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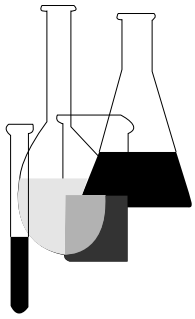
Health Effects Test Guidelines

OPPTS 870.8600 Developmental Neurotoxicity Screen



“Public Draft”

INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

Public Draft Access Information: This draft guideline is part of a series of related harmonized guidelines that need to be considered as a unit. *For copies:* These guidelines are available electronically from the EPA Public Access Gopher (gopher.epa.gov) under the heading ‘‘Environmental Test Methods and Guidelines’’ or in paper by contacting the OPP Public Docket at (703) 305–5805 or by e-mail: guidelines@epamail.epa.gov.

To Submit Comments: Interested persons are invited to submit comments. By mail: Public Docket and Freedom of Information Section, Office of Pesticide Programs, Field Operations Division (7506C), Environmental Protection Agency, 401 M St. SW., Washington, DC 20460. In person: bring to: Rm. 1132, Crystal Mall #2, 1921 Jefferson Davis Highway, Arlington, VA. Comments may also be submitted electronically by sending electronic mail (e-mail) to: guidelines@epamail.epa.gov.

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OPPTS 870.8600 Developmental neurotoxicity screen.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background.** The source material used in developing this harmonized OPPTS test guideline is the OPPT guideline under 40 CFR 795.250 Developmental Neurotoxicity Screen.

(b) **Purpose.** In the assessment and evaluation of the toxic characteristics of a chemical, it is important to determine when acceptable exposures in the adult may not be acceptable to a developing organism. This test is designed to provide information on the potential functional and morphologic hazards to the nervous system which may arise in the offspring from exposure of the mother during pregnancy and lactation.

(c) **Principle of the test method.** The test substance is administered to several groups of pregnant animals during gestation and lactation, one dose level being used per group. Offspring are randomly selected from within litters for neurotoxicity evaluation. The evaluation includes observation to detect gross neurological and behavioral abnormalities, determination of motor activity, neuropathological evaluation, and brain weights. Measurements are carried out periodically during both postnatal development and adulthood.

(d) **Test procedures**—(1) **Animal selection**—(i) **Species and strain.** Testing should be performed in the Sprague Dawley rat.

(ii) **Age.** Young adult animals (nulliparous females) should be used.

(iii) **Sex.** Pregnant females should be used at each dose level.

(iv) **Number of animals.** The objective is for a sufficient number of pregnant rats to be exposed to ensure that an adequate number of offspring are produced for neurotoxicity evaluation. At least 20 litters are recommended at each dose level. This number assumes a coefficient of variation of 20 to 25 percent for most behavioral tests. If, based upon experience with historical control data or data for positive controls in a given laboratory, the coefficient of variation for a given task is higher than 20 to 25 percent, calculation of appropriate sample sizes to detect a 20 percent change from control values with 80 percent power would need to be done. For most designs, calculations can be made according to Dixon and Massey under paragraph (f)(5) of this guideline, Neter and Wasserman under paragraph (f)(10) of this guideline, Sokal and Rohlf under paragraph (f)(11) of this guideline, or Jensen under paragraph (f)(8) of this guideline.

(A) On day–4 after birth, the size of each litter should be adjusted by eliminating extra pups by random selection to yield, as nearly as possible, four males and four females per litter. Whenever the number of male or female pups prevents having four of each sex per litter, partial adjustment (for example, five males and three females) is permitted. Adjustments are not appropriate for litters of less than eight pups. Elimination of runts only is not appropriate. Individual pups should be identified uniquely after standardization of litters. A method that may be used can be found under paragraph (f)(1) of this guideline.

(B) After standardization of litters, males and females should be randomly assigned to one of each of three behavioral tasks. Alternatively, more than one of the behavioral tasks may be conducted in the same animal. In the latter case, a minimum of 1 to 2 days should separate the tests when conducted at about the same age.

(C) One male and one female should be randomly selected from each litter for sacrifice at weaning as specified in paragraph (d)(8) of this guideline.

