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Running Head: Adverse Outcome Pathway Modeling Strategies

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Defining and Modeling Known Adverse Outcome Pathways: Domoic Acid and Neuronal Signaling as a Case Study

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ABSTRACT

An adverse outcome pathway (AOP) is a sequence of key events from a molecular-level initiating event and an ensuing cascade of steps to an adverse outcome with population level significance. To implement a predictive strategy for ecotoxicology, the multiscale nature of an AOP requires computational models to link salient processes (e.g., in chemical uptake, toxicokinetics, toxicodynamics, and population dynamics). A case study with domoic acid was used to demonstrate strategies and enable generic recommendations for developing computational models in an effort to move toward a toxicity testing paradigm focused on toxicity pathway perturbations applicable to ecological risk assessment. Domoic acid, an algal toxin with adverse effects on both wildlife and humans, is a potent agonist for kainate receptors (ionotropic glutamate receptors whose activation leads to the influx of Na^{+} and Ca^{2+}). Increased Ca^{2+} concentrations result in neuronal excitotoxicity and cell death primarily in the hippocampus, which produces seizures, impairs learning and memory, and alters behavior in some species. Altered neuronal Ca^{2+} is a key process in domoic acid toxicity which can be evaluated in vitro. Further, results of these assays would be amenable to mechanistic modeling for identifying domoic acid concentrations and Ca^{2+} perturbations that are normal, adaptive, or clearly toxic. In vitro assays with outputs amenable to measurement in exposed populations can link in vitro to in vivo conditions, and toxicokinetic information will aid in linking in vitro results to the individual organism. Development of an AOP required an iterative process with three important outcomes: a critically reviewed, stressor-specific AOP; identification of key processes suitable for evaluation with in vitro assays; and strategies for model development.

Key words: Hippocampus, Neurobehavioral Algal Toxin Calcium

INTRODUCTION

Regulatory toxicology has relied largely upon whole-animal studies and measures of apical endpoints to quantify chemical exposure concentrations that result in different levels of effect [1]. In addition, classic toxicity studies have been used to assess dose-response relationships and estimate chemical concentrations that are unlikely to produce adverse outcomes. Human health risk assessment focuses on minimizing individual-level adverse effects, whereas ecological risk assessments focus on population-level effects, and only in the case of threatened and endangered species are individual-level effects of concern. Thus, adverse outcomes relevant for ecological risk assessment focus more frequently on development, survival, growth, and/or reproduction [2]. With thousands of manmade chemicals that need to be evaluated for regulatory purposes [3], obtaining whole-animal or population-level data is impractical, and a predictive strategy has been recommended by the National Research Council (NRC) based on in vitro toxicity assays which predict cellular level effects that can be extrapolated to effects on individuals [1].

To implement a predictive strategy for ecological risk assessment, results from in vitro toxicity assays focused on cellular responses to molecular initiating events will need to be extrapolated to effects upon organisms and ultimately to populations. A conceptual framework that links a molecular-level initiating event with adverse effects relevant for risk assessment has been called an adverse outcome pathway (AOP) [2,4]. The first step is to evaluate organism exposure to a chemical(s) in the environment. This includes anthropogenic introduction of a chemical toxicant, or the natural formation of a toxin in the environment, and subsequent distribution (i.e., fate and transport) to individual organisms. Once a chemical enters an organism, disposition of the toxic moiety to target cells must be understood as a chemical may be metabolized, resulting in metabolites that are more toxic than the original chemical. After a toxic

chemical reaches a target tissue, a molecular initiating event occurs that results in a cellular response, which has been called a toxicity pathway (TP) [1]. Finally, the sequence of events between cellular response and adverse outcome upon an individual organism or population of organisms is an AOP. Each of the steps described above requires review of the existing literature, articulation of what is known, and the identification of data necessary to inform regulatory management decisions.

At the core of the NRC vision and predictive ecotoxicology lies the use of in vitro assays. These assays will need to be developed to detect perturbations of normal functioning in a target cell/tissue. They should be sensitive for testing a wide dose range, including low doses below a threshold for perturbation; doses with adaptive responses; and doses with adverse responses. In order to provide a mechanistically sound basis for extrapolating in vitro assay results to in vivo responses, computational models will be needed that connect pathway perturbations with biological processes that occur at higher levels of organization (tissue, organism, and population). This type of computational modeling in predictive ecotoxicology is still relatively limited and new models will be needed to fill specific gaps.

