Methods to Identify and Characterize Developmental Neurotoxicity for Human Health Risk Assessment. III: Pharmacokinetic and Pharmacodynamic Considerations

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We review pharmacokinetic and pharmacodynamic factors that should be considered in the design and interpretation of developmental neurotoxicity studies. Toxicologic effects on the developing nervous system depend on the delivered dose, exposure duration, and developmental stage at which exposure occurred. Several pharmacokinetic processes (absorption, distribution, metabolism, and excretion) govern chemical disposition within the dam and the nervous system of the offspring. In addition, unique physical features such as the presence or absence of a placental barrier and the gradual development of the blood–brain barrier influence chemical disposition and thus modulate developmental neurotoxicity. Neonatal exposure may depend on maternal pharmacokinetic processes and transfer of the xenobiotic through the milk, although direct exposure may occur through other routes (e.g., inhalation). Measurement of the xenobiotic in milk and evaluation of biomarkers of exposure or effect following exposure can confirm or characterize neonatal exposure. Physiologically based pharmacokinetic and pharmacodynamic models that incorporate these and other determinants can estimate tissue dose and biologic response following in utero or neonatal exposure. These models can characterize dose–response relationships and improve extrapolation of results from animal studies to humans. In addition, pharmacologic data allow an experimenter to determine whether exposure to the test chemical is adequate, whether exposure occurs during critical periods of nervous system development, whether route and duration of exposure are appropriate, and whether developmental neurotoxicity can be differentiated from direct actions of the xenobiotic. Key words: developmental neurotoxicity, pharmacodynamics, pharmacokinetics, physiologically based pharmacokinetic modeling, rat. — Environ Health Perspect 109(supp 1):101–111 (2001).

Developmental neurotoxicity is any effect of a toxicant on the developing nervous system before or after birth that interferes with normal nervous system structure or function. Exposure to xenobiotics during development can cause adverse structural, functional, neurochemical, or behavioral effects. Studies conducted on laboratory animals can be used to detect or characterize developmental neurotoxicity typically focus on behavioral and pathologic effects and rarely involve consideration of pharmacokinetic or pharmacodynamic processes. These studies have been used as the basis for human health risk assessment for regulatory purposes for many years (1–3), and are discussed in companion articles in this journal (4,5). The value of developmental neurotoxicity studies conducted in laboratory animals can be increased by consideration of pharmacokinetic processes (e.g., absorption, distribution, metabolism, and elimination processes) and pharmacodynamic effects (e.g., physiologic, biochemical, and molecular effects) of a chemical on the organism in study design and interpretation. We outline pharmacokinetic and pharmacodynamic data that can be used to improve risk assessment of developmental neurotoxicants. Pharmacologic data can verify or characterize exposure, refine the dose–response curve, determine chemical delivery to a target site, and identify effects on physiologic systems resulting from exposure to the study compound. These pharmacokinetic and pharmacodynamic data collected from animals may be used to replace or modify default assumptions related to dose–response assessment and to extrapolate from animal data to human scenarios, thus reducing uncertainty in risk assessment (6). Here we focus on studies conducted in the rat, the test species most commonly used in developmental neurotoxicity studies, although we refer to other species to illustrate pharmacologic factors that should be considered to improve experimental design of developmental neurotoxicity studies.

For several reasons, pharmacokinetic and pharmacodynamic processes often are more complicated in the developing nervous system than in the adult. For example, dynamic physiologic changes occur in the maternal–placental–fetal unit during pregnancy and in the offspring during postnatal development. Brain growth is rapid before and after birth, and access of chemicals to the brain may change as the blood–brain barrier (BBB) develops and matures. Exposure to the conceptus often depends on transfer of the toxicant from the dam to the developing animal via the circulation in utero and the milk during lactation. Thus, exposure to the developing nervous system is a function of the dose administered to the dam and the pharmacokinetics of the compound in the maternal, placental, and embryonic/fetal (or neonatal) circulations. Changes in either maternal or offspring pharmacokinetics could therefore affect the extent to which the developing nervous system is exposed. In addition, the intrinsic vulnerability of the nervous system...
to toxic agents varies with developmental stage at which exposure occurs, i.e., there are critical periods, both pre- and postnatally, for disruption of structural and functional nervous system development ([7]; for review see Rice and Barone (8) and Adams et al. (9)]. There may be a heightened sensitivity of the immature nervous system to toxic insult as a result of various developmental processes such as cell formation, migration, development of neurites, and establishment of intercellular connections. There may also be greater exposure of the immature (vs. mature) nervous system to toxic chemicals. The pharmacokinetics and toxic effects of a test compound should therefore be evaluated during the relevant period of development. Pharmacokinetic and pharmacodynamic data might be used to address questions such as whether gestational exposure to the developing nervous system occurs in utero, and whether the compound is transferred to the milk, creating lactational exposure. If exposure is confirmed based on pharmacokinetic data or if neurotoxicity is observed in the developing animal, the next step may be dose characterization in the dam and offspring. To characterize exposure and dose response for study design or interpretation, a pharmacokinetic profile in the dam and embryo/fetus or pup can be generated based on available data or through pharmacokinetic modeling. The ultimate goal in risk assessment is to describe the entire exposure, target tissue dose, and toxic response continuum for more accurate extrapolation to humans (Figure 1).