(2) **Control group.** A concurrent control group should be used. This group should be a sham treated group, or, if a vehicle is used in administering the test substance, a vehicle control group. Animals in the control groups should be handled in an identical manner to test group animals. The vehicle should neither be developmentally toxic nor have effects on reproduction.

(3) **Dose levels and dose selection.** (i) At least three dose levels plus a control (vehicle control, if a vehicle is used) should be used.

(ii) If the substance has been shown to be developmentally toxic either in a standard developmental toxicity study or a pilot study, the highest dose level should be the maximum dose which will not induce in utero or neonatal deaths or malformations sufficient to preclude a meaningful evaluation of neurotoxicity.

(iii) In the absence of standard developmental toxicity, unless limited by the physicochemical nature or biologicial properties of the substance, the highest dose level should induce some overt maternal toxicity but should not result in a reduction in weight gain exceeding 20 percent during gestation and lactation.

(iv) The lowest dose should not produce any grossly observable evidence of either maternal or developmental neurotoxicity.

(v) The intermediate doses should be equally spaced between the highest and lowest dose.

(4) **Dosing period.** Day–0 in the test is the day on which a vaginal plug and/or sperm are observed. The dose period should cover the period from day–6 of gestation through weaning (21 days postnatally).

(5) **Administration of test substance.** The test substance or vehicle should be administered orally by intubation. The test substance should be administered at the same time each day. The animals should be weighed periodically and the dosage based on the most recent weight determination.

(6) **Observation of dams.** (i) A gross examination of the dams should be made at least once each day, before daily treatment. The animals should be observed by trained technicians who are blind with respect to the animal's treatment, using standardized procedures to maximize inter-observer reliability. Where possible, it is advisable that the same observer be used to evaluate the animals in a given study. If this is not possible, some demonstration of interobserver reliability is required.

(ii) During the treatment and observation periods, cage-side observations should include:

(A) Any responses with respect to body position, activity level, coordination of movement, and gait.

(B) Any unusual or bizarre behavior including, but not limited to, headflicking, head searching, compulsive biting or licking, self-mutilation, circling, and walking backwards.

(C) The presence of convulsions, tremors, increased levels of lacrimation and/or red-colored tears, increased levels of salivation, piloerection, pupillary dilation or constriction, unusual respiration (shouldow, labored, dyspneic, gasping, and retching) and/or mouth breathing, diarrhea, excessive or diminished urination, or vocalization.

(iii) Signs of toxicity should be recorded as they are observed, including the time of onset, the degree and duration.

(iv) Animals should be weighed at least weekly.

(v) The day of delivery of litters should be recorded.

(7) **Study conduct**—(i) **Observation of offspring.** (A) All offspring should be examined cage-side daily for gross signs of mortality and morbidity.

(B) All offspring should be examined outside the cage for gross signs of toxicity whenever they are weighed or removed from their cages for behavioral testing. The offspring should be observed by trained technicians, who are unaware of the animal's treatment, using standardized procedures to maximize interobserver reliability. Where possible, it is advisable that the same observer be used to evaluate the animals in a given

study. If this is not possible, some demonstration of interobserver reliability is required. At a minimum, the end points outlined in paragraph (d)(6)(ii) of this guideline should be monitored as appropriate for the developmental stage being observed.

(C) Any gross signs of toxicity in the offspring should be recorded as they are observed, including the time of onset, the degree, and duration.

(ii) **Developmental landmarks.** Live pups should be counted and litters weighed by weighing each individual pup at birth, or soon thereafter, and on days 4, 7, 13, 17, and 21, and biweekly thereafter. The age of the pups at the time of the appearance of the following developmental landmarks should be determined:

(A) **Vaginal opening.** General procedure for this determination may be found under paragraph (f)(1) of this guideline.

(B) **Testes descent.** General procedure for this determination may be found under paragraph (f)(1) of this guideline.

(iii) **Motor activity.** (A) Motor activity should be monitored specifically on days 13, 17, 21, 45 (±2 days), and 60 (±2 days). Motor activity should be monitored by an automated activity recording apparatus. The device used should be capable of detecting both increases and decreases in activity, i.e. baseline activity as measured by the device should not be so low as to preclude decreases nor so high as to preclude increases. Each device should be tested by standard procedures to ensure, to the extent possible, reliability of operation across devices and testing of animals within dose groups should be balanced across devices.

