OECD GUIDELINE FOR THE TESTING OF CHEMICALS

PROPOSAL FOR A NEW GUIDELINE 426

Developmental Neurotoxicity Study

INTRODUCTION

1.   In Copenhagen in June 1995, an OECD Working Group on Reproduction and Developmental Toxicity discussed the need to update existing OECD Test Guidelines for reproduction and developmental toxicity, and the development of new Guidelines for endpoints not yet covered (1). The Working Group recommended that a Guideline for developmental neurotoxicity should be written, based on a US guideline (2). On 17th - 18th June 1996, a second Consultation Meeting was held in Copenhagen to provide the Secretariat with guidance on the outline of a new guideline on developmental neurotoxicity, including the major elements, e.g., the placing of the test in overall testing strategy, details concerning choice of animal species, dosing period, testing period, end points to be assessed, and criteria for evaluating results (3). A US neurotoxicity risk assessment guideline was published in 1998 (4). An OECD Expert Consultation Meeting and ILSI Risk Science Institute Workshop were held back-to-back on 23rd-25th of October 2000. These meetings were held to discuss the scientific and technical issues related to the current Test Guideline. Recommendations from the ILSI Workshop (5)(6)(7) and the outcome of the OECD Expert Consultation Meeting (8) were considered in the development of this guideline.

INITIAL CONSIDERATIONS

2.   A number of chemicals are known to produce developmental neurotoxic effects in humans and other species (9)(10)(11)(12)(13). In the assessment and evaluation of the toxic characteristics of a chemical substance or mixture ("test substance"), determination of the potential for developmental neurotoxicity is important. Developmental neurotoxicity studies are designed to develop data, including dose-response characterizations, on the potential functional and morphological effects on the developing nervous system of the offspring that may arise from exposure during pregnancy or during early life.

3.   A developmental neurotoxicity study can be conducted as a separate study, or as a follow-up to a standard developmental toxicity and/or adult neurotoxicity study (e.g., Test Guidelines 415, 416, 424), or as an add-on study. Developmental neurotoxicity testing is conducted to further characterise neurological effects observed in other studies, and should be considered if the substance has been shown to cause neuropathology or neurotoxicity in adults, to be a hormonally-active material *in vivo* (e.g., pituitary, thyroid, sex hormones), or to cause other types of toxicity, suggestive of nervous system involvement at a developmental stage. Additional studies could be performed to further characterize effects seen with this Test Guideline.

PRINCIPLE OF THE TEST

4.   This protocol is designed to be performed as an independent study. However, observations and measurements described here can also be incorporated into a perinatal developmental toxicity, or added on to a one-or two-generation reproduction study. When the developmental neurotoxicity study is

incorporated within or attached to another study, it is imperative to preserve the integrity of both study types.

5.   The test substance is administered to pregnant animals during gestation and lactation. Dams are tested to assess effects in pregnant and lactating females and/or to provide comparative information (dams versus offspring). Offspring are randomly selected from within litters for neurotoxicity evaluation. The evaluation consists of observations to detect gross neurologic and behavioural abnormalities, including sexual maturation; and the assessment of physical development, reflex ontogeny, motor activity, motor and sensory function, and learning and memory; and the evaluation of brain weights and neuropathology during postnatal development and adulthood.

6.   When the protocol is conducted as a stand-alone study, additional available animals in each group could be used for specific neurobehavioral, neuropathological, neurochemical or electrophysiological procedures that may supplement the data obtained from the examinations recommended by this guideline (14)(15)(16)(17).   The supplemental procedures can be particularly useful when empirical observation, anticipated effects, or mechanism/mode of action indicate a specific type of neurotoxicity. These procedures may be used in the dams as well as in the pups.

PREPARATIONS FOR THE TEST

Selection of animal species

7.   The preferred test species is the rat; other species can be used when appropriate. The use of another species should be justified based on toxicological, pharmacokinetic, and/or other data. Justification must include availability of species-specific postnatal neurobehavioral and neuropathological assessments. If there was an earlier test that raised concerns, the species/strain that raised a concern should be considered. Because of the differing performance attributes of different rat strains, there should be evidence that the strain selected for use has adequate fecundity and responsiveness. The reliability and sensitivity of the selected rat strain, or other species, to detect developmental neurotoxicity should be documented.

Housing and feeding conditions

8.   The temperature in the experimental animal room should be $22^{\circ}C$ ($\underline{+}3^{\circ}$). Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning, the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. It is also possible to reverse the light cycle prior to mating and for the duration of the study, in order to perform the assessments of functional and behavioral endpoints during the dark period (under red light), i.e. during the time the animals are normally active (18). For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water.

9.   Animals may be housed individually or be caged in small groups of the same sex. Mating procedures should be carried out in cages suitable for the purpose. After evidence of copulation, or no later than day 15 of pregnancy, mated animals should be caged separately in delivery or maternity cages. Cages should be arranged in such a way that possible effects due to cage placement are minimised. Mated females should be provided with appropriate and defined nesting materials when parturition is near. It is well known that inappropriate handling or stress during pregnancy can result in adverse outcomes, including prenatal developmental loss and altered fetal and postnatal development. To guard against foetal loss from factors which are not treatment-related, animals should be carefully handled during pregnancy and stress from outside factors such as excessive outside noise or excessive variances in temperature and humidity should be avoided.

Preparation of the animals

10.   Healthy animals should be used, which have been acclimated to laboratory conditions and have not been subjected to previous experimental procedures, unless the study is incorporated in another study (see paragraph 4). The test animals should be characterised as to species, strain, source, sex, weight and age. Each animal should be assigned and marked with a unique identification number. The animals of all test groups should, as nearly as practicable, be of uniform weight and age, and should be representative of the species and strain under study. Young adult nulliparous female animals should be used at each dose level. Care should be taken to avoid mating of siblings. Gestation Day 0 is the day on which a vaginal plug and/or sperm are observed. Adequate acclimation time (e.g., 2-3 days) should be allowed when purchasing time-pregnant animals from a supplier. Mated females should be assigned in an unbiased way to the control and treatment groups, and as far as possible, they should be evenly distributed among the groups (e.g., a stratified random procedure is recommended to provide even distribution among all groups, e.g., by body weight). Females inseminated by the same male should be equalised across groups.

PROCEDURE

Number and sex of animals

11.   Each test and control group should contain a sufficient number of pregnant females to be exposed to the test substance to ensure that an adequate number of offspring are produced for neurotoxicity evaluation. A total of 20 litters are recommended at each dose level. Groups of fewer than 16 pregnant animals may not be appropriate to substantiate claims of a negative result. Replicate and staggered-group dosing designs are allowed if total numbers of litters per group are achieved, and appropriate statistical models are used, to account for replicates.