Factors That Modify in Utero Exposure of the Developing Nervous System

Factors that determine whether prenatal exposure to a xenobiotic will cause developmental toxicity include the pharmacodynamic action of the xenobiotic on the conceptus, the prenatal developmental stage at which exposure occurs, and the concentration of the active form of the xenobiotic reaching the conceptus in utero. Chemicals may produce developmental neurotoxicity directly by acting on the fetus or indirectly by inducing maternal effects. Most indirect effects result from altered delivery of oxygen, glucose, amino acids, specific coenzymes, and other substrates to the fetus. Indirect effects can arise from placental insufficiency, by direct effects on maternal glucose regulation (e.g., by hypoglycemic agents), or by alterations in maternal ventilatory control (e.g., opiate-induced hyperventilation or hypoventilation). Some xenobiotics are likely to act through more than one mechanism. For example, ethanol has specific teratogenic effects in both the embryonic and fetal period but may also alter maternal nutrition and disturb fetal acid-base balance (10).

The nature and incidence of developmental effects of chemicals depend on the developmental stage insulted (11). In general, exposures to certain chemicals during pre-differentiation early in gestation do not produce congenital malformations but instead lead to embryonic death or abortion (12). Similarly, the embryo is highly susceptible to teratogenic insult during organogenesis because organ development is continual within this period (11). After organogenesis, the developing nervous system can remain susceptible to chemical insult (8,13). Although major brain structures are largely completed during organogenesis, neuronal migration occurs and neuronal connections continue to be formed through early neonatal life; thus, brain organization is an important consideration during the fetal and postnatal periods (14).

The concentration of the active form of a xenobiotic reaching the conceptus in utero is another important determinant of effect and is influenced by maternal and fetal metabolism. Numerous xenobiotics are known to exert their toxicities only after metabolic activation by various enzymes within the body [reviewed by Juchau (15)]. Biotransformation may vary among species or individuals based on genetic or physiologic variables such as pregnancy, disease, or chronic drug therapy. Therefore, establishing whether metabolism is quantitatively and qualitatively similar among different species is important when making interspecies extrapolations. For example, differing susceptibilities of various inbred mouse strains, and possibly human individuals, to the developmental effects of phenytoin may involve genetically determined differences in phenytoin biotransformation (16,17), and different sensitivities to retinoid developmental toxicity among species can be attributed partly to differences in metabolism (18).

The activity of hepatic microsomal drug-metabolizing enzymes increases during human pregnancy, leading to an increase in hepatic clearance of certain drugs and chemicals (19). Conversely, the hepatic microsomal metabolizing enzyme activity in rats decreases during gestation to approximately 50% of control levels (for review see Oesterheld (20); however, the total metabolic capacity of the rat liver may remain constant or can increase during pregnancy because liver hypertrophy leads to increased (~40%) liver weights in pregnant rats (21). The human fetus possesses a well-developed complex of drug-metabolizing enzymes, albeit less active than those of the adult, whereas fetuses of common laboratory animals may be deficient in drug-metabolizing activity (22–28). This difference between human and rat fetus enzyme activity may be a disadvantage of the rat model. Even though levels of hepatic and extrapulmonary cytochrome P450 enzymes are lower in the lar fetuses than in the dam, some investigators have hypothesized that these levels may be sufficient to catalyze deleterious biotransformation reactions (29). Fetal tissues and placenta are deficient in A-esterases and carboxylesterase (30), two enzymes that may limit detoxification of organophosphates and pyrethroid insecticides in the fetus.

Plasma protein binding is another important determinant of xenobiotic disposition and action. Significant changes in plasma protein composition can occur during pregnancy (31,32). Both albumin and α-1 acid glycoprotein fractions are reduced during pregnancy, and the pharmacokinetics of highly protein-bound acidic and basic drugs are affected. Plasma protein levels of the developing offspring undergo changes during development (33) that may lead to increased levels of free (i.e., protein-unbound) drugs. Differences in the degree of protein binding between maternal and umbilical cord plasma have been demonstrated. In some cases these differences in plasma protein binding may be sufficient to account for a difference in total drug concentration between maternal and fetal plasma (32). Xenobiotic binding to fetal or neonatal plasma proteins may displace endogenous substrates that are normally bound to these proteins. For example, the competitive displacement of bilirubin from

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**Figure 1.** Overview of pharmacokinetic processes that occur during nervous system development. AUC represents the area under the concentration–time curve.
Factors That Modify Postnatal Exposure of the Developing Nervous System

