 weeks	0 (0), 100 (21.43)	0.406, 0.252 BG 0.358, 0.325 CB 0.479, 0.602 cortex 0.258, 0.79 HC 0.396, 0.308 HT 0.634, 0.306 MB 0.609, 1.28 MO	0.01, 0.077	–	–
Niu et al. (2009)	Wistar albino rat (adult P0 generation, ♀); drinking water from GD0 to PND21 F1 (8); 12 weeks after lactation	*F1* 0 (0), 67.87 (10.88)	–	*F1* 0.59, 2.78	–	–

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Study	Treatment[1]	Fluoride Concentration ppm (mg/kg-d)	Internal Doses[2]			
			Brain or Other Tissue	Serum/ Plasma	Urine	Bone
Reddy and Karnati (2015)	Wistar rat (adult, 6 ♂); intraperitoneal injection for 14 days	0, 9.05 mg/kg bw (single injection)	0.43, 0.77 gastrocnemius muscle tissue	–	–	–
Rumiantsev et al. (1988)	Rat (adult); gavage for 30 days	0 (0), 33.32 (5)	–	–	14, 744 µg/24 hr urine	2.3, 10.6 mg% thigh bone
Whitford et al. (2009)	Sprague Dawley rat (adult, 8 ♀); drinking water for 8 months	0 (0), 33.2 (3) 85.6 (6), 155.2 (12)	0.082, 3.9, 6.42, 18.1 µmol/kg ww	0.32, 15.27, 25.72, 97.56 µmol/L	–	150, 5408, 9497, 14,847 ash femur distal epiphysis

CB = cerebellum; CC = cerebral cortex; HC = hippocampus; MO = medulla oblongata; BG = basal ganglia; MB = mid-brain; HT = hypothalamus.
[1]For two studies (Niu et al. 2009 and Rumiantsev et al. 1988), number of animals and/or sex of animals not available.
[2]Unless otherwise noted, units for brain or other tissue and bone are provided as µg/g (units in study may have been µg/g, mg/kg, or ppm), and units for serum/plasma and urine are provided as mg/L (units in study may have been µg/mL or ppm).

Table U-2. Comparison of Drinking Water Concentration and Levels of Fluoride in Serum or Plasma

Study	Treatment	Fluoride Concentration ppm (mg/kg-d)	Internal Doses (Serum or Plasma)	Risk of Bias	Reporting Quality of LOD, Analytical Methods, and Timing of Sample Collection
Balayssac et al. (2002)	Sprague Dawley rat (adult, 10 ♂); drinking water for 104 days	0 (0), 75 (10.393), 150 (20.787)	1, 5.5, 10.9 µM (equivalent to 0.019, 0.1045, 0.2071 mg/L)	++	**LOD:** The method was linear from 0.01 to 100 mg/L fluoride with all values within this range.
				++	**Analytical method:** Samples were collected after slight anesthesia. A combined fluoride-selective electrode ISEC 301F linked to a Meterlab PHM250® ion analyzer was used to measure plasma F concentrations.
				−	**Timing:** Plasma F levels were measured at the end of the experiment, but when the experiment ended was not clear, as at least one outcome appears to have been measured 8 days after exposure ended (based on Figure 1).
	Lou/C rat (adult, 12 ♂); drinking water for 195 days	0 (0), 150 (20.787)	1.1, 12.3 µM (equivalent to 0.0209, 0.2337 mg/L)	++	**LOD:** The method was linear from 0.01 to 100 mg/L fluoride with all values within this range.
				++	**Analytical method:** Samples were collected after slight anesthesia. A combined fluoride-selective electrode ISEC 301F linked to a Meterlab PHM250® ion analyzer was used to measure plasma F concentration.
				+	**Timing:** Plasma F levels were measured at the end of the experiment, which appears to be at the end of treatment based on the time that the outcome was measured throughout treatment.
Banji et al. (2013)	Wistar rat (adult P0 generation, 6 ♀); drinking water from GD6–PND15	0 (0), 61.37 (9.05)	*P0* 0.12, 0.32 (day 15) mg/L 0.14, 0.42 (day 30) mg/L	−	**LOD:** No LOD was provided.
				+	**Analytical method:** Fluoride levels were determined using a potentiometer with an ion selective electrode. No other specifics were provided.
				+	**Timing:** Serum fluoride levels were determined by collecting 0.5 mL blood from the tail vein of the dam on GD20 (corresponding to 15th day of treatment) and PND11 (corresponding with 30th day of treatment), but was not measured at termination of treatment (PND15) or in the pups at the end of treatment.