12.   On postnatal day (PND) 4 (day of delivery is PND 0), the size of each litter should be adjusted by eliminating extra pups by random selection to yield a uniform litter size for all litters (19). The litter size should not exceed the average litter size for the strain of rodents used (e.g., 8 - 12). The litter should have, as nearly as possible, equal numbers of male and female pups. Selective elimination of pups, e.g. based upon body weight, is not appropriate. After standardisation of litters and prior to further testing of functional end points, individual pups that are scheduled to be tested should be identified uniquely (20).

Assignment of animals for functional and behavioural tests, brain weights, and neuropathological evaluations

13.   The guideline allows various approaches with respect to the assignment of animals exposed *in utero* and through lactation to functional and behavioural tests, sexual maturation, brain weight determination, and neuropathological evaluation (21).

14.   Pups are selected from each dose group and assigned for endpoint assessments on or after PND 4. Selection of pups should be performed so that equal numbers of males and females from each dose group are obtained for all tests. For motor activity testing the same pair of male and female littermates should be tested at all preweaning ages (see paragraph 35). For all other tests the same or separate pairs of male and female litter mates may be assigned to different behavioral tests. Different pups may need to be assigned to weanling versus adult tests of cognitive function in order to avoid confounding the effects of age and prior training on these measurements (22)(23). Repeated testing of the same animals for cognitive testing at both ages is allowed if such testing does not prevent the detection of an effect due to treatment. At weaning, pups not selected for testing can be humanely disposed of. The statistical unit of measure should be the number of litters and not the total number of pups.

15.   There are different ways to assign pups to the pre-weaning and post-weaning examinations, cognitive tests, pathological examinations etc. Table 1 and 2 are examples that may be adapted to the litter size or other experimental requirements. The example presented in Table 1 is described below and supplemented by some background information. One set of pups (20 pups/sex/dose level), may be used for pre-weaning testing of behavioral ontogeny. Out of these animals 10 pups/sex/dose level, selected randomly from as many different litters as possible, will be humanely killed at day 22 (the day after the last day of lactational exposure) or at some earlier time point between PND 11 and PND 22, if appropriate. After adequate fixation, the brains will be weighed and saved in fixative for subsequent histopathologic evaluation. Brain weight data can instead be collected using unfixed brains from a separate set of 10 males and 10 females per dose level that are not used for histopathologic evaluation. Another set of 20 animals/sex/dose level may be used for post-weaning examinations (functional tests, motor activity, sexual maturation and auditory startle). For cognitive function testing in adolescents and young adults a minimum of 10 pups/sex/dose level will be used. Depending on the cognitive function tests the use of a higher number of animals should be considered (for animal assignments see Table 2). Out of these animals 10 animals/sex/doselevel, selected randomly, will be anesthetized and fixed via perfusion at study termination (approximately PND 70). After additional fixation in situ, the brain will be removed and processed for neuropathological evaluation. Whole-brain weight measurements will be performed when the brains are removed, prior to further processing, or using fresh tissue from a separate set of non-perfused animals (10/sex/dose level).

Table 1: Example for assignment of animals for functional/behavioural tests, neuropathology evaluation, and brain weights, as described in paragraphs 13, 14, 15. (PND = postnatal day)[a].

Pup no. m	Pup no. f	No. of pups assigned to test	Examination / Test
1	5	~20 m + ~20 f 10 m + 10 f [b] 6 m + 6 f [b] 10 m + 10 f [b]	**Pre-weaning investigations** Behavioral ontogeny Day 22 brain weight Day 22 Neuropathology Day 22 morphometry (Optional)
2	6	~20 m + ~20 f ~20 m + ~20 f ~20 m + ~20 f ~20 m + ~20 f 10 m + 10 f [b] 6 m + 6 f [b] 10 m + 10 f [b]	**Post-weaning investigations** Behavioral / functional tests Motor activity Sexual maturation Auditory startle Day 60 brain weight Day 60 Neuropathology Day 60 morphometry (Optional)
3	7	10 m + 10 f [b, c]	Cognitive function test (adolescents)
3	7	10 m + 10 f [b, c]	Cognitive function test (young adults)
4	8	- -	Reserve animals for replacements or additional tests

Notes to Table 1:
Note a) for this example litters are culled to 4 males + 4 females; male pups are numbered 1 through 4, females pups 5 through 8.
Note b) from as many different litters as possible
Note c) different pups to be used for tests in adolescents and young adults (e.g. even / odd litters)

Table 2: Example for assignment of animals for functional/behavioural tests, neuropathology evaluation, and brain weights, as described in paragraphs 13, 14, 15. (PND = postnatal day)[a].

Pup no. m	Pup no. f	No. of pups assigned to test	Examination / Test
1	2		**Pre-weaning investigations**
		~20 m + ~20 f	Behavioral ontogeny
		10 m + 10 f [b]	Day 22 brain weight
		6 m + 6 f [b]	Day 22 Neuropathology
		10 m + 10 f [b]	Day 22 morphometry (Optional)
2	6		**Post-weaning investigations**
		~20 m + ~20 f	Behavioral / functional tests
		~20 m + ~20 f	Motor activity
		~20 m + ~20 f	Sexual maturation
		~20 m + ~20 f	Auditory startle
		~20 m + ~20 f	Cognitive function test (adolescents)
		10 m + 10 f [b]	Day 60 brain weight
		6 m + 6 f [b]	Day 60 Neuropathology
		10 m + 10 f [b]	Day 60 morphometry (Optional)
3	7	~20 m + ~20 f	Cognitive function test (young adults)
4	8	- -	Reserve animals for replacements or additional tests

Notes to Table 2

Note a) for this example litters are culled to 4 males + 4 females; male pups are numbered 1 through 4, females pups 5 through 8.

Note b) from as many different litters as possible

Dosage

16.   At least three dose levels and a concurrent control should be used. The dose levels should be spaced to produce a gradation of toxic effects. Unless limited by the physico-chemical nature or biological properties of the substance, the highest dose level should be chosen with the aim to induce some maternal toxicity (e.g., clinical signs, decreased body weight gain (not more than 10%) and/or evidence of toxicity in a target organ). Alternatively, pilot studies or preliminary range-finding studies should be performed to determine the highest dosage to be used which should produce a minimal degree of maternal toxicity. If the test substance has been shown to be developmentally toxic either in a standard developmental toxicity study or in a pilot study, the highest dose level should be the maximum dose which will not induce *in utero* or neonatal death or malformations, sufficient to preclude a meaningful evaluation of neurotoxicity. The lowest dose level should aim to not produce any evidence of either maternal or developmental toxicity or neurotoxicity. A descending sequence of dose levels should be selected with a view to demonstrating any dose-related response and a no-observed-adverse effect level (NOAEL), or doses near the limit of detection that would allow the determination of a benchmark dose. Two- to four-fold intervals are frequently optimal for setting the descending dose levels, and the addition of a fourth test group is often preferable to using very large intervals (e.g. more than a factor of 10) between dosages.