Once parturition occurs, exposure of offspring to a test chemical and its metabolites generally occurs through the milk. Although some preformed, endogenous, systemic substances are found in milk (59), a selective blood–milk barrier exists for the mammary ducts of most species studied. Various factors affect the entry of compounds into breast milk, including molecular size, lipophilicity, and pH (for review see Wilson (56) and Wilson et al. (57)). The transfer of drugs, chemicals, and micromolecular nutrients from blood to the milk occurs via mechanisms similar to transport mechanisms of other membranes. Such mechanisms include diffusion through water-filled pores, diffusion of lipid-soluble compounds through lipid membranes, and active, or carrier-mediated, transport. Passive diffusion is affected mainly by the chemical or drug disposition in lactating mothers, the physicochemical properties of the molecule, and the protein and lipid content of breast milk. In general, lipophilic chemicals penetrate membrane barriers easily and are preferentially concentrated in the milk fat globules, which can lead to a high ratio of chemical in milk compared to plasma. Because lipophilic compounds partition into fat, the fat content of breast milk is a major determinant of the chemical level in whole milk. The concentration of fat and other nutrients in the milk varies among species. Mature human milk contains approximately 3–5% fat, 0.8–0.9% protein, 6.9–7.2% carbohydrate calculated as lactose, and 0.2% mineral constituents expressed as ash (58). The concentration of protein (8–12%), fat (11–15%), iron, and copper in rat milk is higher than that found in human breast milk (59). Within each species, fat and other nutrient concentrations vary throughout lactation period postpartially and diurnally (59).

The pK$_a$ of a weak acid or base is a primary determinant of its ability to enter

### Table 1. Placental morphology and classification in humans and common laboratory animals

| Placental type          | Tissue layers |
|-------------------------|---------------|
|                         | mb | mce | mct | me | is | tr | tct | fce | rb |
| Epitheliocorial          | +  | +   | +   | +  | +  | +  | +   | +   | rb |
| Endotheliocorial         | +  | –   | –   | –  | +  | +  | +   | +   | rb |
| Hemochorial              | mono | – | – | – | + | + | + | + | rb |
|                         | tri | – | – | – | +++ | + | + | + | rb |

| Species            |
|--------------------|
| Sheep, pig         |
| Dog, cat           |
| Human, guinea pig  |
| Rabbit, Rat, mouse |

Abbriviations: +, number of cell layers; rb, fetal blood; fce, fetal capillary endothelium; tct, fetal connective tissue; is, intervillosous space; mb, maternal blood; mce, maternal capillary endothelium; mct, maternal connective tissue; me, maternal epithelium; mb, trophoblast. Maternal blood is represented by a shaded column, as are intervillous space and fetal blood. The placentas of all species listed contain these layers. Columns between these layers represent cell layers (if any) that separate mb, is, and rb. Data modified from Page (180).
infant, with the dose rate being the amount ingested over time in the mother’s milk. However, the offspring blood levels of chemical also depend on the degree of absorption in the gastrointestinal tract, as well as distribution, metabolism, and excretion. The milk-to-plasma (M:P) ratio, which compares milk drug concentration with maternal plasma drug concentration, serves as an index of the extent of drug excretion in the milk. The impact of these various determinants has been quantified, and pharmacokinetic models have been established for predicting the behavior of unstudied chemicals (57,74–77).

These principles have been used successfully to describe M:P partitioning of many chemicals. However, in vivo studies in a variety of species have identified some drugs that are found in milk at higher-than-predicted concentrations. Drugs for which this is true include aminopyrine (78), N4-acetylated p-aminophenolic acid (79), N4-acetylated sulphanilamide (80), acyclovir (81), cimetidine (82–85), and nitrofurantoin (86). The unpredictable M:P ratios observed for these compounds may be due to an active transport mechanism.

An additional concern that should be considered in lactational exposure studies is the potential for the xenobiotic to alter milk secretion and composition. Such interference can occur in mammary development, secretion by mammary alveolar cells, hormonal support for lactation, or nutrient transport [for review see Neville and Walsh (87)].

Methods Used to Confirm Exposure of the Developing Nervous System

Several factors should be considered when designing studies to assess whether placental transfer of a chemical might occur. Single time-point measurements of either maternal or fetal chemical concentrations are difficult to interpret in developmental neurotoxicity studies for various reasons. First, some xenobiotics may be transported more readily across the placenta during late gestation than during early gestation. Second, placental transfer may vary over time, depending on the dosing paradigm used (i.e., bolus injection vs. dietary exposure). In addition, it is difficult to predict whether a bolus dose or continuous infusion of a chemical given to the dam will lead to a higher concentration within the embryo/fetus; either may produce a higher exposure, depending on the substance administered. Substances that are eliminated rapidly from the maternal circulation tend to lead to fetal plasma concentrations that are only a fraction of the maternal peak concentration.

Current research techniques to measure placental transfer include the use of the chronically cannulated sheep, rabbit, or monkey, the isolated perfused human placenta or placental lobe, and the small-animal placenta (rabbit, guinea pig, and even rats) as a variety of transfer rates for chemicals (88). Concentration gradients across the sheep placenta are often larger than in primates, especially for less lipid-soluble chemicals (89). The large litter size of rabbits allows individual fetuses to be removed in sequence to measure chemical concentrations in both plasma and tissue (90,91). Small laboratory animals may be used to characterize distribution of the toxicant, with either autoradiography (92) or extraction and chemical assay (93) used to detect the toxicant.