Study	Treatment	Fluoride Concentration ppm (mg/kg-d)	Internal Doses (Serum or Plasma)	Risk of Bias	Reporting Quality of LOD, Analytical Methods, and Timing of Sample Collection
Ekambaram and Paul (2001)	Wistar rat (adult, 8 ♀); drinking water for 60 days	0 (0), 226.24 (36.26)	0.22, 1.94 mg/L	−	**LOD:** No LOD was provided.
				++	**Analytical method:** Serum fluoride was determined by the method using a fluoride ion specific electrode and a Fisher "accumet" model 425 pH/mV digital meter. One mL of serum was mixed with 10 mL of total ionic strength adjusting buffer in a small plastic beaker. The solution was mixed thoroughly by using a magnetic stirrer. Then the electrode was immersed in the sample solution. After the reading stabilized, the millivolt reading was recorded and expressed in mg/L.
				−	**Timing:** When the blood was collected for F analysis was not stated. Blood was stated to be collected only at sacrifice for acetylcholinesterase measurement, but how long it was between treatment and sacrifice was not reported.
Ekambaram and Paul (2002)	Wistar rat (adult, 8 ♀); drinking water for 60 days	0.2 (0.04), 226 (40.4)	0.22, 1.94 mg/L	−	**LOD:** No LOD was provided.
				++	**Analytical method:** Serum fluoride was determined by the method using a fluoride ion specific electrode and a Fisher "accumet" model 425 pH/mV digital meter. One mL of serum was mixed with 10 mL of total ionic strength adjusting buffer in a small plastic beaker. The solution was mixed thoroughly by using a magnetic stirrer. Then the electrode was immersed in the sample solution. After the reading stabilized, the millivolt reading was recorded and expressed in mg/L.
				−	**Timing:** Blood was stated to be collected at sacrifice, but how long after treatment the animals were sacrificed was not noted.
Gao et al. (2009a)	Sprague Dawley rat (adult, 8 ♂♀); gavage for 3 months	0 (0), 62.68 (9.05)	0.018, 7.135 μg/mL (i.e., mg/L)	+	**LOD:** No LOD was provided in the publication, but the study cites another publication for the methods used, which reported an LOD of 0.012 μg/mL (number from "Determination for fluoride in serum-ion selective electrode method (WS / T 212-2001)")
				+	**Analytical method:** Rats were decapitated, and blood samples were taken and stored in a polyethylene tube with plug. Blood samples were centrifuged immediately to extract blood serum samples, which were stored in a refrigerator at −35 °C. All serum fluoride concentrations were determined using method "Determination for fluoride in serum-ion selective electrode method (WS / T 212-2001)" for two weeks to complete the measurement.
				−	**Timing:** Blood was stated to be collected at sacrifice, but how long after treatment the animals were sacrificed was not noted. Authors noted that animals were sacrificed after the learning tests were complete, but did not indicate how many days after treatment this was.

Study	Treatment	Fluoride Concentration ppm (mg/kg-d)	Internal Doses (Serum or Plasma)	Risk of Bias	Reporting Quality of LOD, Analytical Methods, and Timing of Sample Collection
Mullenix et al. (1995)	Sprague Dawley rat (adult, 20 ♀); drinking water for 6 weeks	0 (0), 100 (21.43)	0.01, 0.077 mg/L	−	**LOD:** No LODs were provided.
				+	**Analytical method:** All plasma fluoride concentrations were determined using an ion-specific electrode, following hexamethyldisiloxane diffusion method.
				−	**Timing:** For the adult studies, when the blood samples were obtained was not reported.
Mullenix et al. (1995)	Sprague Dawley rat (weanling, 6–19 ♂♀); drinking water for 6 or 20 weeks	0, 75, 100, 125, 175		−	**LOD:** No LODs were provided.
				+	**Analytical method:** All plasma fluoride concentrations were determined using an ion-specific electrode, following hexamethyldisiloxane diffusion method.
				−	**Timing:** Fluoride levels were measured 3 weeks after exposure.
Niu et al. (2009)	Wistar albino rat (adult P0 generation, ♀); drinking water from GD0 to PND21 F1 (8); 12 weeks after lactation	*F1* 0 (0), 67.87 (10.88)	*F1* 0.59, 2.78 mg/L **units unclear, listed as mg/L, μg/dL, and μg/dL in article	−	**LOD:** No LODs were provided.
				−	**Analytical method:** Analytical methods were not reported.
				−	**Timing:** How or when blood samples were collected for analysis was not reported. Animals were sacrificed after the learning tests were complete, but authors gave no indication of how many days after treatment this was.