Toxicology has a rich history of the use of AOP models, also known as exposure-dose-response models and mode-of-action models, for improving risk assessment. These are mostly chemical-specific and entail detailed descriptions of chemical disposition, i.e., toxicokinetics and toxicodynamics. For example, a cancer risk assessment for vinyl chloride used a physiologically based toxicokinetic model to relate outcomes across various mammalian species with the rate of formation of the reactive epoxide metabolite in liver [5,6]. Computational models useful for ecological risk assessment include models developed to predict reproductive endpoints such as basal oocyte maturation in salmon (*Oncorhynchus kisutch*) [7]; and changes in the production of

vitellogenin (Vtg, a precursor to a major egg yolk protein) [8-10]. To predict the effect of hypoxia on Atlantic croaker (*Micropogonias undulatus*) fecundity, Murphy et al. [11] used model predictions of cumulative Vtg production and an assumption that cumulative Vtg production in Atlantic croaker is directly related to fecundity based on a statistical model relating fathead minnow plasma Vtg concentrations with changes in fecundity [12]. Fecundity can then be used as input into a population dynamic model to predict effects upon a population of fish [13]. Models such as these will be needed to relate in vitro assay results to relevant environmental conditions and ecological endpoints in an AOP.

As an extension of the toxicity testing principles described by the NRC [1] for ecological risk assessment, Workgroup 1 was asked to recommend strategies for how computational models of AOPs can be developed from the extant literature, and to anchor these strategies by developing a case study. The case study focused upon excitatory neurotoxicity mediated through chemical interactions with GABA (γ-aminobutyric acid) and glutamate neurotransmitter systems (**Figure 1**). In particular, we chose to use the algal toxin domoic acid because of its adverse effects on both wildlife and humans. Recognition of the domoic acid environmental problem and extensive scientific study has been both recent and intense involving diverse scientific disciplines (e.g., oceanography, public health, toxicology, medicinal chemistry and ecology). A rich peer-reviewed literature base exists that can be mined for the development of an AOP for domoic acid, and the molecular initiating event and relevant ecological risk endpoints are known. Throughout the case study, the following questions were kept in mind. At what concentration will biologic perturbation(s) be likely to alter normal processes beyond adaptive capacities and lead to an adverse outcome? What in vitro tests can be developed to evaluate pathway perturbations? How can these in vitro test systems be described by computational modeling to better assess

perturbations across a wide range of concentrations? What kinds of data are needed to connect TPs to an AOP?

The ultimate goal of the case study was to demonstrate strategies that support recommendations for developing computational models of known AOPs that facilitate moving beyond the current toxicity testing paradigm focused on chemical-specific toxicity to a focus on biological system perturbations relevant for ecological risk assessment. A nine-member workgroup was convened from disciplines of neurotoxicology, wildlife biology, ecotoxicology, and engineering to develop strategies for computational model development in support of predictive ecotoxicology. The workgroup specifically developed: a strategy for systematically mining the literature for relevant information; strategies for constructing a conceptual framework for a multi-scale AOP model with integration of data/information from disparate sources; an approach to identify critical data needs for transforming a conceptual model framework into a dynamic, computational model; and a tractable research strategy for evaluating predictive capabilities of a model and refining it for utility in ecological risk assessment. The purpose of the case study was to guide members through the process of developing practical strategies and recommendations.

CASE STUDY

Domoic acid is an amino acid originally isolated from the marine red alga (*Chondria armata* Kutz.) in 1958 [14]. Domoic acid was later confirmed to be the causative agent in an accidental poisoning in 1987 on Prince Edward Island, Canada, when more than 100 people became ill and three people died after ingesting contaminated blue mussels, *Mytilus edulis*, [15]. The clinical symptoms of domoic acid poisoning included confusion and selective short-term memory loss. Thus, the term amnesic shellfish poisoning (ASP) was introduced [16]. The source of domoic

acid was traced to a bloom of marine diatoms from the genus *Pseudo-nitzschia* (PN) [17], and up to nine species of PN are potential domoic acid producers [18]. The 1987 poisoning on Prince Edward Island received considerable international attention in part because of the widespread occurrence of PN in temperate ocean regions and the recognition that domoic acid poisoning is likely a worldwide problem. In recent years, outbreaks of domoic acid have been documented in New Zealand [19], Japan [20], Denmark [21], Scotland [22,23], France [24], Spain [25,26], Portugal [27] and Ireland [28]. In North America, reoccurring outbreaks of domoic acid have occurred on the U.S. West Coast since 1991. Outbreaks of domoic acid near Monterey Bay, California,USA, killed hundreds of brown pelicans (*Pelecanus occidentalis*), cormorants (*Phalacrocorus penicillatus*) and several species of marine mammals including California sea lions (*Zalophus californianus*) [29].