(B) Each animal should be tested individually. The test session should be long enough to demonstrate habituation of motor activity in control animals, i.e. to approach asymptotic levels by the last 20 percent of the session. Animals' activity counts should be collected in equal time periods of no greater than 10 min duration. All sessions should have the same duration. Treatment groups should be counterbalanced across test times.

(C) Efforts should be made to ensure that variations in the test conditions are minimal and are not systematically related to treatment. Among the variables which can affect motor activity are sound level, size, and shape of the test cage, temperature, relative humidity, lighting conditions, odors, use of home cage or novel test cage, and environmental distractions.

(D) Additional information on the conduct of a motor activity study may be obtained in the TSCA motor activity guideline, in OPPTS 870.6200.

(iv) **Auditory startle test.** An auditory startle habituation test should be performed on the offspring on day–22 and day–60. Details on the conduct of this testing may be obtained under paragraph (f)(1) of this guide-

line. In performing the auditory startle task, the mean response amplitude on each block of 10 trials (5 blocks of 10 trials per session on each day of testing) should be made. While use of prepulse inhibition is not a requirement, it may be used at the discretion of the investigator. Details on the conduct of this testing may be obtained under paragraph (f)(7) of this guideline.

(v) **Active avoidance test.** Active avoidance testing should be conducted beginning at 60 to 61 days of age. Details on the apparatus may be obtained under paragraph (f)(4) of this guideline and on the conduct of testing under paragraph (f)(2) of this guideline, respectively; reviews on active avoidance conditioning can be found under paragraphs (f)(3) and (f)(9) of this guideline. In performing the active avoidance task, the following measures should be made:

(A) Mean number of shuttles during the adaptation period preceding each daily session.

(B) Mean number and latency of avoidances per session, presented in blocks of 10 trials (2 blocks of 10 trials per session across five sessions).

(C) Mean number and latency of escapes per session, presented in blocks of 10 trials as above.

(D) Mean duration of shocks per session, presented in blocks of 10 trials as above.

(E) Mean number of shuttles during the intertrial intervals.

(8) **Postmortem evaluation**—(i) **Age of animals.** One male and one female per litter should be sacrificed at weaning and the remainder following the last behavioral measures. Neuropathology and brain weight determinations should be made on animals sacrificed at weaning and after the last behavioral measures.

(ii) **Neuropathology.** Details for the conduct of neuropathology evaluation may be obtained in the TSCA neuropathology guideline in OPPTS 870.6200 of this chapter. At least six offspring per dose group should be randomly selected from each sacrificed group (weaning and adulthood) for neuropathologic evaluation. These animals should be balanced across litters, and equal numbers of males and females should be used. The remaining sacrificed animals should be used to determine brain weight. Animals should be perfused in situ by a generally recognized technique. After perfusion, the brain and spinal cord should be removed and gross abnormalities noted. Cross-sections of the following areas should be examined: The forebrain, the center of the cerebrum and midbrain, the cerebellum and pons, and the medulla oblongata; the spinal cord at cervical and lumbar swelling; Gasserian ganglia, dorsal root ganglia, dorsal and ventral root fibers, proximal sciatic nerve (midthigh and sciatic notch), sural nerve (at

knee), and tibial nerve (at knee). Tissue samples from both the central and peripheral nervous system should be further immersion-fixed and stored in appropriate fixative for further examination. After dehydration, tissue specimens should be cleared with xylene and embedded in paraffin or paraplast except for the sural nerve which should be embedded in plastic. A method for plastic embedding is described under paragraph (f)(12) of this guideline. Tissue sections should be prepared from the tissue blocks. The following general testing sequence is recommended for gathering histopathological data:

(A) **General staining.** A general staining procedure should be performed on all tissue specimens in the highest treatment group. Hematoxylin and eosin (H&E) should be used for this purpose. The staining should be differentiated properly to achieve bluish nuclei with pinkish background.

