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Application of Systems Biology in Developmental Neuronal Toxicity

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Authors' Note

Here we provide an overview of our efforts to apply a systems biology approach to understand a particular toxicological problem: potential anesthetic (e.g. ketamine)-induced neurodegeneration in the developing nervous system. Systems biology, as adopted for toxicology, is referred to as systems toxicology and involves the study of system perturbations caused by chemicals or stressors. By monitoring alterations in gene and protein expression, cell signaling processes, pharmacokinetics, behavior and imaging outcomes, it is hoped to more completely define the affected system(s).

Systems biology has been defined as the iterative and integrative study of biological systems as they respond to perturbations [1]. It can be utilized to enhance the understanding of complex biological processes such as apoptosis in the developing brain. High throughput molecular biological approaches including genomics, proteomics and metabolomics provide the fundamental data necessary for the building blocks of biological systems. As these databases grow and become linked together as integrative modules, they provide the intermediate components necessary for use in a systems biology approach. The overall goal is to determine the appropriate placement of these biological modules into a mechanistic scheme that allows for the development of integrated computational models. However, the development of these mathematical models often lags behind the initial definition of the system and this is true for the current example.

The developing nervous system varies in susceptibility to neurotoxic insults depending on stage of development. Because of the complexity and temporal features of the manifestations of developmental neurotoxicity, this area of study may benefit greatly from a systems biology approach. Systems biology, as applied to toxicological problems, might provide a structure around which to arrange information in the form of a biological model. The goals of applying a systems biology approach to developmental neurotoxicology are to predict the functional outcomes of component-to-component relationships using computational models that allow for the directional and quantitative description of the response of the complete organism in response to perturbations of its component systems. These perturbations may come in the form of environmental alterations or exposures to drugs or other chemicals or infectious agents. Thus, the application of a systems biology approach towards understanding issues relevant to developmental neurotoxicology has the potential to help advance the understanding of brain-related biological processes, including neuronal plasticity and toxicity. Achieving these goals will be most challenging.

In this Editorial, we discuss how systems biology, pharmacogenomic and behavioral approaches, as applied to important problems in developmental neurotoxicology, have provided a structure around which we are beginning to arrange information to form a helpful—hopefully predictive--biological model. The approaches that can be used as effective tools in dissecting out mechanisms underlying pharmacological and toxicological phenomena associated with exposures to drugs or environmental toxicants during development will be discussed.

Peri-surgical neurotoxicity continues to garner considerable interest among anesthesiologists and a growing concern about the issue is anticipated from surgeons and toxicologists. Here, we shall focus the discussion on representative general anesthetics--primarily ketamine--as examples of how the systems biology approach can be employed to begin to describe how specific receptor subunits and intracellular signaling events are involved in the expression of anesthetic-induced neurotoxicity during sensitive developmental stages. Four steps employed in a systems biology approach have been reported by Leroy Hood's group [1] and the application of these steps in a developmental neurotoxicity context will be discussed.

Step 1: Available information about the biological system of interest will be described and a preliminary model of how the system functions will be formulated. Evidence in support of a correlation between surgery and subsequent cognitive changes has accumulated [2-4], but at present, causality can not be concluded for either extreme of the life span. Nor have any molecular, cellular, or pathophysiological events linking peri-surgical events to cognitive outcomes been identified in the clinical literature. Challenges include the choice and standardization of the cognitive domain(s) to be tested, the timing of such testing and controlling for the effects of anesthesia and surgery. Meanwhile, a growing body of preclinical data [5-9] implicates the involvement of N-methyl-D-aspartate (NMDA)-type glutamate receptors in the etiology of the neurotoxic effects of several anesthetic agents. Also, the degree to which the nervous system is resistant to neurotoxic insults is highly dependent upon the stage of development [10] at exposure. Based on data from a variety of approaches and different animal models (from *in vitro* to *in vivo*, from rodent to nonhuman primates), it has been postulated that exposure of the developing brain to NMDA antagonists [e.g., ketamine or phencyclidine (PCP)], causes a relatively rapid compensatory up-regulation of NMDA receptors (increase in number), thereby making cells bearing these receptors more vulnerable to the excitotoxic effects of endogenous glutamate after removal of the NMDA receptor antagonists [8-12]. Although comprehensive gene expression and proteomic studies and mathematical modeling have yet to be completed, a biological model has been established as a first step in the process. The model has been perturbed in an iterative manner to confirm some of the biological pathways participating in the brain cell death induced by exposure to certain anesthetics during development.