Toxicokinetics

The seabird and marine mammal deaths in addition to the human poisonings have focused attention on the penetration and persistence of domoic acid in marine food webs and the diverse chemical dosimetry that exists among organisms both within similar and different trophic levels. Among filter feeding invertebrates such as bivalves, most species appear to readily absorb domoic acid during ingestion of PN. However, profound interspecies differences exist in the elimination of domoic acid with some species such as mussels (*Mytilus* sp) and oysters (*Crassostrea gigas*) exhibiting elimination half-lives on the order of hours to a few days while other species such as scallops (*P. magellanicus, P. maximus*) and razor clams (*Siliqua patula*) exhibit elimination half-lives of several months to years [30-34]. In other invertebrates such as decapod crustaceans and cephalopods, exposure occurs through predation on contaminated bivalves or crustaceans such as krill, with highest tissue levels of domoic acid typically occurring

in the hepatopancreas or digestive gland [35,36]. Because of the biological persistence of domoic acid in many invertebrate species, they serve to provide a source of domoic acid well beyond the time period of a PN outbreak, creating the potential for prolonged or repetitive exposures in higher trophic level species. Interestingly, despite clear evidence for bioaccumulation, there have been no documented adverse effects of domoic acid on invertebrates [37].

A thorough understanding of domoic acid toxicokinetics within vertebrate species is lacking. Among vertebrate groups such as birds and mammals, there is little evidence to indicate domoic acid is metabolized to any significant extent. Excretion is typically rapid and appears to occur primarily through urinary elimination [38,39]. In fish and shellfish, there is evidence to suggest domoic acid is metabolized to several different isomers – epi-domoic acid and isodomoic acid A and B [40,41]. However, these domoic acid derivatives are also naturally produced [42] and it remains to be definitely established whether biotransformation of domoic acid occurs. There are few detailed studies on the gastrointestinal absorption of domoic acid. Indirect evidence based on the induction of neurological effects after oral dosing suggests that for most species domoic acid is at least partially absorbed from the gastrointestinal tract [43]. However, urinary elimination data for domoic acid during repeated dosing in rats indicated less than 5 % of the oral dose was being absorbed [44]. In naturally exposed fish such as the northern anchovy (*Engraulis mordax*), domoic acid accumulates in the liver with much lower levels found in muscle and brain tissue [45]. Northern anchovy and Pacific sardines (*Sardinops sagax*) collected simultaneously in Monterey Bay, California, weekly for one year revealed similar occurrence patterns of domoic acid in the viscera, however, anchovies consistently accumulated higher levels than the sardines [46]. The presence of domoic acid in the viscera was closely correlated

to the presence of toxic diatom species in the water, suggesting that the toxin is not retained for long periods of time in the viscera following feeding on toxic cells (toxin levels in the viscera of fish were 250-1800 times higher than those found in body muscle tissue). In Coho salmon (*Oncorhynchus kisutch*) administered an oral gavage dose of domoic acid (10 mg/Kg) the toxin was well absorbed from the gastrointestinal tract with the kidney having the highest peak tissue concentration of 9000 ng/g [47]. The bile:liver concentration ratio was 10:1 after 24 h dosing, suggesting biliary excretion may be an important elimination route in fish [47]. This latter finding may help explain earlier studies in mammals, which did not consider this elimination route and the possibility of significant first pass elimination by the liver via biliary elimination. In salmon, the brain and plasma concentrations of domoic acid were both very low after oral dosing and never exceeded 250 ng/g [47]. Intracoelomic injections of various doses of domoic acid to salmon indicated the fish were susceptible to excitatory neurotoxic effects, with a sensitivity comparable to mammals (EC50 = 2.6 to 5.6 µg/g fish weight) [47]. These findings suggest that the lack of evidence for large scale fish mortalities associated with PN outbreaks is more closely linked to toxicokinetic differences between fish and mammals than toxicodynamic differences.