Study	Treatment	Fluoride Concentration ppm (mg/kg-d)	Internal Doses (Serum or Plasma)	Risk of Bias	Reporting Quality of LOD, Analytical Methods, and Timing of Sample Collection
Whitford et al. (2009)	Sprague Dawley rat (adult, 8 ♀); drinking water for 8 months	0 (0), 33.2 (3), 85.6 (6), 155.2 (12)	0.32, 15.27, 25.72, 97.56 µmol/L (equivalent to 0.006, 0.290, 0.489, 1.854 mg/L)	−	**LOD:** No LODs were provided.
				++	**Analytical method:** Plasma was analyzed for fluoride using an ion-specific electrode (Orion Research, Model 9409) and a reference electrode (Orion, Model 90-01) coupled to a potentiometer (Orion Research, Model 720A) after overnight hexamethyldisiloxane (HMDS)-facilitated diffusion. The next day the alkaline trap, 50 µL of 0.05 N NaOH, was allowed to air dry. The NaOH crystals were dissolved by adding 20 µL of 0.20 N acetic acid to form an acetate buffer system (pH 5.2). This solution was transferred to the sensing surface of the inverted reference electrode and the fluoride electrode was lowered until it contacted the solution. The mV potential was recorded after a stable reading had been obtained, which occurred within 2–4 min. Three fluoride standards, each in triplicate, calculated to bracket the expected amounts of fluoride in the different tissues, were diffused along with the samples. The r2 values for every standard curve exceeded 0.97. Non-diffused standards also were analyzed. These standards were prepared with the same reagents used to prepare the diffused standards and to have the same concentrations as the diffused standards. Comparison of the mV potentials of the diffused and non-diffused standards confirmed fluoride in the diffused standards had been completely trapped and analyzed (range of agreement 96–102%).
				−	**Timing:** Authors noted that blood was collected 4 days after completion of the behavioral studies, but when this was in terms of completion of the fluoride treatment was not clear (presumably 4 days or more after completion of treatment). In addition, authors noted that samples were placed in the refrigerator until analysis, but did not note the length of time until the samples were analyzed.

Appendix V. Background Fluoride Levels in Drinking Water and Diet

Table V-1. Background Fluoride Levels in Drinking Water and Diet

Reference	Route of Administration	Vehicle Control	Background Fluoride in Drinking Water	Background Fluoride in Feed
Balaji et al. (2015)	Drinking water	Nonfluoridated water	NR	NR
Balayssac et al. (2002)	Drinking water	Deionized water	NR	14 ppm
Banji et al. (2013)	Drinking water	Deionized water	NR	NR
Băran-Poesina et al. (2013)	Drinking water	Distilled water	NR	NR
Bartos et al. (2015)	Drinking water	Drinking water	NR	NR
Basha et al. (2011)	Drinking water	Tap water (<1 ppm F)	1 ppm	NR
Basha and Sujitha (2012)	Drinking water	Tap water	NR	NR
Bataineh and Nusier (2006)	Drinking water	Tap water (NaF = 1.2 ppm)	0.54 ppm	NR
Bera et al. (2007)	Gavage	Deionized water	NR	NR
Bhatnagar et al. (2002)	Drinking water	Deionized, defluoridated water	NR	NR
Chen and Geng (2011)	Drinking water	Distilled water	NR	NR
Chioca et al. (2008)	Drinking water	Drinking water (NaF = 1.54 ppm)	0.697 ppm	NR
Dong et al. (2015c)	Drinking water	Drinking water (<0.5 ppm F)	<0.5 ppm	6.2 mg/kg
Dong et al. (2015b)	Drinking water	Drinking water (<0.5 ppm F)	<0.5 ppm	6.2 mg/kg
Dong et al. (2015a)	Drinking water	Drinking water (<0.5 ppm F)	<0.5 ppm	6.2 mg/kg
Ekambaram and Paul (2001)	Drinking water	Drinking water (0.2 ppm F)	0.2 ppm	NR
Ekambaram and Paul (2002)	Drinking water	Drinking water (0.2 ppm F)	0.2 ppm	NR
Ekambaram and Paul (2003)	Drinking water	Tap water	NR	NR
El-lethey et al. (2010)	Drinking water	Distilled water	NR	NR
El-lethey et al. (2011)	Drinking water	Distilled water	NR	NR
El-lethey and Shaheed (2011)	Drinking water	Distilled water	NR	NR
El-lethey and Kamel (2011)	Drinking water	Distilled water	NR	NR
Elliott (1967)	Dietary	Well below 1 ppm in drinking water	<1 ppm	Fluorine content 0.002% in feed