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Step 2: Where possible, the genes and proteins involved in the described pathways should be defined. In order to understand the underlying mechanism of ketamine-induced neurodegeneration, brain tissue from the frontal cortical levels, where the most severe neuronal damage occurs, was selected for RNA isolation and microarray analysis. Here, the damage was characterized by large increases in TUNEL labeling which is thought to be relatively specific for broken DNA strands and which is often indicative of apoptotic processes. Consistent with the TUNEL labeling and other *in vivo* data, a total of 32 genes were found to be involved in apoptosis: 15 genes were up-regulated and 17 genes were down-regulated in ketamine-exposed animals [13]. Apoptosis-related genes consist of those that have two distinct modes of operation: pro-apoptosis or anti-apoptosis. In response to various stimuli such as stress or sustained elevations of intracellular calcium levels, the ultimate fate of a brain cell is determined by the roles of these apoptosis-related genes in regulating the life/death cell balance. While the mechanism(s) underlying ketamine-induced neuronal cell death have not been fully elucidated, our microarray data indicate that the majority (approximately two-thirds) of up-regulated genes are pro-apoptotic in nature (Agt, Clu, Gjb6, Hrk, Igfbp3, Inpp5d, Jun, Mal, Rassf5 and Txnip [13]. On the other hand, about one-half of the 17 genes that are down-regulated are anti-apoptotic genes [13]. Taken together these observations indicate that the frontal cortex is the brain region most vulnerable to ketamine-induced neurotoxicity during development and that the survival of neurons in the early phases of the apoptotic cascade depends on the balance between pro- and anti-apoptotic factors.

Because ketamine is an NMDA receptor antagonist, it was postulated that the localization of the most severe neurodegeneration in the frontal cortex should correspond to alterations in NMDA receptor expression levels in that tissue. To examine potential underlying mechanisms, an oligonucleotide probe complementary to the mRNA encoding the NMDA receptor NR1 subunit was used for *in situ* hybridization. The autoradiographic density (labeling) of NR1 subunit mRNA was increased in the frontal cortex of brains treated with ketamine. Changes in NMDA receptor R2 family genes were also monitored: *Grin2a (NR2A*) and *Grin2c (NR2C*) were significantly up-regulated in ketamine-treated animals whereas no significant effects were observed in *Grin2b* (*NR2B*) or *Grin2d* (*NR2D*) as detected in microarray experiments and subsequently confirmed using TaqMan analyses [13]. It should be noted that NMDA-R2 subunits produce functional receptors only when combined with NMDA-R1 receptor subunits [14] and that heteromeric complexes increase receptor responsiveness to NMDA and yield different functional properties [15]. Our findings are consistent with those of previous *in situ* hybridization and immunoblotting data (protein expression levels) that demonstrated a compensatory up-regulation of NMDA-R1 and NMDA-R2 receptors following prolonged exposure to NMDA receptor antagonists [10,11,16].

Step 3: Associated physiological parameters and pharmacokinetic experiments providing information across important periods of development will be considered. For any animal model it is essential to monitor and control physiological parameters. These parameters are carefully controlled during pediatric anesthesia but can be very difficult to control in rodent models. Thus, the nonhuman primate provides a model that is ideal for these types of experiments. During anesthesia, all physiological parameters including percent oxygen saturation, exhaled carbon dioxide, body temperature, heart rate, blood pressure, glucose, and hematocrit can be monitored and maintained within normal ranges in the same manner as in the pediatric clinic. Because prolonged hypoperfusion can lead to cerebral hypoxia and ischemic-related cell death, it is necessary to maintain normal blood pressure and oxygen saturation [10] and this was readily accomplished in our nonhuman primate studies.

Plasma ketamine concentrations are related to neuronal cell death in in a dose-related fashion, with higher doses causing more death. In perinatal monkeys, steady-state plasma ketamine concentrations of 10-25 ug/ml were achieved during prolonged periods (up to 24 hours) of anesthesia. These plasma levels of ketamine are necessary to maintain anesthesia in this experimental model. However, it is important to note that monkeys at different stages of development require different ketamine plasma concentrations to maintain anesthesia. For example, PND 35 animals required a higher plasma concentration of ketamine to maintain the same level of anesthesia as PND 5 animals. Another important finding was that even though the plasma concentrations of ketamine were highest in the PND 35 monkeys, there was no evidence of increased neuronal cell death whereas in PND 5 animals neuronal cell loss was significant [10,17].