The concentration of xenobiotic in the fetus may be estimated from the placental transfer rate and the rate of direct elimination by the fetus using the concentration-time course of the chemical in the mother. Depending on the particular xenobiotic, pharmacokinetic endpoints of interest can include comparisons of peak (or trough) fetal and maternal concentrations, area under the concentration-time curve (AUC), and mean steady-state concentrations in the fetus and dam expressed as a fetal-to-maternal ratio. A transplacental binding gradient may account partly for the differences in placental transfer and teratogenic potency between all-trans and 13-cis-retinoic acid in the mouse (93). A pH gradient across the placenta also appears to play a role in the extent of placental transfer of some substances. The embryonic compartment is alkaline relative to maternal plasma during early rodent organogenesis, and several teratogenic weak acids can accumulate in the embryo during this period, presumably by ion trapping (94). This concept has been formalized in a physiologically based pharmacokinetic (PBPK) model (95).

Experimental animal studies evaluating placental (and lactational) transfer often rely on the use of high-purity radiolabeled chemicals to determine xenobiotic distribution patterns in the dam and fetus or neonate. The use of radiolabeled chemicals also facilitates mass balance determinations. Measurement of radiochemicals often relies on whole-body autoradiography techniques using small numbers of animals. Because individual radiolabeled isotopes are followed, interpretation of these studies requires some knowledge of the metabolism to ensure that the radiolabel remains with the metabolite of interest. Studies examining the transfer and distribution of nonradiolabeled chemicals require the development of sensitive analytic methodologies to evaluate the parent compound and its metabolites.

Exposure to the developing animal in utero can be confirmed using analytic techniques to measure the parent compound or active...
metabolite in plasma, blood, or amniotic fluid. Evidence of exposure in nursing neonates can be demonstrated by determining the concentration of the test substance and its important metabolites in pup blood or tissue during the lactation period or by measuring chemical-specific biomarkers of exposure or effect in pups (e.g., cholinesterase inhibition or carboxylesterase inhibition). The measurement of toxicant concentrations in milk is often used as a surrogate to confirm neonatal exposure. Changes in the magnitude of exposure over time can be estimated by sampling either the maternal milk or stomach contents of the pups at several time points during the lactation period. Each of these sampling procedures is hindered by the preference for a terminal sacrifice of the experimental animal for adequate sampling. An alternative approach is to employ cross-fostering in the experimental design to distinguish between body burden of a drug or chemical resulting from gestational or lactational routes of exposure. Exposure in the developing animal can be confirmed by measuring a biomarker of exposure or effect rather than the actual toxicant.

**Characterization of Exposure**

In studies where only maternal animals are treated and there is little or no opportunity for other types of postnatal exposure (such as in a gavage study), exposure in pups is mediated by the presence of the test substance or its metabolites in milk, the presence of normal lactation function and behavior on the part of both dam and pups, and the volume of milk consumed by the pups. In this type of treatment scenario the amount of test substance delivered to the pups via the milk does not remain constant because levels in milk depend upon the delivered dose, route of administration, duration of exposure, gestational stage, and species. Therefore, characterization of exposure is a critical element in defining the toxicologic dose–response (or concentration–response) relationship for proper interpretation and extrapolation of study results. For example, placental transfer of the antiepileptic drug phenytoin varies significantly among animal species as well as with gestational age and route of administration (97). The gestational stage-dependent kinetics of the herbicide 2,4,5-T in pregnant mice would be expected to produce increasing embryo or fetal exposures over the course of pregnancy at the same maternally administered dose (98). The dose-dependent kinetics of the developmental neurotoxicants phenytoin and valproic acid appear to be accentuated in pregnant animals (99,100). These findings indicate that maternally administered dose may be a poor surrogate for concentrations in the developing organism and emphasize the importance of exposure monitoring in the evaluation of developmental neurotoxicity.

Exposure characterization is particularly important in extrapolating study results to humans. In some cases peak exposure is linked to an adverse effect, and in other cases the total exposure over time is linked to the adverse effect; thus, different pharmacokinetic patterns may be important in predicting outcome, depending on the study compound (101,102). For example, maximal maternal plasma and embryo concentrations (Cmax) correlate with the development of neural tube defects in animals treated with the neuroteratogen valproic acid (103). Conversely, the AUC values for retinoids correlate best with their teratogenic activity (104,105). There is some evidence that peak concentrations may play a crucial role in the neurobehavioral effects of early lead exposure in monkeys (106). For some developmental toxicants such as 2-methoxyethanol, the critical pharmacokinetic parameter (e.g., Cmax vs. AUC) apparently depends on the organ system and developmental period during which exposure occurs (107). These data suggest that the pattern of exposure is a key determinant of developmental toxicity, which could have important implications for risk assessment in humans (108,109).

The level of exposure characterization may be limited by practical considerations in a routine developmental neurotoxicity study but ideally could at least include quantification of the test compound (and any major metabolites) in maternal plasma over time. However, more proximate measures of offspring exposure may be essential for some compounds. For example, lactational exposure following administration of the same maternal dose of methylmercury or inorganic mercury to mice produced much higher brain mercury concentrations in the pups of dams given methylmercury despite similar pup plasma mercury concentrations in the two groups (110). Thus, plasma-level data must be viewed in the context of the disposition of a compound within the organism, and all the pharmacokinetic processes that determine the concentrations of the active substance at the target must be considered. Although such information is most useful if analogous data are available for humans, a lack of human data does not preclude its use. For example, it can be incorporated into physiologically based models to improve the estimation of target organ exposure in humans. The concept of using PBPK models to better describe the disposition of a xenobiotic in both rodents and humans has been discussed in the context of developmental risk assessment by Welsch et al. (111) and is discussed in more detail later in this article.