17.		Dose levels should be selected taking into account all existing toxicity data as well as additional information on metabolism and toxicokinetics of the test substance or related materials. This information may also assist in demonstrating the adequacy of the dosing regimen. In the case of anticipated direct exposure of human infants, other than through milk, direct dosing of pups should be considered (24)

18.		The concurrent control group should be a sham-treated control group or a vehicle-control group if a vehicle is used in administering the test substance. All animals should normally be administered the same volume of either test substance or vehicle on a body weight basis. If a vehicle or other additive is used to facilitate dosing, consideration should be given to the following characteristics: effects on the absorption, distribution, metabolism, or retention of the test substance; effects on the chemical properties of the test substance which may alter its toxic characteristics; and effects on the food or water consumption or the nutritional status of the animals. The vehicle should neither be developmentally toxic nor have effects on reproduction. Animals in the control group(s) should be handled in an identical manner to test group animals.

Administration of doses

19.		The test substance or vehicle should be administered by the route most relevant to potential human exposure, and based on available metabolism and distribution information in the test animals. The route of administration will generally be oral (e.g., gavage, dietary, via drinking water), but other routes (e.g., dermal; inhalation) may be used depending on the characteristics and anticipated or known human exposure routes. Justification should be provided for the route of administration chosen. The test substance should be administered at approximately the same time every day.

20.		The dose administered to each animal should normally be based on the most recent individual body weight determination. However, caution should be exercised when adjusting the doses during the last third of pregnancy. If excess toxicity is noted in the treated dams, those animals should be humanely killed. If several pregnant animals in the same dose group show signs of excess toxicity, that group should be terminated.

21.		The test substances or vehicle should, as a minimum, be administered daily to mated females from the time of implantation (gestation day 6) throughout lactation (PND 21), so that the pups are exposed to the test substance during pre- and post-natal neurological development.[*] Dosing durations should be adjusted for other species to ensure exposure during the all early periods of brain development (i.e., equivalent to prenatal and early postnatal human brain growth). Dosing may begin from the initiation of pregnancy (gestation day 0 = GD 0) although consideration should be given to the potential of the test substance to cause pre-implantation loss. Dosing should not occur on the day of parturition in those animals which have not completely delivered their offspring. In general, it is assumed that exposure of the pups will occur through the maternal milk; however, direct dosing of pups should be considered in the case of anticipated direct exposure of human infants other than through milk, based on exposure and pharmacokinetic information (24)

---

[*] Administration of the test substance at gestation day 0 (GD 0) may lead to a risk of preimplantation loss. Administration beginning at GD 6 would avoid this risk, but the developmental stages between GD 0 and 6 would not be treated. When a laboratory purchases time-mated animals, it is impractical to begin dosing at GD 0, and thus GD 6 would be a good starting date. The testing laboratory should set the dosing regimen according to relevant information about the effects of the test substance, prior experience, and logistical considerations, this may include extension of dosing past weaning

OBSERVATIONS

Observations on dams

22.   All dams should be carefully observed at least once daily with respect to their health condition as well as at least daily for morbidity and mortality.

23.   During the treatment and observation periods, more detailed clinical observations should be conducted periodically (at least twice weekly). The animals should be observed outside the home cage by trained technicians who are unaware of the animals' treatment, using standardized procedures to minimise animal stress, observer bias and maximise inter-observer reliability.   Where possible, it is advisable that the observations in a given study be made by the same technician.

24.   The presence of observed signs should be recorded. Whenever feasible, the magnitude of the observed signs should also be recorded. Clinical observations should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and secretions and autonomic activity (e.g., lacrimation, piloerection, pupil size, unusual respiratory pattern and/or mouth breathing, and any unusual signs of urination or defecation).

25.   Any unusual responses with respect to body position, activity level (e.g., decreased or increased exploration of the standard area) and co-ordination of movement should also be noted. Changes in gait, (e.g., waddling, ataxia), posture (e.g., hunched-back) and reactivity to handling, placing or other environmental stimuli, as well as the presence of clonic or tonic movements, convulsions, or tremors, stereotypies (e.g., excessive grooming, unusual head movements, repetitive circling) or bizarre behaviour (e.g., biting or excessive licking, self-mutilation, walking backwards, vocalization) or aggression should be recorded.

26.   Signs of toxicity should be recorded, including the day of onset, time of day, degree, and duration.

27.   Animals should be weighed at the time of dosing at least weekly throughout the study, on or near the day of delivery, and on PND 21 (weaning). For gavage studies dams should be weighed at least twice weekly. Doses should be adjusted at the time of each body weight determination, as appropriate. Food consumption should be measured if exposure is via the diet. Water consumption should be measured if exposure is via the water supply.

Observations on offspring

28.   All offspring should be carefully observed at least daily for signs of toxicity and for morbidity and mortality.

29.   During the treatment and observation periods, more detailed clinical observations of the offspring should be conducted at least once weekly, when pups are weighed (see paragraph 33). The offspring should be observed by trained technicians who are unaware of the animals' treatment, using standardized procedures to minimise bias and maximise inter-observer reliability. Where possible, it is advisable that the observations are made by the same technician. At a minimum, the endpoints described in paragraphs 24 and 25 should be monitored as appropriate for the developmental stage being observed.

30.   All signs of toxicity in the offspring should be recorded, including the day of onset, time of day, degree, and duration.

Developmental landmarks

31.   Body weight may be the best indicator of physical development. Changes in preweaning landmarks of development such as pinna reflex, incisor eruption etc are highly correlated with body weight (25)(26). Measurement of developmental landmarks is, therefore, recommended only when there is prior evidence that these endpoints will provide additional information. In case the measurement of developmental landmarks is appropriate, Table 3 presents the minimum number of times when measurements should be performed. Depending on the anticipated effects, and the results of the initial measurements, it may be advisable to add additional time points or to perform the measurements in other developmental stages.

32.   In certain instances, such as delayed or premature delivery, or when there is a high variability in duration of pregnancy within groups, it is advisable to use post-coital age instead of postnatal age when assessing physical development. If pups are tested on PND 21, it is recommended that this testing be carried out prior to actual weaning to avoid a confounding effect by the stress associated with weaning. In addition, any postweaning testing of pups should not occur within two to three days of weaning.

Table 3: Timing of the assessment of physical and developmental landmarks and functional/behavioural endpoints.

Age Periods / End points	Preweaning [Before PND 21]	Adolescence [PND 21-59 (a)]	Young adults [PND 60-75]
Physical and developmental landmarks			
Physical development Body weight	weekly (a)	weekly	weekly
Brain weight	at PND22 (b)	optional at PND22	at termination
Neuropathology	at PND 22 (b)	optional at PND22	at termination
Sexual maturation	--	as appropriate	--
Other developmental landmarks (c)	as appropriate	--	--
Behavioral ontogeny	At least two measures	--	--
Functional/behavioural endpoints			
Motor activity (including habituation)	1 – 3 times	once	once
Motor and sensory function	--	once	once
Learning and memory	--	once	once

Notes to Table 3

Note a) It is recommended that pups not be tested on the day of weaning (generally PND 21), in order to avoid acute effects and to avoid testing a period of stress due to the weaning

process, but that they be tested 2-3 days postweaning (PND 23-24). Body weights should be measured at least twice weekly when directly dosing pups.