In the rodent model [11] plasma and brain tissue ketamine levels peaked within 5 min of ketamine injection (ip) and dropped to approximately zero within six hours after the last dose. Assessment of nucleosomal DNA fragmentation (a potential apoptotic marker) revealed that this marker was not significantly affected 5 min – 4 hour after ketamine administration. However, after longer withdrawal times (6 hours or more) when plasma and brain ketamine levels were approximately zero, the neuronal apoptotic marker was significantly increased. These data indicate that ketamine-induced neural damage is not due to direct toxic effects of ketamine, but through some secondary mechanisms, such as altered NMDA receptor expression.

Step 4: Various global datasets, complex behavioral capabilities and dynamic imaging data will be integrated to determine if they support the model. Discrepancies will be identified and hypotheses-driven studies will be conducted in order to address them. Thus, data generated via iteration of the third and fourth steps will be used to reformulate the model in light of new data. To identify biomarkers such as apoptotic pathway signatures and to determine their validity for predicting subsequent cognitive outcomes related to toxicant exposure during development, PET/CT and/or PET/MRI imaging slong with cognitive behavioral assays should be considered important steps.

Currently, the effects of specific anesthetics and surgical variables on subsequent cognitive performance are still not completely understood, nor have any molecular imaging and pathophysiological measurements linking peri-operative events with cognitive outcomes been discerned from the human data. It is proposed that molecular imaging with isotope-labeled biomarkers (radio-tracers) will help detect neurotoxicity in neonates, infants, and young and adult monkeys and humans alike. The high-resolution positron emission tomography scanner (microPET) combined with CT or MRI can provide *in vivo* molecular imaging at a sufficient resolution to resolve both major structures and neuronal activities in the rodent, nonhuman primate and human brain. To determine whether prolonged pediatric anesthetic exposure is likely to be associated with subsequent long-term cognitive deficits, anesthetic-induced neurodegeneration can be repeatedly assessed *in vivo* by monitoring changes in the uptake (binding) of specific radiotracers [e.g., [^{18}F]-Benzodiazepine Receptor ligand (PBRS)] that can highlight adverse events such as neurotoxicity and gliosis. Caspase inhibitors and other apoptosis markers (e.g., Annexin V) can be monitored in specific regions of interest in the rodent and monkey brain and hopefully in humans. The severity

of anesthetic-induced neural damage (e.g., apoptosis) can then be determined by the size of the area in which the radioactive tracers are found and the concentration of the tracers in those areas [18-19]. Cognitive function can be assessed in the same animals in parallel using behavioral instruments such as the National Center for Toxicological Research (NCTR) Operant Test Battery (OTB) [20-21] which includes tasks for monitoring aspects of learning, motivation, color and position discrimination, and memory. The demonstration that several measures of OTB performance correlate highly with measures of intelligence in children [22] serves to highlight the relevance of such measures. The time course of treatment-related cognitive deficits can, thus, at least in theory, be associated with underlying biochemical changes in brain. Recent observations have demonstrated that a single 24-h episode of ketamine anesthesia, occurring during a sensitive period of brain development, can result in very long-lasting deficits in brain function in primates [23]. These data provide proof-of-concept that general anesthesia during critical periods of brain development can result in subsequent functional deficits. The use of dynamic molecular imaging approaches, when used in parallel with sophisticated behavioral assessments, could greatly decrease the uncertainty in extrapolating pre-clinical data to the human condition.

Summary

This Editorial provides an overview of our efforts to apply a systems biology approach to help understand a particular toxicological problem: anesthetic-induced neurodegeneration in the developing nervous system. By monitoring alterations in several critical processes it is hoped that we will be able to define the affected system(s) in an integrated manner.

Although more studies are needed in order to build a quantitative model, some general developmental neurotoxicology pathways have been identified using the four steps of a systems biology approach. Further elucidation of the precise pathway(s) and developmental stages associated with susceptibility to anesthetic agents will also require additional studies.

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