Toxicology

Individuals that were severely intoxicated during the 1987 poisoning incident presented clinical symptoms indicating the gastrointestinal tract and the cardiovascular and nervous systems were compromised. The primary neurological symptoms observed were seizures and loss of memory indicating neuronal hyperexcitability and excitotoxicity as the most probable TP for domoic acid. Histopathological hallmarks for deceased patients were neuronal death in several brain regions, among them the hippocampus, nucleus accumbus, thalamus, olfactory,

septal and subfrontal cortical areas. In the hippocampus, which plays major roles in learning and spatial memory, the CA1, CA3 and CA4 but not CA2 regions were seriously affected (reviewed in Pulido [48]). Sea lions intoxicated with domoic acid were subsequently found to have persistent seizures and their brain histopathology showed cell death in hippocampus (CA1, CA3 and CA4 regions) and other limbic structures [49]. Experimental rodent studies also showed similar histopathological findings [50,51].

Domoic acid is an agonist for presynaptic and postsynaptic kainate receptors (Figure 1). Kainate receptors together with NMDA (N-methyl D-aspartate) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors belong to the family of ionotropic glutamate receptors whose activation leads to the influx of Na^+ and Ca^{2+} [52]. Glutamate is the main excitatory neurotransmitter in vertebrates and invertebrates. In combination with the inhibitory GABA neurotransmitter, glutamate contributes to the control of neural excitability. Kainate receptors are localized both at pre- and postsynaptic sites. At presynaptic sites, they directly affect transmitter release from both excitatory and inhibitory neuron terminals. At postsynaptic sites, kainate receptors lead to cell depolarization, which would bring the neuron closer to its spike firing threshold. By having this dual localization, kainate receptors help in the control of neuronal excitability. However, sustained activation of postsynaptic kainate receptors by domoic acid results in massive ion flux, and excessive release of glutamate from excitatory terminals. The released glutamate may in turn activate NMDA receptors, which have lost their physiologic Mg^{2+} block due to domoic acid-induced depolarization. The final event is an increase of NMDA-mediated Ca^{2+} flux and subsequent activation of intracellular prooxidative cascades and ion imbalances eventually leading to excitotoxicity-mediated neuronal death [53,54].

Kainate receptors are widely expressed in the hippocampus. Glutamatergic granule cells in

the hippocampus express these receptors, suggesting that cell death found after domoic acid intoxication may be produced by hyper-stimulation of NMDA receptor after glutamate is released in excess. In agreement with this hypothesis, the seriously damaged CA3 area of the hippocampus receives projections from hippocampal granule cells. Qiu and Curras-Collazo [55] elegantly demonstrated that domoic acid first targets kainate receptors in the hippocampus by blocking its effects in vivo with a kainate receptor antagonist. The sequential involvement of distinct glutamate receptors was confirmed and further elucidated in rat mixed cortical cell and hippocampal slice cultures [56,57].

The progression of the neurotoxicity pathway for domoic acid from merely activating kainate receptors to the activation of both kainate and NMDA receptors might determine different neural AOPs manifested as seizures and neuronal death. These events in vivo were reproduced in vitro in a series of studies using primary cultures of rodent cerebellar granule cells, an in vitro model mainly constituted by glutamatergic neurons that express both NMDA and kainate receptors [53,58,59]. In this system, domoic acid increased glutamate release, intracellular calcium and cell death that were prevented by kainate and NMDA receptor antagonists [54,60,61]. Whether cell death was necrotic or apoptotic depended on domoic acid concentration [62]. The parallel responses observed in vivo and in vitro support the NRC notion that in vitro toxicity assays can have useful predictive value for extrapolating effects to individuals [1].

Conceptual framework for excitatory neurotoxin AOP

An AOP spans multiple levels of biological organization to link molecular initiating events (i.e., target cell perturbations) with adverse outcomes relevant for ecological risk assessment. The core of any AOP model is the TP, which by definition extends only to the cellular level (**Figure 2**). The TP model must be developed with sufficient detail to adequately describe key

cellular responses such that, when sufficiently perturbed, loss of function and eventual cell death can be accurately predicted [1]. During TP development it is important to remember that key cellular functions must be measureable via in vitro testing, since the majority of testing and data generation in the future is expected to be in vitro in nature. For domoic acid toxicity, the most consistent and prominent adverse outcome is memory loss, with the underlying etiology reasonably well defined as: domoic acid exposure → excitatory neurotoxicity → hippocampal lesions → memory loss and neurobehavioral changes. This relationship between exposure and outcome provides a foundation for a conceptual AOP framework that links a key molecular initiating event (domoic acid-induced excitatory toxicity) with adverse outcomes that are relevant for individual health (loss of memory and/or critical behavioral responses to environmental stimuli) and ultimately ecological risk assessment (survival, growth, and reproduction) (Figure 2).