Note b) Brain weights and neuropathology may be assessed at some earlier time (e.g., PND 11), if appropriate.

Note c) Other developmental landmarks in addition to the body weight (e.g., eye opening); should be recorded when appropriate.

Physical development

33. Live pups should be counted and sexed (e.g., ano-genital distance), and each pup within a litter should be weighed individually at birth or soon thereafter, at least weekly throughout lactation, and at least once every two weeks thereafter. When sexual maturation is evaluated, the age of the animal when vaginal patency (27) or preputial separation (28) occurs should be determined for at least one male and one female per litter. For example, in Sprague-Dawley rats, approximate ranges in age for vaginal patency and preputial separation ranges are, respectively, PND 32-35 and PND 42-46.

Behavioral ontogeny

34. Ontogeny of selected behaviours should be measured in at least one pup/sex/litter during the appropriate age period, with the same pups being used on all test days for all behaviours assessed. The measurement days should be spaced evenly over that period to define either the normal or treatment-related change in ontogeny of that behaviour (29). The following are some examples of behaviours for which their ontogeny could be assessed: air righting, negative geotaxis, and motor activity (29)(30)(31).

Motor activity

35. Motor activity should be monitored (32)(33)(34) during the preweaning and adult age periods. The test session should be long enough to demonstrate intra-session habituation for non-treated controls (35)(36). Use of motor activity to measure behavioural ontogeny (see paragraph 34) is strongly recommended. If used as a test of behavioural ontogeny, then testing should utilize the same animals for all preweaning test sessions. Testing should be frequent enough to assess the ontogeny of intra-session habituation (35). This may require three or more time periods prior to, and including the day of weaning (e.g., PND13, 17, 21). Testing of the same animals, or littermates, should also occur at an adult age close to study termination. Testing on additional days may be done as necessary. Motor activity should be monitored by an automated activity recording apparatus which must be capable of detecting both increases and decreases in activity, (i.e., baseline activity as measured by the device must not be so low as to preclude detection of decreases nor so high as to preclude detection of increases in activity). Each device should be tested by standard procedures to ensure, to the extent possible, reliability of operation across devices and across days. To the extent possible, treatment groups should be balanced across devices. Each animal should be tested individually. Treatment groups should be counter-balanced across test times to avoid confounding by circadian rhythms of activity. Efforts should be made to ensure that variations in the test conditions are minimal and are not systematically related to treatment. Among the variables that can affect motor activity are sound level, size and shape of the test cage, temperature, relative humidity, light conditions, odours, use of home cage or novel test cage, and environmental distractions.

Motor and sensory function

36. Motor and sensory function (neurological function) should be examined in detail at least once for the adolescent period and once during the young adult period. Sufficient testing should be conducted to ensure an

adequate sampling of sensory modalities (e.g., somatosensory, vestibular) and motor functions (e.g., strength, coordination). A few examples of tests for motor and sensory function are extensor thrust response, righting reaction, auditory startle, and evoked potentials (31)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46).

Learning and memory tests

37.   A test of associative learning and memory should be conducted postweaning and for young adults (PND 60-75). The same or separate test(s) may be used at these two stages of development. Some flexibility is allowed in the choice of test(s) for learning and memory in weanling and adult rats. However, the test(s) must be designed so as to fulfil two criteria. First, learning should be assessed either as a change across several repeated learning trials or sessions, or, in tests involving a single trial, with reference to a condition that controls for non-associative effects of the training experience. Second, the test(s) should include some measure of memory (short-term or long-term) in addition to original learning (acquisition), but note that this measure of memory cannot be reported in the absence of a measure of acquisition obtained from the same test. If the test(s) of learning and memory reveal(s) an effect of the test substance, additional tests to rule out alternative interpretations based on alterations in sensory, motivational, and/or motor capacities may be considered. In addition to the above two criteria, it is recommended that the test of learning and memory be chosen on the basis of its demonstrated sensitivity to the class of compound under investigation, if such information is available in the literature. In the absence of such information, examples of tests that could be made to meet the above criteria include: passive avoidance (34)(47), delayed-matching-to-position for the adult rat (48) and for the infant rat (49), olfactory conditioning (34)(50), Morris water maze (51)(52)(53)(54), radial arm maze (51)(55) T-maze (34), and acquisition and retention of schedule-controlled behaviour (22)(23)(56). Additional tests are described in the literature for weanling (23) and adult (57) rats.

Post-mortem examination

38.   Maternal animals can be euthanized and discarded after weaning of the offspring on or after the pups are weaned on PND 21.

39.   Neuropathological evaluation of the offspring will be conducted using the brains of animals sacrificed at PND 22 or, if determined to be more appropriate, at an earlier time point between PND 11 and PND 22, as well as at study termination. The brains of animals sacrificed on PND 22 or earlier may be fixed either by in situ immersion or by perfusion. Animals sacrificed at study termination should be fixed by perfusion.

Processing of tissue samples

40.   All gross abnormalities apparent at the time of necropsy should be noted. Tissue samples taken should represent all major regions of the nervous system. The tissue samples should be retained in an appropriate fixative and processed according to standardized published histological protocols (ii) (58)(59)(60). Paraffin embedding is acceptable for tissues of the CNS and PNS, but plastic embedding is considered appropriate when a higher degree of resolution is required (e.g., for peripheral nerves when a peripheral neuropathy is suspected and/or for morphometric analysis of peripheral nerves).

Qualitative examination.

41.   The purposes of the qualitative examination are: i) to identify regions within the nervous system exhibiting evidence of neuropathological alterations; ii) to identify types of neuropathological alterations resulting from exposure to the test substance; and, iii) to determine the range of severity of the neuropathological alterations. Representative histological sections from the tissue samples should be examined microscopically by an appropriately trained pathologist for evidence of neuropathological alterations. All

neuropathologic alterations should be assigned a subjective grade indicating severity. The following stepwise procedure is recommended for the qualitative analysis. First, sections from the high dose group are compared with those of the control group. If no evidence of neuropathological alterations are found in animals of the high dose group, no further analysis is required. If evidence of neuropathological alterations are found in the high dose group, then animals from the intermediate and low dose groups are examined. A hematoxylin and eosin stain may be sufficient for evaluating brain sections from animals sacrificed at PND 22 or earlier. However, a myelin stain (e.g., luxol fast blue / cresyl violet) and a silver stain (e.g., Bielschowsky's or Bodians stains) are recommended for sections of PNS and CNS tissues from animals killed at study termination. Subject to the professional judgement of the pathologist and the kind of lesions observed, additional stains may be considered appropriate to identify and characterize particular types of alterations (e.g., GFAP).

