LETTER TO THE EDITOR

Response to: Use of the Pup as the Statistical Unit in Developmental Neurotoxicity Studies: Overlooked Model or Poor Research Design?

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Regarding the “Letter to the Editor” from Drs Hardy and Stedeford concerning our article “Co-exposure of neonatal mice to a flame retardant PBDE 99 (2,2′,4,4′,5-pentabromodiphenyl ether) and methyl mercury enhances developmental neurotoxic defects.” The main comments and criticism are the use of the experimental design and its evaluation.

Although developmental toxicology is a relatively new science, it is firmly rooted in teratology. Consequently statistical evaluation is related to the situation where the mother is exposed and her fetus and offspring is exposed to a chemical via the placenta and the mother’s milk. In such cases, not only is the genetic variable important but so too is uptake and distribution of the chemical in the mother, placental transfer, maternal, and fetal metabolism, etc. Exposure to a chemical will in this way also occur during an undefined period of brain development. In order to predict when a chemical can exert its effect and to study its mechanistic effects it is essential to identify critical stages when chemical agents can be harmful. Gestation is divided into two main periods, the embryonic and the fetal. In humans the embryonic period constitutes 20% of the whole gestational period and the fetal period 80%. In research animals, such as mouse and rat, it is the converse, that is, the embryonic period constitutes 80% of gestation and the fetal period 20%. The rapid growth and development of the brain (Davison and Dobbing, 1968), “the brain growth spurt” (BGS) does not take place at the same time in all mammalian species. In the human, the BGS begins in the third trimester of pregnancy and continues throughout the first 2 years of life. In mouse and rat, the BGS is neonatal, spanning the first 3–4 weeks of life.

In our investigations in mice we identified a defined critical period during neonatal brain development when exposure to low doses of neurotoxic agents caused persistent neuro-behavioral and neurochemical aberrations. This critical window was around postnatal day 10. In studies using nicotine we reported that this agent causes permanent disturbances in the cholinergic nicotinic receptors and aberrant behavioral response to nicotine at adult age. This adult reaction to nicotine, a hypoactive response, was the opposite of that observed in control animals and in animals exposed to nicotine before or after the critical period. At no time during the neonatal period were low-affinity nicotine-binding sites (corresponding to α7) found in the cerebral cortex following nicotine treatment, but the persistence of the effect on receptors and adult behavioral response was evident only in adult mice exposed on postnatal days 10–14 (Eriksson et al., 2000). We have shown this defined critical window of susceptibility and induction of persistent effects on spontaneous behavior following exposure to nicotine (Eriksson et al., 2000), 1,1,1-trichloro-2,2-bis(chloropheny1)-ethane (DDT) (Eriksson et al., 1992), polychlorinated biphenyls (PCBs) (Eriksson 1998), diisopropylfluorophosphate (Ahlbom et al., 1995), PBDE 99 (Eriksson et al., 2002), and PBDE 209 (Viberg et al., 2003). Our studies demonstrated that the presence of any of these agents during this critical stage of development induced these persistent effects.

Our study design is as follows: Pregnant mice from a commercial breeder are randomly selected. Their offspring are individually treated with the parent substance during the neonatal period, which appears postnatally, and the mothers are left untreated. The mice are tested as adults after random selection from three to five different litters. In our analysis the mice are the experimental unit. In traditional behavioral teratology one uses either the mother or the litter as the statistical experimental unit. Such an experimental set-up is quite different from ours, as not only has the genetic influence to be considered but also the facts stated above and that the offspring are influenced by the behavior of the exposed mothers. In such cases “the litter generally should be considered the statistical unit” (page 7, paragraph 3, line 9, in Kimmel, C. A. (1990). Current status of behavioral teratology: Science and regulation. CRC Rev. Toxicol. 19:1–10). The papers that Drs Hardy and Stedeford quote are all related to developmental exposure of the mother. Actually their examples also used fetal weight as a variable to illustrate litter effects. Correction for litter effects is made so as not to overestimate the statistical evaluation, that is, perceive effects that are not truly statistically significant (increase in type 1 error).
Spontaneous Behavior of Adult Mice Exposed Neonatally to PBDE 99