To construct the AOP, it will be important to consider and integrate the weight of evidence from diverse studies (acute/chronic; lab/field; lab animals/marine mammals) that support causal, mechanistic, inferential, and correlational relationships across these multiple levels of biological organization. The core of an AOP for the neurotoxicity of domoic acid is the proposed excitatory toxicity pathway for hippocampal neurons. A challenge in developing this excitatory neurotoxicity AOP is the diversity of structures, functions and interactions of the various cell types found in vertebrate and invertebrate nervous systems. The central nervous system (CNS, the brain and spinal cord) is composed of four major cell types that interact in dynamic structural and biochemical contexts to generate organ function: neurons (cells that generate action potentials); astrocytes (cells that maintain metabolic and ionic organ homeostasis); oligodendrocytes (myelinating cells); and microglia (monocyte-derived cells). Neurons are

responsible for the perception of sensory stimuli and the coordination of cellular, tissue, and organismal responses to stimuli from the environment. Neurotoxic processes encompass more than cytotoxic effects, as toxicants usually produce sub-lethal, functional impairments at low or moderate exposure levels. Various cells may demonstrate different sensitivities to toxicants, as well as present different developmental windows of vulnerability. Furthermore, a neurotoxicant that alters the activities of a particular cell type also induces secondary changes in the interactions between that responsive cell and other cell types [63].

The cellular response pathway perturbed by domoic acid is glutamatergic neurotransmission within excitatory neurons. Activation of kainate-receptors by glutamate opens ion channels in the glutamatergic neuron, and allows a flux of Na^+ and Ca^{2+} from the extracellular to the intracellular spaces. In normal function, the intake of sufficient amounts of Ca^{2+} (and Na^+) causes membrane depolarization and propagation of an impulse along the neuron. With glutamate-induced hyperstimulation of neurons, excitatory neurons accumulate excess Ca^{2+}, initiating second messenger cascades, and at high enough levels of excitation and cellular Ca^{2+} this leads to cell death. With high-level exposures to domoic acid, the enhancement of Ca^{2+} intake occurs through an initial stimulation of the kainate receptors. While excess Ca^{2+} is toxic, Ca^{2+} is an essential component of excitatory CNS-signaling. In assessing likely adversity of intracellular Ca^{2+}, it becomes important to distinguish required levels of Ca^{2+} from that combination of increased intracellular Ca^{2+} and time of exposure that are expected to have adverse consequences for glutamatergic cell function.

In the new toxicity testing paradigm [1], appropriate cellular systems need to be developed that are amenable to computational modeling to predict expected dose-response behaviors for adaptive (low level changes in Ca^{2+}) and excessive, prolonged perturbations of Ca^{2+}. The in

vitro assays may include receptor-binding assays (e.g., determining whether a toxin inhibits [^{3}H]kainate binding), fluorimetric assays quantifying intracellular Ca^{2+} during treatment with domoic acid; and cell viability assays. In contrast to the NRC report [1], in vitro assay systems for ecological risk assessment may need to be simultaneously developed using cells/cell lines for a variety of environmental species. The design of an assay system would rely heavily on the current understanding of the biology of Ca^{2+} signaling in neuronal cells and the state-of-the-art in evaluating cellular Ca^{2+} dynamics in vitro (see **Figure 3**).

Dose-response models for glutamatergic neuronal function

The in vitro pathway assays will provide quantitative results over a broad range of in vitro exposure concentrations. These data sets will be amenable to more extensive dose response modeling than data sets from most in vivo assays. The detailed dose-response curves from in vitro assays would also be more amenable to statistical analysis for evaluating effective thresholds and possible non-linear characteristics of responses at low levels of response. More importantly, the breadth of data from the studies and the careful control of experimental conditions in a well-defined assay at an appropriate level of cellular detail provide the grist for developing dose-response models with much greater fidelity to the biology, in this case the biology of glutamatergic neurons and the consequences of excessive Ca^{2+} loads on these cells.

