BACKGROUND

The National Toxicology Program (NTP) has developed a new flexible study design, termed the modified one generation (MOG) study that enables the assessment of high-quality Developmental and Reproductive Toxicity (DART) studies alongside obtaining information on doses and target organ toxicity for a perinatal cancer bioassay (Foster, 2014). This design offers a unique and pragmatic approach to assess various developmental windows of susceptibility in diverse end-points in littermates following perinatal-postnatal exposure to a chemical or an environmental toxicant using animals that have already been generated for the cancer assessment. In the MOG, time-mated females are typically administered the test material from gestation day (GD) 6 through weaning at postnatal day (PND) 28. The subsequent F1 offspring are then continuously administered the test article through adulthood via the same route of exposure as the dams. F1 animals after PND 4 can be allocated to various cohorts or “cassettes” and are assessed on different end-points throughout the study and for gross and histopathology examination. The cassettes in a typical MOG include (i) sub-chronic cohort (ii) teratology cohort and (iii) breeding and littering cohort.

The purpose of this document is to provide an outline to include an additional cassette to the MOG, that we term the developmental neurotoxicity testing (DNT) arm. The DNT arm is designed to use animals that are already being generated in the study to comprehensively assess potential toxicant-related adverse effects of peri- and postnatal exposure on the development and function of the nervous system that are currently not examined in any other cassette of the MOG.

The battery of tests mainly captures neurobehavioral and neuropathologic effects in offspring during sensitive periods of neurologic development and during adulthood. Although the DNT-arm has been adapted to the MOG paradigm, at minimum, it incorporates most of the key features of current DNT Guidelines (EPA- DNT Test guidelines and OECD 426), thereby being a valuable source of quantitative and qualitative information for risk assessment.

This document provides a conceptual framework and rationale for the studies in the DNT-arm of the MOG. It is not designed to provide in-depth specifications for the conduct of these studies.

THE DNT-ARM OF THE MOG: DESIGN

This design comprises 2 interrelated parts: (I) The Dose-Range Finder (DRF) (II) DNT-Arm of the MOG

I. The Dose-Range Finding Study (DRF: Dosing GD 6 - PND 28)

The primary purpose of the DRF is to determine the maximal dose level that is tolerated by the dam (dosing GD6 - PND 28) through pregnancy, littering and weaning that has minimal to no impact on pup survival. Although this is typically the main driver for dose-selection in the subsequent MOG phase, there may be cases where certain neurotoxicity end-points provide additional useful information or may be dose-limiting. For example, continual seizures in the dam may preclude selection of a certain dose for the subsequent MOG. Currently, clinical observations are the only end-points for indicators of neurotoxicity in the DRF. However, there may be potential early indications of regional neuropathological alterations in the brain at high doses, which may not be reflected by clinical
observations. This, in turn, may trigger the need for a more in-depth assessment of these specific brain regions in the subsequent MOG (e.g. special staining).

Based on this rationale, a limited neurohistopathological examination of the brain has been added to the DRF with the recognition that in most cases, these end-points will likely not drive dose-setting in the subsequent MOG, but instead, may contribute towards refining the DNT-arm of the main MOG. The brains will be examined in 6 controls and 6 surviving high-dose PND 28 offspring (3 males and 3 females from different litters). For the assessment, transverse brain sections will be used except for cerebellum, which will be sagittal. The two stains which will be used are (i) hematoxylin and eosin (H & E), which is the routine first-tier screening for NTP studies, and (ii) the Kluver and Barrera stain (Luxol Fast Blue/cresyl violet) stain to assess patterns of myelination during development (Figure 1).

Following the assessment of a limited number of studies, there will be a comprehensive evaluation and a decision as to whether including routine limited neuropathological assessment is a valuable addition to all DRFs in NTP studies.