42.   Morphometric evaluation is strongly recommended as it may assist in the detection of a treatment-related effect, even in the absence of histopathologic alterations. Sampling and preparation of nervous tissue is to be compatible with morphometric evaluation. Data from morphometric evaluation is valuable in the interpretion of treatment-related differences in brain weight or morphology. Morphometric evaluations may include, for example, linear or areal measurements of specific brain regions. Stereology may be used to identify treatment-related effects on parameters such as volume or cell number for specific neuroanatomic regions (61)(62)(63)(64)(65)(66).

43.   The brains should be examined for any evidence of treatment-related neuropathological alterations and adequate samples should be taken from all major brain regions [e.g., olfactory bulbs, cerebral cortex, hippocampus, basal ganglia, thalamus, hypothalamus, midbrain (tectum, tegmentum, and cerebral peduncles), pons, medulla oblongata, and cerebellum to ensure a thorough examination. Sections in the same plane should be evaluated in all animals.

44.   Neuropathological evaluation should include an examination for indications of developmental insult to the brain (7)(67)(69)(70)(71)(72), in addition to the cellular alterations (e.g., neuronal vacuolation, degeneration, necrosis) and tissue changes (e.g., gliosis, leukocytic infiltration, cystic formation), typical of the adult brain. In this regard, it is important that treatment-related effects be distinguished from normal developmental events known to occur at a developmental stage corresponding to the time of sacrifice. Examples of significant alterations indicative of developmental insult include, but are not restricted to:

- alterations in the gross size or shape of the olfactory bulbs, cerebrum or cerebellum;

- alterations in the relative size of various brain regions, including decreases or increases in the size of regions resulting from the loss or persistence of normally transient populations of cells or axonal projections (e.g., external germinal layer of cerebellum, corpus callosum);

- alterations in proliferation, migration, and differentiation, as indicated by areas of excessive apoptosis or necrosis, clusters or dispersed populations of ectopic, disoriented or malformed neurons or alterations in the relative size of various layers of cortical structures;

- alterations in patterns of myelination, including an overall size reduction or altered staining of myelinated structures, although such alterations may not be readily apparent in the brains of animals sacrificed on or before PND 22;

- evidence of hydrocephalus, in particular enlargement of the ventricles, stenosis of the cerebral aqueduct and thinning of the cerebral hemispheres.

45.   For animals sacrificed at study termination, representative histological sections from the tissue samples should be examined microscopically for evidence of neuropathological alterations. The nervous

system should be thoroughly examined for evidence of any treatment-related neuropathological alterations. Particular attention should be paid to regions known to be sensitive to neurotoxic insult or those regions likely to be affected based on the results of functional tests (73).

Analysis of the dose-response relationship of neuropathologic alterations.

46.   If any treatment-related neuropathological alterations are found in the qualitative examination, the dose-dependence of the incidence, frequency and severity grade of the lesions should be determined, based on an evaluation of all animals from all dose groups. All regions of the brain that exhibit any evidence of neuropathologic alteration should be included in this evaluation. For each type of lesion, the characteristics used to define each severity grade should be described, indicating the features used to differentiate each grade. The frequency of each type of lesion and its severity grade should be recorded and a statistical analysis performed to evaluate dose-response relationships. While the use of coded slides is not required, the nature of the dose-response relationship can be substantiated by a subsequent evaluation where treatment information is encoded so as to be unknown to the pathologist when the slides are scored.

DATA AND REPORTING

Data

47.   Data should be reported individually and summarised in tabular form, showing for each test group the types of change and the number of dams, offspring by sex, and litters displaying each type of change. If direct postnatal exposure of the offspring has been performed, the route of exposure should be reported

Evaluation and interpretation of results

48.   A perinatal developmental neurotoxicity study will provide information on the effects of repeated exposure to a substance during in utero and early postnatal development. Since emphasis is placed on both general toxicity and developmental neurotoxicity endpoints, the results of the study will allow for the discrimination between neurodevelopmental effects occurring in the absence of general maternal toxicity, and those which are only expressed at levels that are also toxic to the maternal animal. Due to the complex interrelationships among study design, statistical analysis, and biological significance of the data, adequate interpretation of developmental neurotoxicity data will involve a great deal of scientific judgment. The interpretation of test results should be done using a weight of evidence approach (16)(74)(75). Data from all studies relevant to the evaluation of developmental neurotoxicity, including human studies, case reports, and experimental animal studies (e.g., toxicokinetic data, structure-activity information, data from other toxicity studies) should be included in this characterization. This includes the relationship between the doses of the test substance and the presence or absence, incidence, and extent of any neurotoxic effect for each sex (16)(76).

49.   Evaluation of data should include a discussion of both the biological and statistical significance. Statistical analysis should be viewed as a tool that guides rather than determines the data interpretation. Lack of statistical significance should not be the sole rationale for concluding a lack of treatment related effect, just as statistical significance should not be the sole justification for concluding a treatment-related effect. To guard against possible false negative findings and the inherent difficulties in 'proving a negative'', available positive and historical control data should be discussed, especially when there are no treatment-related effects. The evaluation should include the relationship, if any, between observed neuropathological and behavioural alterations.

50.   All results should be analyzed using statistical models appropriate to the experimental design. The choice of a parametric or a nonparametric analysis should be justified by considering factors such as the

nature of the data (transformed or not) and their distribution, as well as the relative robustness of the statistical test, etc. The study purpose and design should guide the choice of statistical analyses to minimize Type I (false positive) and Type II (false negative) (77)(78). Developmental studies using multiparous species testing multiple pups per litter must include litter in the statistical model to guard against an inflated Type I error rates (79)(80)(81)(82) . Experiments should be designed such that littermates are not treated as independent observations. Any endpoint repeatedly measured in the same subject should be analyzed using statistical models that account for the non-independence of those measures.

Test report

47.   The test report must include the following information:

Test substance:
- physical nature and, where relevant, physiochemical properties;
- identification data, including source;
- purity of the preparation, and known and/or anticipated impurities

Vehicle (if appropriate):
- justification for choice of vehicle, if other than water or physiological saline solution

Test animals:
- species and strain used
- supplier of test animals
- number, age at start, and sex of animals;
- source, housing conditions, diet, etc.;
- individual weights of animals at the start of the test.

Test conditions:
- rationale for dose level selection;
- rationale for dosing route and period;
- specifications of the doses administered, including details of the vehicle, volume and physical form of the material administered;
- details of test substance formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation;
- details of the administration of the test substance;
- conversion from diet/drinking water or inhalation test substance concentration (ppm) to the actual dose (mg/kg body weight/day), if applicable;
- environmental conditions;
- details of food and water quality.
- dates of study start and end

Observations and test procedures:
- a detailed description of the procedures used to standardize observations and procedures as well as operational definitions for scoring observations;
- a list of all test procedured used;
- details of the behavioural/functional, pathological, neurochemical or electrophysiological procedures used, including information and details on automated devices;
- procedures for calibrating and ensuring the equivalence of devices and the balancing of treatment groups in testing procedures;
- a short justification explaining any decisions involving professional judgement.