What level of biological detail will be required to have confidence in quantitative predictions from these models? The first challenge is to include in the pathway model sufficient detail to account for the major contributors to the outcome and of the biological system itself. With nerve impulse transmission, an electrochemical description was provided by Hodgkin and Huxley [64] and has been elaborated extensively [65,66]. Activation of an action potential by glutamate brings a burst of calcium into the cell. Technically, it should be possible to build a description

around the core elements of a Hodgkin-Huxley model to include the incremental changes in calcium with the successive activations of the Ca-channels. In addition, the dynamics of the description of the electrochemical processes would still need to link to a description of the control of intracellular calcium that drives the adverse responses in these neurons. The model developed to describe calcium fluxes in vascular smooth muscle cells [67], included several sub-compartments for calcium sequestration within the cells – mitochondria, sarcoplasmic reticulum, and bound cytosolic forms (see Figure 3). The kinetics of movement of calcium among these pools was partially derived from studies with radio-labeled calcium. An alternative to a full description would likely focus on calcium control in the neurons, with an uptake component determined from specific studies of calcium fluxes after glutamate stimulation in cells similar to those used for the proposed in vitro toxicity assay. Some computational studies of calcium fluxes in neurons are available [68], and have been simulated using neuronal cell models made available through academic programs at Duke and Yale. A tutorial at the Neuron web-site (http://neuron.duke.edu) discusses modeling of calcium in presynaptic compartments. Another excellent tutorial for modeling Ca^{2+} in cells has been developed by Blackwell (http://www.brains-minds-media.org/archive/224). Actual development of the TP model structure for calcium transients in response to domoic acid stimulation of glutamatergic receptors was considered to be outside the scope of this workshop.

The overall structure of the computational model of the glutamatergic neuronal signaling pathway is likely to have some key elements. Once developed, this model structure should be useful for a variety of toxicants with glutamatergic neuronal targets, not only domoic acid, and will lay the foundation for toxicity pathways that affect other neuronal signaling pathways related to ion-channel function and neuronal viability with persistent stimulation. The process of

validating these pathway assays includes coordinated development of the tools to look at the normal Ca^{2+} signaling. Outputs of the in vitro pathway model include: concentrations of the test compound below which changes are indistinguishable from normal cell behaviors; concentrations causing alterations within the bounds of normal variation; adaptive changes with some changes in Ca^{2+}, but below levels causing adverse responses at the cellular level; and concentrations causing overtly adverse responses, i.e., cytotoxicity, apoptosis, etc.

Predictive in vitro assays for excitatory neurotoxins

When in vitro systems are designed for neurotoxicity testing relevant to an AOP, appropriate endpoints must be selected (see Supplemental Data). Three major classes of endpoints can be measured in culture: cell viability and cell death on either an individual cell or cell population level; generic cell functions that are not specific to neurons or glia, such as respiration, plasma membrane function, Ca^{2+} homeostasis, and oxidative stress responses; and differentiated cell functions, such as axonal transport, synapse function, myelination, neurotransmitter uptake and metabolism. With respect to domoic acid-like neurotoxicity, three components that must be present are: kainate receptors, NMDA receptors and a glutamate-releasing system. Furthermore, the culture system has to be easy to prepare and maintain, and amenable to use in high-throughput platforms. Methods to prepare primary cultures of rodent cerebral or cerebellar granule cells are currently available that fulfill these requirements. These cultures are prepared from 16 to 18-d old embryos or from 8-d old rat pups [57,58]. These culture systems are enriched in glutamatergic, cholinergic and GABAergic neurons. The neurons mature at 6 to 10 days in culture expressing functional receptors for NMDA and kainate [54,57,59,61,69] and releasing glutamate under depolarization [53]. When glutamate surpasses a concentration threshold, cell death occurs by a mechanism including NMDA receptors [53] or oxidative stress

[54]. The cultures can be kept in vitro for up to two weeks. Future development of immortalized cell lines is needed to increase reliability and decrease reliance on animals.