Figure 1. Brain section stained with Kluver-Barrera (Luxol fast blue and Cresyl violet) stain for the simultaneous evaluation of myelin (stains blue) as well as Nissl substance (stains purple) within the neuronal cell body. The luxol fast blue provides information about the myelination status within the neuronal tissues. The cresyl violet assesses the overall cellularity (neurons and glia) within the brain. In addition, the Kluver-Barrera stain also identifies lipofuscin (stains steel blue) accumulated within the aging neuronal bodies, provides a good contrast between certain neuronal sub-sites, and aids in simple linear morphometry of the brain.
II. The Developmental Neurotoxicity Testing (DNT) arm (in red) of the MOG (PND 28 - ~ PND 100)

The DNT-arm has been adapted to the MOG using current DNT Guideline studies as a reference in an effort to identify neurotoxic effects of developmental chemical exposures using neurobehavioral assays and neuropathology, which are currently not being assessed, by other cassettes. In the MOG design, timed-mated female rats are dosed with the test article with an appropriate control starting GD 6 continuously through pregnancy and weaning with at least 20 litters/group. The subsequent F1 offspring are then continuously administered with the test article via the same route of exposure as the dams. The F1 pups shall be culled to 4 males and 4 females per dose group/litter on PND 4. With the addition of the DNT-arm, one male and one female may be assigned to up to four cassettes (i) sub-chronic (ii) DNT (iii) teratology and (iv) breeding and littering to obtain a total of 20 animals/sex/group (1/litter) per cassette with one exception. Of the 20 M and 20 F from the sub-chronic cohort, 10M and 10F shall be assigned to the DNT cohort for neurohistopathology assessment at PND 28 (indicated by blue arrow and text in the figure above). Figure 2 below provides a schematic representation of the DNT- Arm of the MOG. The major end-points that will be assessed in the DNT-arm are listed in Table 1.

Schematic of the MOG with maximum possible cohorts

Timed - Pregnant Female HSD Rats - 3 dose groups + control
Continuous dosing (start with ~25 dams/dose group - calculations based on obtaining 20 viable dams)

Figure 2: Schematic of the MOG phase for routine DNT Assessment. One male and one female from each litter (to obtain a total of 20 M and 20 F) shall be recruited to each of the above cohorts (sub-chronic, DNT, teratology, Breeding/Littering) with one exception. Of the 20 M and 20 F from the sub-chronic cohort, 10M and 10F shall be assigned to the DNT cohort for neurohistopathology assessment at PND 28 (indicated by blue arrow and text in the figure above). Note: Cohort selection for the MOG is to be determined by the Study Design Team. Abbreviations: G= gestational phase; L = Lactational phase; M = Mating; GD = Gestational Day; PND = Postnatal Day
Table 1: Timing of assessment of physical and developmental landmarks and neurobehavioral end-points (adapted from OECD 426)

<table>
<thead>
<tr>
<th>Assessment</th>
<th>Endpoint</th>
<th>Pre-Weaning</th>
<th>Weaning</th>
<th>Adults</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLINICAL OBSERVATIONS AND HISTOPATHOLOGY</td>
<td>Clinical Observations &amp; Body Weight</td>
<td>As appropriate</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>All animals</td>
<td>As appropriate</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Detailed Clinical Observations</td>
<td></td>
<td>As appropriate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20/sex (1/sex/litter)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Brain weight &amp; Neurohistopathology</td>
<td>~PND 28*</td>
<td>Study termination</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10/sex (1/sex/litter) (PND 28)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20/sex (1/sex/litter) (TSAC)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sexual Maturation (all pups)</td>
<td></td>
<td></td>
<td>As appropriate</td>
</tr>
<tr>
<td>NEUROBEHAVIORAL END-POINTS</td>
<td>Motor Activity (Including habituation)</td>
<td>~PND 28*</td>
<td>~PND 60-70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20/sex (1/sex/litter)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Motor and Sensory Function</td>
<td>~PND 28*</td>
<td>~ PND 60-70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Prepulse Inhibition of Auditory Startle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20/sex (1/sex/litter)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Learning &amp; Memory</td>
<td></td>
<td></td>
<td>~ PND 60-70</td>
</tr>
<tr>
<td></td>
<td>Morris Water Maze</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20/sex (1/sex/litter)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

* post-weaning testing of pups should not occur during the two days after weaning (Assessment marked as PND 28 will occur on PND 31-33)
Clinical Observations

All dams and offspring will be carefully observed at least daily for signs of toxicity, morbidity and mortality similar to that in the DNT Guideline studies (OECD 426).