Results (individual and summary):
- the number of animals at the start of the study and the number at the end of the study
- the number of animals used for each test method;
- identification number of each animal and the litter from which it came;
- litter size and mean weight at birth by sex;
- body weight and body weight change data, including terminal body weight for dams and offspring;
- food consumption and water consumption if appropriate (e.g., if chemical is administered through diet or water);
- toxic response data by sex and dose level, including signs of toxicity or mortality, including time and cause of death, if appropriate;
- nature, severity, duration, day of onset, time of day, and subsequent course of the detailed clinical observations;
- score on each developmental landmark (weight, sexual maturation and reflex ontogeny) at each observation time;
- a detailed description of all behavioural, functional, neuropathological, neurochemical, electrophysiological findings, including both increases and decreases from controls;
- necropsy findings;
- brain weights;
- any diagnoses derived from neurological signs and lesions, including naturally-occurring diseases or conditions;
- absorption and metabolism data, including complementary data from a separate toxicokinetic study, if available;
- statistical treatment of results, including: statistical models used to analyze the data, and the results, regardless of whether they were significant or not.
- list of study personnel, including professional training

Discussion of results:
- dose response information;
- relationship of any other toxic effects to a conclusion about the neurotoxic potential of the test chemical;
- impact of any toxicokinetic information on the conclusions
- similarities of effects to any known neurotoxicants
- relationships, if any, between neuropathological and functional effects
- no-observed-adverse effect level or benchmark dose for dams and offspring.

Conclusions:
- a discussion of the overall interpretation of the data based on the results, including a conclusion of whether or not the chemical caused developmental neurotoxicity.

LITERATURE

(1)   Organisation for Economic Co-operation and Development (1995) Draft Report of the OECD *Ad Hoc* Working Group on Reproduction and Developmental Toxicity. Copenhagen, Denmark, 13th-14th June 1995.

(2)   U.S. EPA (1998) U.S. Environmental Protection Agency Health Effects Test Guidelines. OPPTS 870.6300. Developmental Neurotoxicity Study. EPA Document 712-C-98-239. Washington, DC:U.S. Environmental Protection Agency.

(3)   Organisation for Economic Co-operation and Development (1996) Final Report of the Consultation Meeting on Developmental Neurotoxicity. Copenhagen, Denmark, 17th-18th June 1996.

(4)   USEPA (1998). Developmental Neurotoxicity Study. OPPTS 870.6300.   USEPA/OPPTS, Washington, DC. EPA 712-C-98-239. 14 pp.

(5)   Cory-Slechta DA, Crofton KM, Foran JA, Ross JF, Sheets LP, Weiss B, Mileson B. Methods to identify and characterize developmental neurotoxicity for human health risk assessment. I: behavioral effects. Environ Health Perspect. 109 (Suppl 1):79-91(2001).

(6)   Dorman DC, Allen SL, Byczkowski JZ, Claudio L, Fisher JE Jr, Fisher JW, Harry GJ, Li AA, Makris SL, Padilla S, Sultatos LG, Mileson BE. Methods to identify and characterize developmental neurotoxicity for human health risk assessment. III: pharmacokinetic and pharmacodynamic considerations. Environ Health Perspect. 109 (Suppl 1):101-111 (2001).

(7)   Garman RH, Fix AS, Jortner BS, Jensen KF, Hardisty JF, Claudio L, Ferenc S. (2001) Methods to identify and characterize developmental neurotoxicity for human health risk assessment II: Neuropathology. Environmental Health Perspectives 109 (Suppl 1):93-100.

(8)   OECD (2003) Report of the Expert Consultation Meeting in Developmental Neurotoxicity Testing. Washington, US, 23-25 October 2000.

(9)   Kimmel, C. A., Rees, D. C., and Francis, E. Z. (1990) Qualitative and quantitative comparability of human and animal developmental neurotoxicity. Neurotox. Teratol. (Special Issue), 12: 173-292.

(10)   Tilson, H. A. and Cranmer, J. M. (1986) Neurotoxicology in the fetus and child, Neurotoxicology 7: 1.

(11)   Spencer,P.S., Schaumburg,H.H. and Ludolph, A.C. (eds), *Experimental and Clinical Neurotoxicology, 2nd Edition,* ISBN: 0195084772 Oxford University Press, New York, March 2000

(12)   Mendola P, Selevan SG, Gutter S, Rice D. Environmental factors associated with a spectrum of neurodevelopmental deficits. Ment Retard Dev Disabil Res Rev 2002;8(3):188-197.

(13)   Slikker,W.B. and Chang,L.W. (eds), *Handbook of Developmental Neurotoxicology*, Hardcover, 1st ed., 748pp., ISBN: 0126488606, Academic Press, January 1998

(14)   OECD (1997). Test Guideline 424: Neurotoxicity Study in Rodents

(15)   World Health Organization (WHO) (1986) *Principles and Methods for the Assessment of Neurotoxicity Associated with Exposure to Chemicals*, (Environmental Health Criteria 60), Albany, New York: World Health Organization Publications Center USA.

(16)   World Health Organization (WHO) (2001) *Neurotoxicity Risk Assessment for Human Health: Principles and Approaches,* (Environmental Health Criteria 223), World Health Organization Publications, Geneva.

(17)   Chang.L.W. and Slikker,W. (1995), Neurotoxicology: Approaches and Methods, Hardcover, 1st ed., 851pp., ISBN: 012168055X, Academic Press, Inc.

(18) De Cabo, C., Viveros, M.P. (1997) Effects of neonatal naltrexone on neurological and somatic development in rats of both genders. Neurotoxicology and Teratology 19 (6) 499-509.

(19) Agnish ND, Keller KA. The rationale for culling of rodent litters. Fundam Appl Toxicol. 1997 Jul;38(1):2-6.

(20) Avery, D.L. and Spyker, J.M. (1977) Foot tattoo of neonatal mice. Lab. Animal Sci. 27:110-112.

(21) Wier, P.J., Guerriero, F.J. and Walker, R.F. (1989) Implementation of a primary screen for developmental neurotoxicity. Fundam. Appl. Toxicol. 13:118-136.

(22) Spear, N.E. and Campbell, B.A. (eds.) (1979) *Ontogeny of Learning and Memory*. New Jersey: Erlbaum.

(23) Krasnegor, N.A. (ed.) (1986) *Perinatal Development: A Psychobiological Perspective*. Orlando: Academic Press.

(24) Zoetis,T. and Walls,I., (eds). Principles and Practices for Direct Dosing of Pre-Weaning Mammals in Toxicity Testing and Research. ILSI Press, Washington, DC (in press).

(25) ICH (1993) ICH Harmonised Tripartite Guideline: Detection of Toxicity to Reproduction for Medical Products. Intenational Conference on Harmonization of Technical Requirements for Registration of Phamaceutical for Human Use.