(B) **Special stains.** Based on the results of the general staining, selected sites and cellular components should be further evaluated by use of specific techniques. If H&E screening does not provide such information, a battery of stains should be used to assess the following components in all appropriate required samples: Neuronal body (e.g., Einarson's gallocyanin), axon (e.g., Kluver's Luxol Fast Blue), and neurofibrils (e.g., Bielchosky). In addition, nerve fiber teasing should be used. A section of normal tissue should be included in each staining to assure that adequate staining has occurred. Any changes should be noted and representative photographs should be taken. If lesions are observed, the special techniques should be repeated in the next lower treatment group until no further lesions are detectable.

(C) **Alternative technique.** If the anatomical locus of expected neuropathology is well-defined, epoxy-embedded sections stained with toluidine blue may be used for small sized tissue samples. This technique obviates the need for special stains.

(iii) **Brain weight.** At least 10 animals that are not sacrificed for histopathology should be used to determine brain weight. The animals should be decapitated and the brains carefully removed, blotted, chilled, and weighed. The following dissection should be performed on an ice-cooled glass plate: First, the rhombencephalon is separated by a transverse section from the rest of the brain and dissected into the cerebellum and the medulla oblongata/pons. A transverse section is made at the level of the ''optic chiasma'' which delimits the anterior part of the hypothalamus and passes through the anterior commissure. The cortex is peeled from the posterior section and added to the anterior section. This divides the brain into four sections, the telencephalon, the diencephalon/mid-brain, the medulla oblongata/pons, and the cerebellum. Sections should be weighed as soon as possible after dissection to avoid drying. Detailed methodology is available under paragraph (f)(6) of this guideline.

(e) **Data reporting and evaluation.** In addition to the reporting requirements specified in 40 CFR part 792, subpart J, the final test report should include the following information.

(1) **Description of system and test methods.** (i) A detailed description of the procedures used to standardize observation and operational definitions for scoring observations.

(ii) Positive control data from the laboratory performing the test that demonstrate the sensitivity of the procedures being used. These data do not have to be from studies using prenatal exposures. However, the laboratory must demonstrate competence in testing neonatal animals perinatally exposed to chemicals and establish test norms for the appropriate age group.

(iii) Procedures for calibrating and assuring the equivalence of devices and balancing treatment groups.

(iv) A short justification explaining any decisions where professional judgement is involved such as fixation technique and choice of stains.

(2) **Results.** The following information should be arranged by test group dose level.

(i) Data for each animal should be provided in tabular form showing:

(A) Its identification number and litter from which it came.

(B) Its body weight and score on each developmental landmark at each observation time; total session activity counts and intrasession subtotals on each day measured; auditory startle response magnitude session counts and intrasession subtotals on each day measured; avoidance session counts and intrasession counts on each day measured; time and cause of death (if appropriate); locations, nature or frequency, and severity of the lesions; total brain weight; absolute weight of each of the four sections; and weight of each section as a percentage of total brain weight. A commonly used scale such as 1+, 2+, 3+, and 4+ for degree of severity of lesions ranging from very slight to extensive may be used for morphologic evaluation. Any diagnoses derived from neurologic signs and lesions, including naturally occurring diseases or conditions, should also be recorded.

(ii) Summary data for each group should include:

(A) The number of animals at the start of the test.

(B) Body weights of the dams during gestation and lactation.

(C) Litter size and mean weight at birth.

(D) The number of animals showing each observation score at each observation time.

(E) The percentage of animals showing each abnormal sign at each observation time.

(F) The mean and standard deviation for each continuous end point at each observation time. These will include body weight, motor activity counts, acoustic startle responses, performance in active avoidance tests, and brain weights (both absolute and relative).

(G) The number of animals in which any lesion was found.

(H) The number of animals affected by each different type of lesion, the average grade of each type of lesion, and the frequency of each different type and/or location of lesions.

(3) **Evaluation of data.** An evaluation of the test results should be made. The evaluation should include the relationship between the doses of the test substance and the presence or absence, incidence, and severity of any neurotoxic effect. The evaluation should include appropriate statistical analyses. The choice of analyses should consider tests appropriate to the experimental design and needed adjustments for multiple comparisons.

(f) **References.** The following references should be consulted for additional background information on this test guideline.

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