Detailed Clinical Observations in lieu of the Functional Observational Battery (FOB)

DNT-guideline documents suggest the incorporation of a functional observational battery (FOB) (Moser, 1990; 2000), which are an adaptation of adult neurotoxicity screening (EPA 1998). However, recent review of studies, which were performed in accordance with the DNT- guidelines, revealed that the FOB was not found to be effective in capturing indicators of neurotoxicity (Raffaele et al., 2010, Graham et al., 2012). At best, it appears to be a collection of observational methods that do not resemble a neurological exam or even the best practices for measuring the parameters it includes. The assessments were found to be largely subjective and were about functions of little or unknown importance to brain integrity, except at the extremes (convulsions or tremors), which can be identified by clinical observations (Graham et al., 2012).

Hence, the NTP will conduct detailed clinical observations in lieu of a FOB during the treatment and observation periods, periodically on twenty dams per dose group (at least twice during the gestational dosing period and twice during the lactational dosing period) and in the offspring (at least one pup/sex/litter). The animals will be observed at least daily outside the home cage by trained technicians who are unaware of the animals’ treatment, using standardized procedures to minimize animal stress and observer bias, and maximize inter-observer reliability. The presence of observed signs will be recorded. Whenever feasible, the magnitude of the observed signs will also be recorded.

Some examples of clinical observations include, but are not limited to, changes in skin, fur, eyes, mucous membranes, occurrence of 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). 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, tremors, stereotypies (e.g., excessive grooming, unusual head movements, repetitive circling), bizarre behavior (e.g., biting or excessive licking, self-mutilation, walking backwards, vocalization), or aggression will be recorded.

Neurohistopathology

Neurohistopathological evaluation and brain weight measurements shall be conducted on PND 28 and study termination (~PND 90-100). For offspring terminated at PND 28, only brain tissues will be evaluated; for animals killed at termination, both central nervous system (CNS) tissues and peripheral nervous system (PNS) tissues will be evaluated. Brains of animals killed on PND 28 will be immersion fixed. Animals killed at study termination will be perfusion fixed per NTP DNT specifications. All aspects of the preparation of tissue samples, from the perfusion of animals, through the dissection of tissue samples, tissue processing, and staining of slides will employ a counterbalanced design such that each batch contains representative samples from each dose group.
As a default, the following 2 stains shall be used:
(A) Kluver and Barrera stain (Luxol Fast Blue/cresyl violet) (Kluver and Barrera 1953); see Figure 1 for description
(B) Hematoxylin and Eosin (H & E)

Based on neurohistopathological and/or clinical observations (e.g. seizures) in the DRF, the need for additional stains shall be determined on a case-by-case basis. Some examples of alternate stains that may be used are listed in Table 2 below:

Table 2: List of stains to examine neuronal morphology (Nissl) and cell death (Fluoro-Jade), glial activation or hypertrophy (GFAP), or the role of inflammation (IBA1) in response to neuronal activity or injury.

<table>
<thead>
<tr>
<th>Stain</th>
<th>Cell type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thionine (Nissl)</td>
<td>Cell/neuron morphology</td>
</tr>
<tr>
<td>Fluoro-Jade (or silver stain)</td>
<td>Neuronal degeneration</td>
</tr>
<tr>
<td>Ionized calcium binding adaptor molecule (IBA1)</td>
<td>Microglia/macrophages</td>
</tr>
<tr>
<td>Glial fibrillary acidic protein (GFAP)</td>
<td>Astrocytes</td>
</tr>
<tr>
<td>Microtubule Associated Protein 2 (MAP-2)</td>
<td>Dendritic damage</td>
</tr>
</tbody>
</table>

Neurobehavioral end-points

The DNT guidelines require the following three major categories of neurobehavioral testing:

1. Motor Activity
2. Motor and Sensory Function
3. Learning and Memory

Hence, the DNT- arm of the MOG will include the following:

1. Motor Activity

   Spontaneous locomotor activity is generally considered a sensitive indicator of neuronal function, representing the peak of neural integration, which has been used for decades to evaluate effects of chemical and physical treatments (Tilson and Mitchell 1984). Measurements that are made in automated systems provide objective and quantitative data, and are required by the U.S. EPA and OECD test guidelines (EPA 1998, OECD 2007). There are many automated chambers commercially available, and detection systems include photocell based, field sensing, mechanical, or electronic/video tracking (Reiter and MacPhail 1982). Despite its advantages as a sensitive measure of nervous system effects, changes in motor activity cannot be attributed to a specific neuronal substrate (Moser et al., 2011).