**Biochemical Markers of Exposure and Effect in Developing Animals**

A biomarker of effect can be defined as a measurable biochemical or physiologic alteration within an organism recognized to have a potential impact on health (112). A biomarker of exposure may be a predictive indicator that does not impact health. Examination of biomarkers of effect following exposure of a laboratory animal to a neurotoxicant can be used to identify the lowest dose capable of eliciting the potential adverse effect and provide information on the mechanism of action of the toxicant. A number of biomarkers have been used to identify insult in the adult nervous system, including astrocyte expression of glial fibrillary acidic protein (GFAP), induction of stress proteins, increased density of apoptotic cells, increased neuronal degeneration, and alterations in receptor density or function throughout the brain (113,114) [for review, see Costa (115)]. The applicability of studies characterizing biomarkers of effect in the developing nervous system depends on a clear understanding of the differences between juvenile and adult brains and adequate documentation and validation studies. Therefore, the utility of the methods and techniques used to identify specific biomarkers of effect in the adult brain to evaluate the toxicity of compounds on the nervous systems of developing animals remains to be seen.
Analysis of potential biomarkers of exposure or effect in the developing nervous system will be complicated by the rapid brain growth and neuronal development that occur during prenatal and postnatal periods. Some of the biomarkers of effect in adults may occur normally in the developing brain as a result of basic processes that underlie nervous system growth and development. These processes—including cell replication, migration, and differentiation; apoptosis; myelination of neurons; and synapse formation—occur in a precisely ordered sequence in each region of the brain, both in utero and postnatally, and can be identified by molecular markers. Exposure to a neurotoxicant may alter the pattern of brain development, or the normal pattern of development may be shifted in time as a result of such exposure.

The biochemical and structural changes that occur in the normal developing nervous system are enormous. Markers of effect of low specificity (e.g., brain weight determinations) may be useful for evaluating toxic actions in the developing nervous system but are less useful in evaluating toxic actions in the adult nervous system. For example, measures of brain weight, protein or lipid concentration, and RNA or DNA content all produce simple but accurate measures of brain development and the schedule of that development either in the whole brain or selected regions. More dynamic measures include incorporation of precursors into macromolecules such as proteins, lipids, RNA, or DNA, indicating both amount and turnover in nervous system tissue (116). Alterations in these biochemical parameters may signal either a change in pattern of brain development or a shift in time of development. Distinguishing between these two situations using biochemical measures can be a challenge. A description of the normal or baseline pattern of ontologic expression of the biochemical target throughout development should be available before any of the methods to evaluate biochemical markers of effect described above are used for routine analysis of developmental neurotoxicant action. For example, an evaluation of changes in protein or lipid content in a given brain region will not be useful for assessing developmental neurotoxicity until an analysis of control brain protein or lipid content at specific time points is available.

Assays to evaluate more specific biochemical and pharmacologic markers of exposure or effect can be conducted using brain tissue homogenate preparations or histologic sections. Examples of this approach include measures of specific protein or mRNA expression used as sentinels of appropriate brain development: myelin gene expression and undernourishment (117); muscarinic receptor expression and parathion exposure (118); astroglial markers and cocaine exposure (119); neurogranin and myelin basic protein expression and exposure to PCBs (120); and chemical-induced hypothyroidism (122). Tissue homogenates can be assayed to determine the levels of neurotransmitters, receptor density, or receptor affinity in given regions of the brain. The function of these receptors may also be assessed by analyzing the second messenger activity linked to a given receptor type in tissue homogenates, such as adenylate cyclase activity or ion flux linked to receptor activation (122,123). Many novel histologic methods have been developed that allow detection and characterization of alterations in specific regions of the brain. Among the potentially useful methods for identifying and localizing biochemical markers of effect in the developing animal brain are silver stain for identification of neuronal degeneration, and terminal deoxynucleotidyl transferase-mediated deoxyuridine diphosphate nick-end labeling for detection of apoptotic cells. Immunohistochemical labeling of GFAP, heat-shock proteins, and other developmentally regulated proteins can also be assessed (124). One must always be aware that the sensitivity for many of these measures may be much lower in the developing brain than in the adult because of the dynamic and rapidly changing events occurring in the normal development of the nervous system.

Microarray technologies offer a new approach for examining alterations in gene expression following chemical exposure (125). This approach allows determination of the presence or absence of particular nucleotide sequences or the relative abundance of an mRNA species in one sample compared with another sample. The application of this technique most relevant to toxicity testing is in determining the mRNA expression patterns of numerous genes at a single time. Although it is tempting to think of this procedure as one for high throughput of samples, this technology instead provides a wealth of information on a limited number of samples. From such information, new hypotheses can be developed and subsequently tested with more traditional techniques such as reverse transcription-polymerase chain reaction. Given the vast number of genes regulated during development, each according to a distinct developmental temporal pattern of expression, choosing any one of two (or more) ages at which to examine chemical-induced alterations in development is difficult. Measurements conducted at one developmental stage cannot distinguish between an increase or decrease in a gene or simply a change, delay, or acceleration in the normal developmental profile. Thus, the need to generate patterns of gene expression during the course of development will require technologic advancements before routine evaluation of larger numbers of samples becomes feasible. Even when these technical limitations are overcome, changes in normal gene expression during development may eclipse the ability to detect toxicant-induced effects.