(26) Lochry,E.A. (1987) Concurrent use of behavioral/functional testing in existing reproductive and developmental toxicity screens: Practical considerations. J. Amer. Coll. Toxicol., 6:433-439.

(27) Adams, J. Buelke-Sam, J., Kimmel, C.A., Nelson, C.J., Reiter, L.W., Sobotka, T.J., Tilson, H.A., Nelson, B.K. (1985) Collaborative behavioral teratology study: Protocol design and testing procedure. *Neurobehavioral Toxicology and Teratology* 7:579-586.

(28) Korenbrot, C.C., Huhtaniemi, I.T., Weiner, R.W. (1977) Preputial separation as an external sign of pubertal development in the male rat. *Biology of Reproduction* 17:298-303.

(29) Spear, L.P. (1990) Neurobehavioral assessment during the early postnatal period. Neurotoxicol. Teratol.. 12(5):489-95.

(30) Altman, J. and Sudarshan, K. (1975) Postnatal development of locomotion in the laboratory rat. *Animal Behavior* 23:896-920.

(31) Adams,J. (1986) Methods in behavioural teratology. In: Handbook of Behavioural Teratology. Riley, E.P. and Vorhees, C.V. (Eds), New York Plenum Press, pp. 67-100.

(32) Reiter, L.W. and MacPhail, R.C. (1979) Motor activity: A survey of methods with potential use in toxicity testing. *Neurobehaviorial Toxicology* 1: Suppl. 1, pp. 53-56.

(33) Robbins, T.W. (1977) A critique of the methods available for the measurement of spontaneous motor activity, *Handbook of Psychopharmacology*, Vol. 7, Eds. Iverson, L.L., Iverson, D.S., Snyder, S.H. New York: Plenum Press. pp. 37-82.

(34) Crofton, K.M., Peele, D.B., Stanton, M.E. (1993) Developmental neurotoxicity following neonatal exposure to 3,3'-iminodipropionitrile in the rat. *Neurotoxicology and Teratology* 15:117-129.

(35) Ruppert, P.H., Dean, K.F. and Reiter, L.W. (1984) Development of locomotor activity of rat pups in figure-eight mazes. Develop. Psychobiol. 18:247-260.

(36) Crofton, K.M., Howard, J.L., Moser, V.C., Gill, M.W., Reiter, L.W., Tilson, H.A., MacPhail, R.C. (1991) Interlaboratory comparison of motor activity experiments: Implications for neurotoxicological assessments. Neurotoxicol. Teratol. 13:599-609.

(37) Handley, D.E. and Ross, J.F. (1998) A force plate system for measuring low-magnitude reaction forces in small laboratory animals. *Physiol. Behav.* 64(5):661-669.

(38) Crofton, K.M. (1992). Reflex modification and the assessment of sensory dysfunction. In Neurotoxicology, (H.Tilson and C. Mitchell, Eds.). Pp. 181-211. Raven Press, Ltd., New York.

(39) Mattsson, J.L., Boyes, W.K. and Ross, J.F. (1992) Incorporating evoked potentials into neurotoxicity test schemes. In: *Target Organ Toxicology Series: Neurotoxicity*, ed. by H.A. Tilson and C.L. Mitchell, Raven Press, pp. 125-145.

(40) Edwards, P.M. and Parker, V.H. (1977) A simple, sensitive, and objective method for early assessment of acrylamide neuropathy in rats. *Toxicol. Appl. Pharmacol*. 40:589-591.

(41) Bushnell, P.J., Kelly, K.L., Crofton, K.M. (1994) Effects of toluene inhalation on detection of auditory signals in rats. *Neurotoxicology and Teratology* 16:149-160.

(42) Crofton, K.M. and Sheets, L.P. (1989) Evaluation of sensory system function using reflex modification of the startle response. *Journal of the American College of Toxicology* 8:199-211.

(43) Crofton, K.M., Lassiter, T.L., Rebert, C.S. (1994) Solvent-induced ototoxicity in rats: An atypical selective mid-frequency hearing deficit. *Hearing Research* 80:25-30.

(44) Pryor, G.T., Dickinson, J., Howd, R.A., Rebert, C.S. (1983) Transient cognitive deficits and high-frequency hearing loss in weanling rats exposed to toluene. *Neurobehavioral Toxicology and Teratology* 5:53-57.

(45) Stanton, M.E. and Freeman, J.H. (1994) Eyeblink conditioning in the infant rat: An animal model of learning in developmental neurotoxicology. *Environmental Health Perspectives* 102:131-139.

(46) Ison, J.R. (1984) Reflex modification as an objective test for sensory processing following toxicant exposure. *Neurotoxicology and Teratology* 6:437-445.

(47) Peele, D.B., Allison, S.D., and Crofton, K.M. (1990) Learning and memory deficits in rats following exposure to 3,3'-iminopropionitrile. *Toxicology and Applied Pharmacology* 105:321-332.

(48) Bushnell, P.J. (1988) Effects of delay, intertrial interval, delay behavior and trimethyltin on spatial delayed response in rats. *Neurotoxicology and Teratology* 10:237-244.

(49) Green, R.J. and Stanton, M.E. (1989) Differential ontogeny of working memory and reference memory in the rat. *Behavioral Neuroscience* 103:98-105.

(50) Kucharski, D. and Spear, N.E. (1984) Conditioning of aversion to an odor paired with peripheral shock in the developing rat. *Developmental Psychobiology* 17:465-479.

(51)   Akaike, M., Ohno, H., Tsutsumi, S., Omusu, M. (1994) Comparison of four spatial maze learning tests with methylnitrosourea-induced microcephaly rats. *Teratology* 49:83-89.

(52)   Hass, U., Lund, S.P., Simonsen, L., Fries, A.S. (1995) Effects of prenatal exposure to xylene on postnatal development and behavior in rats. *Neurotoxicology and Teratology* 17:341-349.

(53)   Kelly, S.J., Goodlett, C.R., Hulsether, S.A., West, J.R. (1988) Impaired spatial navigation in adult female but not adult male rats exposed to alcohol during the brain growth spurt. *Behaviorial Brain Research* 27:247-257.

(54)   Morris, R. (1984) Developments of a water-maze procedure for studying spatial learning in the rat. *Neurology Science Methods* 11:47-60.

(55)   Akaike, M. (1988) Impaired Biel and Radial arm maze learning in rats with methyl-nitrosurea induced microcephaly. *Neurotoxicology and Teratology* 10:327-332.

(56)   Cory-Slechta, D.A., Weiss, B., Cox, C. (1983) Delayed behavioral toxicity of lead with increasing exposure concentration. *Toxicology and Applied Pharmacology* 71:342-352.

(57)   Miller, D.B. and Eckerman, D.A. (1986) Learning and memory measures. In: *Neurobehavioral Toxicology*, Annau, Z. (ed). Baltimore: Johns Hopkins University Press, pp. 94-149.

(58)   Luna, L.G. (1968) *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology*. (Third Edition) New York: McGraw Hill.