   Motor activity in the DNT-arm of the MOG includes a measure of general activity level, and response and habituation to a novel environment. It will be assessed on ~PND 28 and PND 65±5. Activity will be assessed using an automated photocell device. Ambulatory activity, total activity, rearing, thigmotaxis (orientation of organism in response to stimulus), and pathway tracking (optional) of the animal will be recorded.
2. Motor and Sensory Function

Sensorimotor function is commonly measured by an animal’s motor-dependent startle response to a high intensity acoustic stimulus (pulse). Traditionally, the Acoustic Startle Response (ASR) was used to assess sensorimotor function in rodents but it can also be used in conjunction with prepulse inhibition (PPI). The ability of an organism to react to a stimulus less intensely when pre-exposed to a weaker stimulus is known as PPI and reflects sensorimotor gating. PPI is an important alert and orienting behavior to assess signals in the environment that may be relevant to the organism. PPI of the ASR is the prototypical assay to test for deficits in sensorimotor gating. The procedure comprises three main components: (i) the prepulse, (ii) startle stimulus, and (iii) startle reflex (Figures 4A). With the delivery of a prepulse, the brain will normally activate inhibitory mechanisms to diminish the response to a repetitive stimulus and thus reduce the amplitude of evoked responses to the second stimulus relative to the first (Figure 4B). This may be measured as startle amplitude (behavioral index) or the P20-N40 (positive wave at 20 ms followed by a negative deflection at 40 ms) auditory evoked potential (physiological index).

PPI was originally developed in human neuropsychiatric research as an operational measure for sensory gating (Braff et al., 1992). PPI deficits may represent the interface of “psychosis and cognition” as they seem to predict cognitive impairment (Van den Buuse et al., 2010; Geyer et al., 2006; Fenton et al., 2003). PPI is shown to be disrupted in patients suffering from schizophrenia as well as other mental and neurodegenerative diseases such as autism spectrum disorders (slower habituation), obsessive-compulsive disorder, Tourette's syndrome, Huntington's disease, Parkinson's disease, and Alzheimer's Disease (Swerdlow et al., 1995; Castellanos et al., 1996). PPI deficits can also be induced by many other psychomimetic drugs such as dopamine agonists (e.g. apomorphine) or NMDA antagonists (e.g. ketamine) (Mansbach et al., 2001), environmental modifications and surgical procedures (Valsamis et al., 2011). Hence, this test is designed to capture a broad spectrum of neurobehavioral anomalies relevant to humans.

Major brain circuitry proposed to be involved in PPI:
Limbic cortex, striatum, pallidum or pontine tegmentum "CSPP" circuitry (Swerdlow 2001)
A. B.

Figure 4: Pre-pulse startle (PPI) chamber (A) Apparatus and (B) Schematic Representation of PPI. In the PPI test, rodents are placed in small chamber on a platform that automatically records their startle responses such as acoustic startle amplitude, startle latency, auditory startle habituation, and prepulse startle inhibition.
3. Learning and Memory

Several tasks can be employed to examine cognitive function but there is no single test that can address all aspects of learning and memory. The DNT-guidelines have an option to choose from a number of tests such as passive avoidance, delayed-matching-to-position, olfactory conditioning, Morris water maze, Biel or Cincinnati maze, radial arm maze, T-maze, and acquisition and retention of schedule-controlled behaviour (OECD 426). Hence, it is critical to choose an appropriate test for learning and memory (L&M) that can capture a relatively complex L&M deficit, while at the same time can be run in a GLP testing paradigm.

Although ideally, a battery of tests would best capture L&M, this is not pragmatically feasible. In the case of unknown toxicants, there is little to no information apriori, thereby precluding the ability to select one test. Hence, the NTP has defaulted to using the Morris Water Maze (MWM) to test for Learning & Memory based on the following features:

(1) It does not require pretraining
(2) It is reliable across many procedural variations and a range of tank diameters
(3) It can be used for a number of species [rats, mice, and humans (virtual versions)]
(4) It appears not to be affected by treatment-induced motivational differences that are unrelated to learning (Vorhees et al., 2006).
(5) Largely unaffected by body mass differences
(6) Motivation is intrinsic (since animals find swimming stressful)
(7) While learning is rapid enough to accomplish the task, it is not excessively rapid thereby providing an opportunity to tease out learning deficits
(8) There is moderate interindividual variability with all the animals completing the task and approximately 90-100% mastering the task
(9) Can be used in a GLP- setting

The MWM has been validated with many drugs, neurotoxins, genetic mutations, lesions, infectious agents, and other variables. It is the most widely reported test of L&M in rodents in the scientific literature with well-documented reports.

