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Methodological Considerations in Neurobehavioral Teratology

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SPEAR, L. P. AND S. E. FILE. *Methodological considerations in neurobehavioral teratology.* PHARMACOL BIOCHEM BEHAV **55**(4) 455–457, 1996.—Neurobehavioral teratology is a rapidly expanding field benefitting from recent advances in neurobiology and behavior and from the increasing availability of compounds with specific pharmacological actions. There is evidence that data derived from animal studies are clinically pertinent and hence animal studies are useful in extending clinical findings, in anticipating consequences of early drug exposure and, by determining the underlying neural mechanisms, in developing therapeutic approaches. However, the usefulness of animal studies crucially depends on the reliability and sensitivity of the methods used. We highlight the importance of appropriate selection of the route, dose, frequency, duration and timing of drug administration. We also emphasize the importance of not confounding treatment with litter effects and suggest that either the litter be used as the unit of analysis, or that each litter contribute only one pup to each test condition. We discuss the question of the time of testing and of testing, not only under baseline conditions, but also in conditions in which the offspring are exposed to stressful, pharmacological or cognitive challenges. We hope that future studies will benefit from these considerations and avoid the methodological weaknesses that beset some of the early studies in this field.

Neurobehavioral teratology Animal models Litter effects Fostering Methodology

IT IS extremely difficult to assess the later impacts of prenatal drug or toxin exposure in clinical studies (8,18). Animal studies therefore play a vital role in confirming and extending clinical findings and in anticipating other harmful effects. However, they can do more as they can explore the neural mechanisms underlying behavioral changes and, as a result, will also be able to suggest future therapeutic approaches. The usefulness of this approach of course depends on the extent to which findings from animal models can be generalized to the clinical situation. In a workshop in which data from humans, non-human primates and rodents were compared for the neurobehavioral consequences of exposure early in life to ethanol, phenytoin and environmental toxins, the following conclusion was reached—"at the level of functional category, close agreement was found across species for all the neurotoxic agents reviewed" (20). However, the usefulness of animal studies crucially depends on the reliability and sensitivity of the methods used and in the following sections we highlight some of the most pertinent considerations.

TREATMENT SELECTION

The particular compound chosen will often be pre-selected by the problem under investigation. Thus, if the purpose of the study is to examine the impact of a clinically used drug, drug of abuse or potential environmental toxin, then the same compound to which humans are exposed should be selected. However, if the prime purpose of the study is to examine hypothesized neurobiological mechanisms then use of drugs with more specific pharmacological action might well be an advantage. Even then, caution must be exercised in concluding that any resultant behavioral change necessarily results from the drug's action at its main receptor site. This is because, unfortunately, drugs are seldom as specific as initially heralded and as more and more receptor sub-types are identified formerly unknown sites of action are often revealed. It is therefore important to demonstrate when possible that the effects of an agonist can be reversed by the appropriate receptor antagonist, or vice versa. This is perhaps particularly important in developmental studies because not all receptors will have

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reached adult levels of function and therefore the balance of receptor sensitivity may differ from that seen in adult animals.

The route of administration should be carefully selected for each study. The best solution is to use the same route as that associated with human use, but for various reasons this is often not possible (19). All routes have their own strengths and weaknesses, but perhaps that least suited to prenatal administration is the intraperitoneal route, since it can produce abnormally elevated fetal drug concentrations that are at least 3 times higher than those in the maternal circulation (3).

The selection of an appropriate dose-range is also critical. As in other fields of behavioral pharmacology it is important to use several doses, but the highest dose should generally be at or just below the threshold for producing maternal toxicity (20). Clearly, maternal toxicity is an extreme example of a maternal effect that could indirectly influence the offspring. The question of whether fostering is the most appropriate method of controlling for maternal influences will be considered in a later section.

The frequency of drug administration is pertinent both to the peak concentration of the drug and to the pattern of drug exposure. Again, the best guidance comes from the pattern of human drug exposure. For new compounds this may not be known, but it is more likely that clinically used drugs will be given to achieve constant drug concentrations, whereas the pattern of recreationally used drugs is more likely to be that of intermittent exposure. The importance of this issue is especially clear with stimulants, where different physiological compensations such as sensitization as opposed to tolerance depend in part on whether drug exposure is episodic or continuous (17). Species differences in pharmacokinetics must be considered when determining the frequency of administration and, if the drug is given during the neonatal period, whether the enzymes necessary for drug metabolism are functional.

It is likely that the nature of observed neurobehavioral teratogenic effects will depend on the developmental stage at which drug exposure is given. Altricial rodents (e.g. rats and mice) are born at a less mature stage than humans. Thus the prenatal period is approximately equivalent to the first and second trimesters of human pregnancy and the first 10 postnatal days in the rat approximate to the third trimester (16). There is evidence that as little as one day difference in the timing of drug exposure can markedly influence the behavioral outcome, but, in general, the greatest vulnerability for structural injury occurs early in organogenesis and for functional impairment it is mid to late organogenesis (21). Interpretation of data from postnatal drug administration is easier if the drug is given directly to the pups, because exposing the dams to drugs does not necessarily result in an equal drug dose to all pups and may result in a drug-induced change in maternal behavior or milk availability that could have confounding effects.