**Application of Dosimetry Modeling to Developmental Neurotoxicology**

The precise characterization of dose–response relationships and the extrapolation of results from animals to humans are both issues of paramount concern in toxicology and risk assessment that may be improved by PBPK modeling. A PBPK model is a series of mathematical equations based on organism-specific and chemical-specific information that describe the pharmacokinetic disposition of a foreign chemical within an organism (126,127). Solution of these equations will simulate the concentration of a chemical (as well as metabolites, if desired) with time in the tissue of interest. The power of PBPK modeling therefore lies in its ability to estimate the amount of the active form of a chemical at its target site within the body over time, given virtually any exposure paradigm. Moreover, extrapolation of an animal PBPK model to humans can be attempted through substitution with human physiologic and, where feasible, human chemical-specific information. Therefore, PBPK modeling can improve our understanding of the risks of developmental neurotoxicants by estimating more precisely the exposure of the conceptus or offspring to the active form of the toxicant after maternal exposure. This approach also improves our ability to extrapolate pharmacokinetic data from animals to humans.

PBPK modeling of various chemicals in animals during pregnancy has been reported (95,128–132). For example, O’Flaherty et al. (95) developed a PBPK model in the pregnant rat and mouse for 5,5’-dimethyloxaoxolidine-2,4-dione (DMO) that accounted for physiologic changes that occur throughout gestation. Their model accurately simulated DMO levels in embryo plasma and embryo homogenate on gestational days 10, 11, and 13 after maternal exposure. More sophisticated models could, in theory, simulate levels of active toxicant within various tissues or portions of tissues of the conceptus. Similarly, modeling of lactational transfer in rats and humans has been successful with certain chemicals (133,134). Fisher et al. (134) developed a PBPK model for lactating women that estimated the amount of certain volatile organic chemicals ingested by a nursing infant for a given nursing schedule after a maternal occupational exposure. Important features of the model included a milk compartment that changed in volume in response to a nursing infant.
Pregnancy and lactation PBPK models can design experiments, use information related to mode of action, and identify important data gaps. Under ideal circumstances, pharmacokinetic data obtained in humans would be used to validate PBPK models developed using animal data. Few pharmacokinetic data are available for most chemicals in pregnant or lactating women, although there are exceptions for some drugs, such as antiepileptic agents that must often be administered throughout pregnancy (135–137). Approaches currently used for estimating human exposure have been described (138).

The construction of a PBPK model begins with the description of the body as a series of compartments representing individual tissues or tissue groups (Figure 2). Those tissues represented by compartments are selected for their relevance to the disposition or action of the chemical under consideration. For example, a central nervous system (CNS) depressant requires inclusion of the brain as a compartment, whereas hepatic metabolism of a drug necessitates the use of the liver as a compartment. Similarly, a gestational PBPK model requires compartments for the placenta and the conceptus (95), whereas a lactational model requires a milk compartment (134). Following selection of appropriate compartments, differential equations are written to describe the fate of a chemical as it passes through each tissue. Many examples of such equations have been published. Organism-specific parameters for which values must be supplied to the differential equations include tissue blood flow, organ volume, cardiac output, and in certain instances ventilation rate, all of which can be obtained from the literature. In most instances, pharmacokinetic data obtained in animals can be used to estimate these values, and the specific methodology employed depends to a large extent on the physiochemical and pharmacokinetic characteristics of the chemical under study. If metabolite disposition is to be included in the model, chemical-specific parameters for the parent compound and the metabolites of interest must be determined. Solution of the model requires a computer equipped with software that can solve simultaneous differential equations. Probably the most commonly used software for this purpose has been Advanced Continuous Systems Language (Pharsight Corporation, Mountain View, CA, USA), although many others can be used as well.

Although PBPK modeling is an extremely powerful pharmacokinetic tool, it is not without problems. Building PBPK models can require considerable resources. At a minimum, moderate programming skills are required, and tissue:blood partition coefficients and elimination rate constants can be difficult and time consuming to determine accurately for many types of chemicals. Furthermore, with a rodent gestational model, the limited amount of tissue obtained from a conceptus or the placenta requires the use of a large number of animals. Although validation of animal PBPK models can be accomplished by comparing model predictions with empirical pharmacokinetic data, such data are often missing and unobtainable in humans. An important challenge to modelers is to address these complicating issues so that PBPK modeling can become a more useful tool, routinely used in all areas of toxicology.