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Reference	Route of Administration	Vehicle Control	Background Fluoride in Drinking Water	Background Fluoride in Feed
Flace et al. (2010)	Gavage	Deionized water	NR	NR
Gao et al. (2008a)	Drinking water	Drinking water (<0.5 ppm F)	<0.5 ppm	NR
Gao et al. (2008b)	Drinking water	Drinking water (<1 ppm F)	<1.0 ppm	NR
Gao et al. (2009a)	Drinking water	Drinking water (<0.5 ppm NaF)	<0.23 ppm	NR
Gao et al. (2009b)	Gavage	Deionized water	NR	NR
Gopal et al. (2006)	Gavage	NR	NR	NR
Gui et al. (2010)	Dietary	Tap water (0.5 ppm F)	0.5 ppm	6.2 mg/kg
Han et al. (2014)	Drinking water	Distilled water	NR	NR
Hong et al. (2005)	Drinking water	Deionized water	NR	25.57 mg/kg
Jain et al. (2015)	Drinking water	NR	NR	NR
Jetti et al. (2016)	Drinking water	Tap water (0.5 ppm F)	0.5 ppm	NR
Jiang et al. (2014a)	Drinking water	Tap water (0.34 mg/L F)	0.34 ppm	NR
Jiang et al. (2014b)	Drinking water	Tap water	NR	NR
Kivrak (2012)	Drinking water	Drinking water (0.3 ppm F)	0.3 ppm	NR
Li et al. (2015)	Drinking water	Distilled water	NR	NR
Liu (1989)	Drinking water	Distilled water	NR	2.9 ppm
Liu et al. (2009)	Drinking water	Tap water (<0.5 ppm NaF)	0.23 ppm	NR
Liu et al. (2010)	Drinking water	Drinking water (<0.5 ppm NaF)	0.23 ppm	NR
Liu et al. (2011)	Drinking water	Tap water (<0.5 ppm F)	0.5 ppm	NR
Liu et al. (2014)	Drinking water	Distilled water	NR	NR
Ma et al. (2015)	Drinking water	Distilled water	NR	NR
Mullenix et al. (1995)	Drinking water	Deionized water	NR	<10 ppm
Niu et al. (2008)	Drinking water	Double distilled water	NR	NR
Niu et al. (2009)	Drinking water	Double distilled water	NR	NR
Niu et al. (2014)	Drinking water	Double distilled water	NR	NR
Paul et al. (1998)	Gavage	Tap water	NR	NR
Pereira et al. (2011)	Drinking water	Tap water (0.7 ppm F)	0.7 ppm	NR
Raghu et al. (2013)	Drinking water	Tap water (0.5 ppm F)	0.5 ppm	NR
Reddy and Karnati (2015)	Intraperitoneal	NR	NR	NR
Rumiantsev et al. (1988)	Gavage	NR	NR	NR
Sarkozi et al. (2015)	Gavage	Distilled water	NR	NR

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Reference	Route of Administration	Vehicle Control	Background Fluoride in Drinking Water	Background Fluoride in Feed
Shen et al. (2004)	Drinking water	Tap water	NR	NR
Sun et al. (2008)	Drinking water	Tap water	NR	NR
Wang et al. (2004)	Drinking water	Drinking water (<0.6 ppm F)	0.6 ppm	25.57 mg/kg
Wang et al. (2006)	Drinking water	Free water	NR	NR
Wei et al. (2014)	Drinking water	Tap water (<1 ppm NaF)	0.45 ppm	<5 mg/kg
Whitford et al. (2009)	Drinking water	Distilled drinking water	NR	<0.01 mg/kg-d
Wu et al. (2006)	Drinking water	Distilled water	NR	NR
Wu et al. (2008)	Drinking water	Tap water (0.6 ppm F)	0.6 ppm	NR
Xu et al. (2001)	Drinking water	Distilled fluoride-free water	NR	NR
Zhang et al. (2013)	Gavage	Drinking water (<0.07 ppm NaF)	0.07 ppm	NR
Zhang et al. (1999)	Drinking water	Distilled water	NR	NR
Zhang et al. (2001)	Drinking water	Deionized water	NR	NR
Zhang et al. (2009)	Drinking water	Fluoride-free water	NR	Fluoride-free
Zhu et al. (2012)	Drinking water	Distilled water	NR	NR