LITTER EFFECTS AND FOSTERING

Much of the work in this field uses rats and mice, which have large litters. The question therefore arises as to how the litters should be allocated and analyzed. Where prenatal treatment has been administered, the first decision concerns whether to leave each litter with its own mother and what to do about litter size. Prenatal exposure necessarily mandates concomitant maternal exposure and this prior drug exposure could influence the mother s behavior after parturition when she is raising her litter. Indeed, insults during pregnancy have been shown to change maternal behaviors such as nest building, licking the pups and response to an intruder (6,10,13). These changed maternal-offspring interactions could, in turn, influence the neurobehavioral maturation of the offspring. There is evidence that the physiological and behavioral impacts of prenatal treatments can be influenced by whether the pups are reared by their own mothers or by untreated foster mothers. For example, being reared by an untreated foster mother reduces the impact of prenatal stress or cocaine exposure (4,6). However, such effects are not always found (22). By fostering each litter of treated or control pups to an untreated foster mother, any contribution due to direct drug-induced alterations in maternal behavior can be excluded. Whether this warrants the extra costs must be an individual decision for each research project. In order to further equate postnatal rearing experience it is customary to use standard litter sizes of, for example, 8–10 pups.

Ideally, both male and female pups should be retained in the litter. Dams differentially attend to male and female pups with respect to anogenital licking (12) and this differential attention contributes to adult sexually dimorphic behaviors (11). Thus the gender composition of the litter can disrupt the emergence of sexually dimorphic behaviors (12). Raising male and female rats in single sex litters has also been shown to impair the development of μ-opioid receptors (2) and responses to nociceptive stimuli and novelty (9). A decision must also be made as to whether to test both males and females. Grimm & Frieder (5) concluded that prenatal treatments affected later behavior predominantly in males, whereas early postnatal experience had impacts on both sexes. It is possible that the nature, as well as the severity, of any behavioral changes will also be gender-dependent.

Finally, the most important issue concerns the analysis of the data, taking fully into account the contribution of genetic (i.e. original litter) effects. If multiple offspring from a given litter are considered as independent observations, large sample sizes can be achieved by testing pups from a small number of litters. The evidence is overwhelming that this is not an acceptable procedure. Animals within a litter are much less variable than animals across litters. Analyses of litter effects have shown large and significant differences whether the offspring are tested as preweanlings or adults (1,7). Even inflating the sample size by treating 2 pups per litter as independent observations nearly triples the likelihood of statistical significance (7). Thus it is essential that litter, rather than individual pups, should be considered as the unit of analysis in developmental toxicology. A logically simple approach is to assign only one animal per litter to each test condition. An alternative, that allows an estimate of the contribution of litter effects to a particular experiment, is to assign multiple pups from each litter into each test condition and to consider litter as a nested variable in analyses of variance of the data.

An issue which is crucial to the above considerations is that of selecting an appropriate sample size. Developmental studies are time-consuming and costly to conduct and if the design does not incorporate a sufficient sample size, any negative findings obtained will be difficult to interpret reliably. Power analyses provide a useful means of estimating the necessary sample size (14).

All of the above considerations apply to the assessment of postnatal treatments, but the issue of how best to control for litter effects becomes even more complex. A design which avoids confounding postnatal treatment and litter effects is to assign only one pup per genetic litter to each test group and to leave the pups with their own mothers. However there is evidence that dams may differentially treat pups from different treatments, hence reducing or enhancing the effects of

the experimental treatment. For example, Pearson et al (15) found that 6-OHDA treated pups reared with vehicle-treated pups exhibited fewer long-term consequences than 6-OHDA-treated pups reared solely with other treated pups. Since it is not possible to know *a priori* whether the dam is likely to pay more, or less, attention to a treated pup the only way this can be assessed is to compare litters homogenous or heterogenous for the experimental treatment (15). The alternative approach of assigning each litter to one treatment condition does not necessarily provide a definitive answer, given that the behavioral or physiological characteristics of litters of treated pups could alter normal maternal care, with subsequent effects on offspring functioning.

TIME OF TESTING

The question of adequate sample size is heavily dependent on the behavioral tests selected and it is here that neurobehavioral teratology is able to benefit from the growing sophistication of behavioral techniques. The tests must be reliable and sensitive and it is essential that all behavioral measurements are made by an observer who does not know a particular animal's history of treatment. It is also important to select the most appropriate age at which to test the animals and, if this is not known, to assess the animals at various ages. Following exposure to a toxicant during development there is likely to be a period of secondary neural changes as the nervous system attempts to adapt to and compensate for the initial effects. This has two important implications. Firstly, assessments made early in ontogeny will be more likely to reveal primary deficits than will testing later in life when further adaptations may have developed. Early assessments are cost effective and may also have greater cross-species comparability as most of the clinical data come from assessments of infants or young children. Secondly, an interesting possibility arises from any adaptation and recovery that may occur, since there is likely to be a cost to any such reorganization. Whilst behavior might appear normal under basal testing conditons, deficits might well emerge when the animal is challenged with stressful events, pharmacological agents or difficult tasks.

CONCLUSIONS

None of the points we have discussed in this article are new and all are discussed in greater detail elsewhere (19). However, in compiling this special issue it became clear that there are still many studies that have not fully recognized some of the design implications that we have raised. The most frequent problem we encountered arose from inadequate consideration of the importance of litter effects. This led to referees rejecting studies that were addressing interesting and important questions and to inevitable disappointment. Although it does cost more to conduct a properly designed study with adequate sample size, the conduct of an inappropriately designed study is a waste of money and an unjustified use of experimental animals.

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