**Practical Considerations in the Experimental Design of Pharmacokinetic Studies in Developmental Neurotoxicity**

The selection of route of administration, duration of exposure, and time during nervous system development that the xenobiotic will be administered can influence whether developmental neurotoxicity occurs following xenobiotic exposure. Thus, the decision of when to initiate chemical exposure is critical. Developmental toxicity studies performed typically by exposing pregnant animals to a test substance beginning at uterine implantation and early stages of organogenesis, including neurulation of the embryo (around gestation day 6 in rats). Several studies have identified specific malformations, especially neural tube defects, that appear to have resulted from preimplantation exposures (140). Additionally, there is some indication that very early gestational exposures to cholinesterase-inhibiting chemicals, while not causing such frank malformations as neural tube defects, may produce more subtle alterations in neurologic development (141,142). Thus, maternal dosing beginning at or before conception may be important for testing some xenobiotics. Another reason for initiating exposure before conception is that some xenobiotics require long exposure times to reach pseudo–steady-state conditions in the dam (143).

Postnatal exposure of animals may be critical in the design of developmental neurotoxicity studies and may depend upon the experimental question. Postnatal brain development occurs in both humans and rodents (13,144). For example, a recent magnetic resonance imaging study in children age 3–15 identified evidence of quantifiable spatial changes in the corpus callosum throughout prepubertal childhood (145). Although regional neurogenesis occurs predominantly during gestation in both humans and rats, neurogenesis of hippocampal dentate granule cells and cerebellar neurons continues into the postnatal period in rats compared to time of parturition in humans (13). In each species, the formation of the neural network and connections as well as myelination are significant processes that occur postnatally [for review see Jacobson (146)]. Continuation of dosing after birth may be necessary to ensure exposure to some chemicals during this critical period of brain development. Direct neonatal dosing may be required for developmental neurotoxicity testing of some xenobiotics if lactational transfer does not occur. Additional scenarios in which children may be exposed (e.g., inhalation, chemical exposure)

![Figure 2](image-url)  
**Figure 2.** The conceptual framework for the PBPK model of gestational transfer of a chemical that is potentially neurotoxic from the exposed mother to the fetus. Arrows connecting tissue compartments indicate movement of chemical within the body. X denotes a time-dependent on-off switch. In this model, exposure may occur from intravenous injection or ingestion. $D_{in}$, intravenous dose of chemical (mg/hr). $D_{oral}$, oral dose of chemical (mg/kg/day).
residues in food or water, pica) might be addressed by direct neonatal dosing studies.

Although direct dosing of pups maximizes neonatal exposure to the test compound, it is not necessarily representative of chemical exposure of the human fetus during the third trimester, which depends on the metabolism and pharmacokinetics of the chemical in the mother. Practical considerations associated with direct dosing of pups must be overcome. Logistical problems associated with dosing large numbers of pups in a study can be significant, and there are limitations in the vehicle and volume of solution that can be administered repeatedly to young animals (147). One additional concern is that direct oral dosing of very young pups might injure or stress the pups, which could lead to functional and behavioral effects not associated with exposure to the test chemical. A potential advantage of direct dosing of a test chemical to pups at precisely known dose levels is that this approach allows a more direct comparison of toxic response for adult and juvenile animals (148). In addition, if exposure is limited to the postnatal period, direct dosing of pups allows the use of interlitter dosing designs that can significantly decrease the number of animals required for any one study. This information could be useful in the assessment of age-related sensitivity to xenobiotic exposure.

Another issue to be considered is the anticipated route of human exposure. For food-use pesticides with potential dietary residues, the most appropriate administration route might be oral (gavage or dietary). In some studies dams are treated daily by gavage with a measured bolus dose of test substance that is dispersed or suspended in a nontoxic vehicle (149–151). In a dietary study the test substance is mixed into the feed, which is made available to the animals for ad libitum consumption throughout the dosing period. Consumption of the mixed feed occurs over a number of hours each day, but consumption may be affected by unpalatability of the admix. Dermal and inhalation studies are conducted with limited daily exposure periods of approximately 6 hr for the dams. Similar problems can occur with exposure via the drinking water. Both dietary and drinking water routes of exposure require substantial evaluation to characterize chemical stability in the dosing vehicle. Inhalation developmental neurotoxicity studies can be challenging because maternal separation from pups may have a negative impact on the developing nervous system and there are difficulties associated with exposing neonatal animals. Some investigators have exposed dams and neonatal pups simultaneously using bedding and stainless steel caging held within a larger, well-mixed exposure chamber (149–151). A whole-body exposure system that permits the simultaneous exposure of rat dams and neonatal pups has been developed recently and used for developmental neurotoxicity studies (152,155). In all cases, actual dose delivered to the maternal animal depends on the absorption of the chemical, which may be affected by many factors related to the physiologic properties of the test substance or to the study design.

Fetal tissue (e.g., blood, brain) levels may confirm fetal exposure but may not be useful in determining how to optimize exposure to rat pups; there are few, if any, alternative actions to increase in utero exposure levels. If there is no placental transfer of the parent compound or active metabolite in the rat, an evaluation of differential metabolism may indicate whether exposure of the human fetus is likely, assuming that rat and human placental transfer are qualitatively similar. If the assumption regarding relevance of rat placental transfer to humans is questioned, either a different animal model (such as the mini pig) might be appropriate, or it may be more relevant to omit exposure during gestation and focus on exposing rat pups after birth.