Morris Water Maze (MWM): Maze learning is the most widely used task in behavioral neuroscience to assess acquisition of the task (learning) as well as working memory (short-term memory), long-term memory, and the ability to shift to learn a new task. Conceptually, the task derives from place cells that are neurons in the hippocampus, which identify or represent points in space in an environment (O'Keefe, 1976).

The MWM utilizes the adverse nature of water as a negative reinforcer to facilitate learning to escape via climbing onto a platform. The animal uses a number of spatial orientation features to identify the location of the submerged platform, which is a test of spatial learning/memory. Although the water maze is often described as if it were a single task, it is no more than an apparatus in which a variety of different tasks can be trained (Figure 5A). The simplest water escape learning task which involves learning to find a hidden platform in a single fixed location is often embedded into a series of sometimes quite complicated training and testing protocols to investigate specific theoretical issues. Distinct protocols engage different mechanisms of navigation, learning and memory. In the DNT- arm of the MOG, the animals will be trained and tested in the following:
**Non-Spatial Learning:** The animals will be trained for 2-3 days on **visible platform** to ensure swimming ability, basic vision, and to learn the platform is the goal.

**Spatial Learning:** In this task, the animals will be placed into the water at and facing the sidewalls of the pool, at different start positions across trials with spatial cues, and will need to learn to swim to the correct location where there will be a **hidden platform**. Over time, the animals learn to find the platform with decreasing escape latencies and more direct swim paths (Figure 5B). A tracking system will measure the gradually declining escape latency across trials, and parameters such as path-length, swim-speed, directionality in relation to platform location etc.

**Probe Trials:** During or after training is complete, the experimenter will conduct a probe trial in which the escape platform is removed from the pool and the animal will be allowed to swim for 60 sec. Typically, a well-trained rat will swim to the target quadrant of the pool and repeatedly across the former location of the platform until starting to search elsewhere (Figure 5C). Rats with lesions of the hippocampus and dentate gyrus, subiculum, or combined impairments do poorly in post-training probe tests (Morris et al., 1982, 1990; Sutherland et al., 1983).

**Reversal Learning:** In this phase, after one location has been thoroughly trained, the platform will be moved to a different quadrant of the pool. Because it is hidden, it is not apparent that anything has changed until the animal fails to find the platform in its usual place. Reversal learning reveals whether or not animals can extinguish their initial learning of the platform’s position and acquire a path to the new goal location.

*The MWM will be used as the default Learning and Memory test if no precluding factors exist.*

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**Figure 5:** Schematic Representation of the Morris Water Maze (A) General Set-Up, (B) Examples of paths and latencies during the task and (C) Examples of normal versus impaired paths on this task
**Passive Avoidance:** The passive avoidance task (or inhibitory avoidance) is a fear-aggravated test used to evaluate learning and memory in rodent models of CNS disorders. It is considered an aversive learning task that involves instrumental conditioning where the animal is given negative reinforcement (punished) for making a response. In this task, an aversive stimulus is conditional upon the behavior of the animal. The animal’s response (e.g. entering a dark compartment of a box when placed in an adjacent lighted compartment, or stepping down from a platform onto a grid floor) is followed by a foot shock (Figure 6). As a function of this response–stimulus pairing, the animal learns to avoid (or inhibit) making the response that was followed by the aversive experience as often indicated by freezing behavior (Grossman et al., 1975; McEchron et al., 2000; Nader et al., 2000; Nagel and Kimble, 1976).

**Active/Conditioned Avoidance:** In Conditioned Avoidance Response (CAR) experiments, a rat is placed in a two-compartment shuttle box and presented with a neutral conditioned stimulus (CS) such as a light or tone, followed after a short delay by an aversive unconditioned stimulus (US), such as a foot-shock (Figure 6). Learning is measured as escape latency, avoidance latency, or freezing and the lack of learning is normally measured by escape loses (this may also result in freezing behavior).