(59)   Sheehan DC, Hrapchak BB. (1980) Theory and Practice of Histotechnology - 2nd Edition, CV Mosby Co., St. Louis

(60)   Fix AS, Garman RH (2000) Practical aspects of neuropathology: A technical guide for working with the nervous system. Toxicologic Pathology 28: 122-131

(61)   Geinisman Y, Gundersen HJG, Van Der Zee E, West MJ. (1997) Towards obtaining unbiased estimates of the total number of synapses in a brain region: problems of primary and secondary important. Journal of Neurocytology 26:711-713.

(62)   Howard CV, Reed MG. (1998) Unbiased Stereology: Three-Dimensional Measurement in Microscopy. Springer-Verlag, NewYork .

(63)   Hyman BT, Gomez-Isla T, Irizarry MC. (1998) Stereology: a practical primer for neuropathology. J.Neuropath. Exp. Neurol. 57: 305-310

(64)   Korbo L, Andersen, BB, Ladefoged O, Møller A. (1993) Total numbers of various cell types in rat cerebellar cortex estimated using an unbiased stereological method. Brain Res. 609: 262-268

(65)   Schmitz C. (1997) Towards more readily comprehensible procedures in disector stereology. J. Neurocytol. 26:707-710.

(66)   West MJ. (1999) Stereological methods for estimating the total number of neurons and synapses: issues of precision and bias. Trends Neurosci. 22:51-61

(67) Gavin CE, Kates B, Gerken LA, Rodier PM. (1994) Patterns of growth deficiency in rats exposed in utero to undernutrition, ethanol, or the neuroteratogen methylazoxymethanol (MAM). Teratology 49:113-121.

(68) Goodlett CR, Leo JT, O'Callaghan JP, Mahoney JC, West JR. (1993) Transient cortical astrogliosis induced by alcohol exposure during the neonatal brain growth spurt in rats. Developmental Brain Research 72: 85-97.

(69) Ohno M, Aotani H, Shimada M. (1995) Glial responses to hypoxic/ischemic encephalopathy in neonatal rat cerebrum. Develop. Brain Res. 84:294-298.

(70) Jensen KF, Catalano SM. (1998) Brain morphogenesis and developmental neurotoxicology. Chapter 1 in Handbook of Developmental Neurotoxicology, eds. W. Slikker, Jr. and L.W. Chang. Academic Press, New York, c. pp. 3-41.

(71) Ikonomidou C, Bosch F, Miksa M, Bittigau P, Vöckler J, Dikranian K, Tenkova TI, Stefovska V, Turski L, Olney JW. (1999) Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. Science 283:70-74.

(72) Ikonomidou C, Bittigau P, Ishimaru, MJ, Wozniak DF, Koch C, Genz K, Price MT, Sefovska V, Hörster F, Tenkova T, Dikranian K, Olney JW. (2000) Ethanol-induced apoptotic degeneration and fetal alcohol syndrome. Science 287:1056–1060

(73) Bennet, H.S., Wyrick, A.D., Lee, S.W., McNeil, J.H. (1976) Science and art in the preparing tissues embedded in plastic for light microscopy, with special reference to glycol methacrylate, glass knives and simple stains. *Stain Technology* 51:71-97.

(74) Tilson, HA; MacPhail, RC; Crofton, KC. (1996) Setting exposure standards: a decision process. Environ Health Perspect 104:401-405.

(75) US EPA (2003) Draft Final Guidelines For Carcinogen Risk Assessment (External Review Draft, February 2003). Other NCEA-F-0644A. 03 Mar 2003. U.S. Environmental Protection Agency, Risk Assessment Forum, Washington, DC, 125 pp.

(76) US EPA (1998) Guidelines For Neurotoxicity Risk Assessment. USEPA 630/R-95/001F. 30 Apr 1998. U.S. EPA, Risk Assessment Forum, Washington, DC, 90 pp.

(77) Muller, K.E., Barton, C.N. and Benignus, V.A. (1984). Recommendations for appropriate statistical practice in toxicologic experiments. *NeuroToxicology* 5 (2): 113-126.

(78) Gad, S.C. (1989) Principles of screening in toxicology with special emphasis on applications to Neurotoxicology. J. Amer. Coll. Tox. 8;21-27.

(79) Abby,H. and Howard,E. .(1973) Statistical procedures in developmental studies on a species with multiple offspring. Dev. Psychobiol. 6:329-335.

(80) Haseman, J.K. and Hogan, M.D. (1975). Selection of the experimental unit in teratology studies. *Teratology* 12: 165-172.

(81)   Holson, R.R., and Pearce, B. (1992). Principles and pitfalls in the analysis of prenatal treatment effects in multiparous species. *Neurotox.Teratol.* 14: 221-228.

(82)   Nelson CJ, Felton RP, Kimmel CA, Buelke-Sam J, Adams J. (1985) Collaborative Behavioral Teratology Study: statistical approach. Neurobehav Toxicol Teratol. 1985 Nov-Dec;7(6):587-90.

Additional References, not referred to in the text but relevant as background information:

(A)   Alleva E., and Sorace A. Important hints in behavioural teratology of rodents. Current Pharmaceut. Design 6:99-126 (2000).

(B)   Bignami G. Economical test methods for developmental neurobehavioral toxicity. Environ. Health Perspect. 104: 285-298 (1996).-

(C)   Buelke-Sam J, Kimmel CA, Adams J, Nelson CJ, Vorhees CV, Wright DC, St Omer V, Korol BA, Butcher RE, Geyer MA, Holson JF, Kutscher CL, Wayner MJ. Collaborative Behavioral Teratology Study: results. Neurobehav Toxicol Teratol. 1985 Nov-Dec;7(6):591-624.

(D)   Cox, C. (1994). Statistical issues for animal studies of developmental neurotoxicity. In: *Neurobehavioral Toxicity: Analysis and Interpretation*, Weiss, B. and O'Donoghue, J. (eds.). New York: Raven Press, pp. 93-101.

(E)   Rodier PM, Gramann WJ. (1979) Morphologic effects of interference with cell proliferation in the early fetal period. Neurobehav. Toxicol. 1:129-135.

(F)   Rodier PM. (1998) Structural-functional relationship in experimentally induced brain damage. Prog. Brain Res. 73:335-348

(G)   Ruppert, P.H., Dean, K.F. and Reiter, L.W. (1985) Development of locomotor activity of rat pups exposed to heavy metals, Toxicol.Appl. Pharmacol. 78:69-77.

(H)   Weil, C. S. (1970) Selection of the valid number of sampling units and a consideration of their combination in toxicological studies involving reproduction, teratogenesis or carcinogenesis. Food Cosmet. Toxicol. 8:177-182.

APPENDIX A.

Figure 1: Example of the testing scheme for assignment of animals for functional/behavioural tests, neuropathology evaluation, and brain weights, as described in paragraphs 13, 14, 15. (PND = postnatal day).

20