Rapid changes in organ development and function occur during the neonatal period. The low body weight of neonates compared to adults and the high food consumption per kilogram of body weight mean that tissue levels of test chemicals can reach higher levels in newborns than in adults. Furthermore, the pharmacokinetics of many chemicals are age-specific. For example, mercury, manganese, and many other neurotoxic metals have higher gastrointestinal absorption and less effective renal or biliary excretion in neonates than in adults (154–155).

The metabolic capacity of the newborn animal also differs from that observed in adults. For example, postnatal animals may be deficient in cytochrome P450 enzymes, A-esterases, and carboxylesterase. The deficiency in P450 enzyme activity could protect the young animal from toxicants activated by P450 because the activation potential is less in the young animal than in the adult. This may not be the case for organophosphorus pesticide toxicity because both the activation and detoxification potential in young animals usually is less than in adults (156), producing increased toxicity in the young (157). This increased toxicity stems mainly from an age-related deficiency in detoxification enzymes in the young (158–160).

The adult nervous system is protected from many toxic substances circulating in the blood by the BBB and blood–nerve barriers. For this reason, there have been efforts to deregulate possible neurotoxicants according to their expected inability to enter the nervous system (161). However, such assumptions are problematic in attempts to predict the potential neurotoxicity of a chemical on the developing nervous system at a time when the BBB may not be fully functional. Even in adults, the BBB does not always protect the brain from the passage of toxicants because this barrier is permeable to many xenobiotics. In addition, the BBB is in a dynamic state, with altered permeability occurring during physiologic and pathologic conditions. Besides developmental and pathophysiologic regulation of the BBB, chemical transport into the various brain regions can display regional differences. For example, systemically administered glutamate causes neurotoxicity in the circumventricular organs and other areas of the CNS that are least protected by the BBB (162).

The timing of BBB development is a matter of much controversy, partly because the different markers used to define the barrier appear at different times during development (163,164). Furthermore, the timing of BBB development varies among animal species. For example, the BBB is not fully developed during fetal life in rodents; however, complex tight junctions in endothelial cells are present very early in human fetal development (165). Species differences in functional aspects of the BBB remain to be determined. The BBB marker P-glycoprotein also appears very early in brain endothelial cells, indicating advanced differentiation even at the earliest stages of development (166). In contrast, astrocytic foot processes and the basement membrane develop postnatally. The relatively high permeability of fetal and neonatal BBB has been ascribed to the uptake of plasma solutes by endothelial cells through a vesicular pathway (165). Although controversial because of possible artifacts associated with the use of high volumes of intravascular tracers in developing animals, the BBB apparently does not reach the stage of impermeability seen in adults until some time postnatally, thus increasing susceptibility to blood-borne neurotoxins (164,167–169).

The level of maturity of the BBB is not the only factor that determines the transport of neurotoxicants into the brain. The physicochemical characteristics of blood-borne compounds are also important determinants in their capacity to cross the BBB. Lipophilicity plays a major role in determining the rate at which xenobiotics may enter the CNS compartment. Once inside the CNS, a highly lipophilic substance tends to accumulate because of the high lipid content of myelin and other neural structures. Thus, the extent of exposure of the CNS to a lipophilic toxicant would be higher than the systemic exposure of the organism (170). An ionized compound will not enter the CNS as readily as a compound that is not ionized, in
which case it will enter the brain at a rate proportional to its lipid/water partition coefficient (177). For example, methyl mercury enters the brain much more readily than inorganic mercury (110,179).

Besides lipophilic substances, other substances that structurally resemble nutrients normally taken up by the CNS can gain facilitated or receptor-mediated access through specific transport mechanisms (173,179). Some of these mechanisms normally transport hormones, amino acids, proteins, fatty acids, and trace elements (179). One such transporter mechanism, the transferrin system, is present on the surface of BBB endothelial cells. The transferrin receptor complex transports iron and manganese (176,177). Areas with high levels of iron receive input from areas with high levels of transferrin receptor in the vasculature and neuropil. For example, the iron-rich areas of the substantia nigra and globus pallidus receive input from the nucleus accumbens and caudate putamen, areas rich in transferrin receptors (178). Transferrin and its receptor may also transport other cations such as aluminum (179).

This mode of access of neurotoxic metals into the CNS may also explain earlier observations of enhanced lead absorption during iron deficiency and anemia (180).

Conclusions

Our goal was to review pharmacologic factors that should be considered to improve the design and interpretation of developmental neurotoxicity studies. Many of the pharmacokinetic methods, analytic chemical techniques, and modeling approaches needed to evaluate the pharmacokinetics of a xenobiotic in the developing organ are available to the research investigator. The systematic application of these resources to developmental neurotoxicology remains in its infancy. Our intent was to emphasize the value of collecting pharmacokinetic and pharmacodynamic data in developmental neurotoxicity studies to improve the risk assessment of xenobiotics. Our hope is that this information will inform and stimulate the reader to consider the following questions when designing studies to evaluate potential developmental neurotoxicity of a xenobiotic:

- Was the experiment designed so as to evaluate direct actions of the chemical separately from developmental neurotoxicity?
- Are there data to suggest that species or life-stage differences in xenobiotic pharmacokinetics or biologic response may occur in developing animals following exposure?

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