*Note: These tests for L&M will only be performed in cases that the MWM cannot be conducted. This will be determined by the study design team on a case-by-case basis*
SUMMARY

By incorporating the DNT-arm to the MOG design, we continue to support the ‘‘3Rs’’ through refining our toxicity study designs, replacing certain other standard toxicity studies by incorporating them into the MOG design and reducing overall animal use compared to conducting individual DART, 90-day toxicity and DNT studies. While some of the end-points from a traditional DNT Guideline study need to be adapted to the MOG paradigm (Table 3), the DNT- arm is designed to incorporate most of the key features of current DNT Guidelines studies thereby serving as a robust source of qualitative and quantitative information acceptable for risk assessment.

<table>
<thead>
<tr>
<th>End-Point</th>
<th>NTP’s DNT- Arm of MOG</th>
<th>DNT Guideline Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preferred Test Species</td>
<td>Rat (Harlan Sprague Dawley)</td>
<td>Rat</td>
</tr>
<tr>
<td>Dosing</td>
<td>GD 6- study termination*</td>
<td>GD 6- PND 21</td>
</tr>
<tr>
<td>Time of Weaning</td>
<td>PND 28</td>
<td>PND 21</td>
</tr>
<tr>
<td>Number and Sex of animals</td>
<td>20 litters/dose group</td>
<td>20 litters/dose group</td>
</tr>
<tr>
<td>Clinical Observations &amp; Body Weight</td>
<td>All animals</td>
<td>All animals</td>
</tr>
<tr>
<td>Detailed Clinical Observations</td>
<td>20/sex (1/sex/litter)</td>
<td>20/sex (1/sex/litter)</td>
</tr>
<tr>
<td>Brain Weight &amp; Neurohistopathology</td>
<td>PND 28 and study termination</td>
<td>PND 11-22 and study termination</td>
</tr>
<tr>
<td>Sexual Maturation</td>
<td>20/sex (1/sex/litter)</td>
<td>20/sex (1/sex/litter)</td>
</tr>
<tr>
<td>Behavioral Ontogeny</td>
<td>Separate FOB shall not be conducted **</td>
<td>FOB</td>
</tr>
<tr>
<td>Motor Activity</td>
<td>20/sex (1/sex/litter) pre-weaning and adult</td>
<td>20/sex (1/sex/litter), pre weaning and adult</td>
</tr>
<tr>
<td>Motor and Sensory Function</td>
<td>20/sex (1/sex/litter)</td>
<td>20/sex (1/sex/litter)</td>
</tr>
<tr>
<td>Learning and Memory</td>
<td>20/sex (1/sex/litter)</td>
<td>10/sex (1/sex/litter)#</td>
</tr>
</tbody>
</table>

Table 3. Comparison between the DNT-Arm of the MOG and Guideline DNT Studies (e.g. OECD 426). Adaptations to the OECD 426 Guidelines are in bold print.

*Although continuous dosing will be the default paradigm, the dosing schedule may be altered on a case-by-case basis should the study design team deem necessary based on the specific nomination and known/anticipated exposure pattern.

** Based on reviews evaluating the FOB in DNT Guideline studies and on conversations with experts in the field, it appears that the FOB was not found to be effective; there is large inter-experimenter and inter-laboratory variability thereby making it difficult to interpret the data. Hence, at this time the NTP will not be conducting a separate FOB. However, many of the indicators will be captured in the detailed clinical observations.

#Although the guideline studies require a minimum of 10 animals/sex, it states that “depending on the sensitivity of cognitive function tests, investigation of a large higher number of animals should be considered e.g., up to 1 male and 1 female per litter”.
OTHER ITEMS UNDER CONSIDERATION FOR CONTINUED IMPROVEMENT OF THE DNT-ARM OF THE MOG

The NTP’s proposed DNT-arm of the MOG is comparable to current DNT Guideline studies. Although the overall performance of the DNT guideline studies and its ability to detect effects of concern from a regulatory perspective have been well established, the recent increase in the number of regulatory DNT studies being conducted has refocused attention on this test method (Makris et al., 2009). Some of the concerns raised by critics are that variability of some end points (e.g., motor activity, morphometrics) is too great to be useful (Chemical Manufacturers Association 1987; Nolen 1985; York et al. 2004; Balls et al., 2005). Further retrospective reviews of control data have identified differences among laboratories in data quality and variability, suggesting methods to decrease variability (Crofton et al. 1991, 2004; Raffaele et al. 2003, 2005; Sette et al. 2004).