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of litter</th>
<th>No. of mice</th>
<th>0–20 min</th>
<th>20–40 min</th>
<th>40–60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Three pups per litter</td>
<td>3</td>
<td>9</td>
<td>561 ± 85</td>
<td>299 ± 52</td>
<td>1 ± 3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>9</td>
<td>300 ± 49**</td>
<td>237 ± 40</td>
<td>307 ± 47**</td>
</tr>
<tr>
<td>One pup per litter</td>
<td>9</td>
<td>9</td>
<td>613 ± 99</td>
<td>294 ± 47</td>
<td>7 ± 10</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>9</td>
<td>273 ± 37**</td>
<td>246 ± 42</td>
<td>299 ± 56**</td>
</tr>
</tbody>
</table>

*Note.* The use of randomly selected individuals as a statistical unit, compared with the litter. Pregnant NMRI mice were randomly selected and purchased from a commercial breeder. From 18 different litters, pups of both sexes, aged 10 days were given either one single oral dose of the brominated flame-retardant, PBDE 99 14 μmol (8 mg)kg bw, or a vehicle (20% fat emulsion). Spontaneous motor behavior was studied in 2-month-old male mice. Statistical evaluation was made by using both the litter (n = 9, one pup per litter) as a statistical unit and randomly selected individuals (n = 9, three mice randomly selected from three randomly chosen litters). The locomotion data over three consecutive 20-min periods (Treatment, Time, and Treatment × Time; between subjects, within-subjects and interaction factors, respectively), were submitted to a split-plot ANOVA design (Kirk, 1968). The major advantages with a split-plot design compared to randomized block factorial design are that the estimates of the within-block effects are usually more accurate than estimates of the between-block estimates. Because the average experimental error over all treatments is the same for both designs, the increased precision on within-block effects is obtained by sacrificing precision on between-block. There were significant treatment × time interactions for locomotion [F(2, 32) = 138] in mice randomly selected from three randomly chosen litters, and significant treatment × time interactions for locomotion [F(2, 32) = 180] in using the litter (n = 9, one pup per litter) as a statistical unit. **p ≤ 0.01, PBDE 99 versus the vehicle group in each experimental design and each time period.

The abstract from our presentation at SOT 2005 referred to by Drs Hardy and Stedeford included a comparison of our statistical evaluation using three randomly taken pups per litter from three litters, to be compared with one pup per litter from nine litters. In this investigation 18 different litters were used. Randomly selected pregnant Naval Medical Research Institute (NMRI) mice were purchased from a commercial breeder. Ten-day-old pups were given either one single oral dose of the brominated flame-retardant, PBDE 99, 14 μmol (8 mg)/kg body weight (bw), or the vehicle only (20% fat emulsion). Spontaneous motor behavior was studied in 2-month-old male mice. Statistical evaluation was made by using both the litter (n = 9) as a statistical unit and randomly selected individuals (n = 9, three mice randomly selected from three randomly selected litters). In both designs, the statistical evaluation ANOVA with a split-plot design and Turkey’s pairwise testing were used, revealed a significant aberrant behavior in the PBDE 99 exposed mice, compared with controls, see Table 1. This study showed (1) that in our neonatal animal model there is no difference whether the litter or the randomly selected individuals are used as the statistical unit and (2) that this design does not overestimated any effects (type 1 error). By using this neonatal animal model we can reduce the number of animals by 60–70%, which is of both ethical and economic significance.

The correctness of our evaluation is also corroborated by the fact that we have never failed to reproduce our results (e.g., for DDT, nicotine, and PBDE 99, over seven published articles of each substance) over nearly two decades. Many of our results have been published in peer-reviewed journals: Anesthesiology, Brain Research, Behavioural Brain Research, Developmental Brain Research, Neurobiology of Aging, European Journal of Pharmacology, Environmental Health Perspective, Toxicology, Toxicological Sciences, Toxicology and Applied Pharmacology, etc. What is more, the behavioral profile of the control animals has remained constant over the same 20-year period.

Current routine testing of chemicals does not take this critical period of brain development into consideration, which reveals a scientific gap that influences the uncertainty in establishing safe levels of exposure of individuals (Claudio et al., 2000; Grandjean and Landrigan, 2006).

In the scientific community it is important that different laboratories, using different methodologies, can publish data for scrutiny and interpretation by other scientists. Regarding PBDEs, there are now increasing number of reports indicating that this category of chemicals can be developmental neurotoxic (Costa and Giordano, 2007) and as was very recently reported also by direct exposure on postnatal day 10 (Lee and Moser, 2007). These articles have been published in different journals, using peer review system.

Scientific research at universities is not intended to conduct guideline studies; rather, to produce scientific data that can be discussed by the scientific community. This process can then also be used to develop guidelines for risk assessment of chemicals for the benefit of human health and of the environment.

REFERENCES