These diverse opinions do not invalidate the DNT study but rather highlight the need for ongoing scientifically based evaluation of this test method and the incorporation of appropriate revisions as scientific knowledge advances and as experience with the DNT study warrants (Makris et al., 2009). Hence, in a continued ongoing effort to refine the DNT-arm of the MOG, the NTP is focusing efforts on the following.

1) Automated Assessment of Motor Function

In earlier sections of this document, a measure of general locomotor activity in accordance with current DNT guidelines has been proposed. Although some aspects of motor function may be measured by motor activity, others such as coordination, equilibrium, strength and quantitative assessment of gait, which require smooth integration of both central and peripheral neurons may not be captured. Concerns raised by critiques in the field regarding the variability in motor activity and with regards to certain functional observational tests such as grip strength and footsplay, which are currently being used in DNT guideline studies (Makris et al., 2009).

In response to these concerns, the NTP is exploring a novel method of motor assessment, which provides an integrated assessment of motor function using a high-speed camera to image the ventral side of mice or rats as they walk on a motorized transparent treadmill belt with automated quantification of stance and swing components of stride. The following are some of the parameters that shall be assessed.

- Stride length
- Stance width
- Stance duration
- Swing duration
- Braking duration
- Propulsion duration
- Stride frequency
- Paw Angle

Anticipated advantages of automated gait analysis are as follows:
- Provides more objective output since it is digital compared with some of the more descriptive traditional assessments of motor function, and hence, can be used as a replacement for several traditional functional observational tests, which appear to have large inter-experimenter and inter-laboratory variability (e.g. Inking of paws, grip strength, footsplay, rotarod).
• Compared with the more traditional methods of assessing motor function which tend to be more subjective, automation is expected to decrease experimenter and laboratory variability, thereby resulting in more consistent and robust data.

*Status:* This system is currently under evaluation and may be incorporated in the assessment of motor function in subsequent studies

2) Molecular Markers

As a continued effort to improve neurotoxicity screening, another avenue that is currently being assessed is the potential to include some useful molecular biomarkers. As a first tier screening, it may be useful to assess whether exposure to the chemical is associated with general brain injury. For example, reactive gliosis is a hallmark response of the CNS to injury and comprises the activation of microglia and astrocytes (O'Callaghan, 1991; Norton et al., 1992; O'Callaghan, 1993; Raivich et al., 1999; Streit et al., 1999; Streit, 2000; McGraw et al., 2001; Norenberg, 2004; Sriram & O'Callaghan, 2004; Streit, 2004; Ladeby et al., 2005; O'Callaghan & Sriram, 2005; Streit et al., 2005).

Identification of *in vivo* biomarkers for reactive gliosis is a major advancement for monitoring disease progression of the CNS and to assess the effectiveness of therapeutic interventions (Chen et al., 2008). Specifically, one such marker, translocator protein 18 kDa (TSPO) (also known as the peripheral benzodiazepine receptor) is under evaluation by the NTP. Under normal physiological conditions, TSPO levels are low in the brain but they markedly increase at sites of brain injury and inflammation making it uniquely suited for assessing active gliosis using in vivo imaging modalities such as Positron Emission Tomography (PET) and radio immunoassays (RIAs) in experimental animals and humans (Chen et al., 2008).

*Status:* In addition to TSPO, the NTP is also exploring other molecular markers for screening including but not limited to markers of axonal injury and cell death such as S-100 (marker for reactive gliosis); SBDP150, 145 (markers for necrosis), SBDP 120 (marker for apoptosis) as well as CRMP-2 (marker for neuroregeneration) and synaptotamin-BDP (marker for synaptic damage).

3) Improvement in Data Capturing and Reporting System

Reviews of historical and positive control data from DNT studies have demonstrated the need for more standardized reporting requirements (Crofton et al. 2004, 2008; Makris et al 2009). In an effort to better capture data, the study design team is exploring the possibility of incorporating automated data capture and reporting systems.
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