Increases in intracellular Ca^{2+} concentration and cell membrane depolarization due to activation of NMDA and kainate receptors can be measured by fluorescent probes. Cell viability can be determined by measuring the incorporation of a fluorescent probe through damaged membranes or by determining the reduced activity of mitochondria (MTT assay), which correlates with cell death. All these assays can be performed in 96-well plate cultures with spectrophotometer or fluorimeter plate readers [59,70]. Compounds found to be positive in this system can be challenged with specific kainate receptor antagonists to verify whether they can be catalogued as domoic acid-like toxicants. If so, a prediction could be made that they might share the domoic acid AOP. The quantitative data obtained from this type of system (e.g., binding coefficients, effective concentrations, and time course of effect) could provide a quantitative approach to predicting relative toxicity of chemicals and differentiating sub-threshold, adaptive and adverse levels of stimulation.

Additional considerations in the development of assays for neurotoxicity pathways are their usefulness for extrapolation to other toxicants (see Supplemental Data) and for other target species. Whether the assay incorporates the types of measurements likely to be made in the field will be important for in vitro – in vivo calibration of the AOP model and ultimately improve extension to population-level modeling. In this regard, in vitro toxicity pathway assays should ideally measure neurochemical parameters that are hardy in field situations and thus potentially useful as biomarkers. The calibrating and anchoring of in vitro data with field data should be approached with two goals in mind: designing in vitro test strategies with end points that are practical in field situations and identifying biomarkers that are applicable to multiple species. In

the field, brain tissue may be subject to postmortem degradation from factors such as temperature and time. For example, hours or days may elapse before tissue can be collected from a beached organism in hot summer and then frozen at -80 °C. In a series of recent ecological studies concerned with methylmercury neurotoxicity, several neurochemical biomarkers were shown to be hardy in field situations and measurable in brain tissues obtained from carcasses of mammalian [71-73], marine [74,75], and avian [76] wildlife. For domoic acid neurotoxicity, recommended neurochemical biomarkers that have been shown to resist postmortem degradation include NMDA (3H-MK801 radioligand receptor binding [77]); glutamic acid decarboxylase (GAD) activity (^{14}C enzymatic assay [78]); and glutamine synthetase activity [78]. Key neurochemicals such as GABA(A) receptors and GABA-T activity are not stable postmortem [78], while others such as the kainic acid receptor and glutamate transporters to our knowledge have not yet been extensively evaluated for postmortem stability.

Cellular Response to Adverse Outcome: Establishing Empirical Relationships

Several studies have established that the hippocampus is a major target for domoic acid. Laboratory studies on domoic acid exposed mice [50,79,80], monkeys [81,82], and rats [83], field studies on domoic acid poisoned marine mammals [49,84,85], and autopsies on domoic acid poisoned humans [86] consistently revealed dense degeneration of hippocampal cells (both neurons and glia), particularly within the CA3 and dentate gyrus regions. The cells in these hippocampal regions undergo atrophy, cytoplasmic vacuolization, edema, and swelling. The magnitude of these effects is dose-dependent. A review of hippocampal damage was presented earlier and highlighted the finding that domoic acid induced neurotoxicity across species, exposure scenarios, and study conditions is rather consistent [48]. Perhaps it is not surprising that hippocampal damage (and resultant impacts on memory and learning) are consistent across

species given that this brain region is conserved and comparable (anatomically, neurochemically, physiologically) across mammals, birds, and fish.

The hippocampus is part of the brain limbic system and plays a critical role in long term memory and navigation. Damage to the hippocampus in humans, rats, monkeys [87], birds [88], and fish [89] results in learning and memory impairments that tend to be either visual or spatial in nature. For example, lesions to the fish hippocampal zone (i.e., pallium) impairs temporal learning (active avoidance conditioning test) by approximately 60% and spatial learning (spatial-maze) by approximately 35% [89]. In studies on pigeons [90] and chickadees [91], aspiration of the hippocampus impaired homing performance and ability to relocate well-known places by approximately 25 to 46%. Using localized kainic acid injections to kill hippocampus cells in the rat, Stubley-Weatherly et al. [92] showed that animals had impaired ability in the acquisition of the water maze task and memory impairment on a passive avoidance task. These experimental results, collectively, provide correlative links between hippocampal function and loss of memory and spatial navigation, and support observations listed below that show domoic acid intoxication impacts memory and spatial navigation in diverse organisms. To help complete the AOP, such results (mainly focused on behavioral outcomes) can be used as nodes that can link to higher-level organismal effects (i.e., survival, growth, reproduction) and ultimately population level effects by use of empirical model calculations.

High doses of domoic acid cause seizures and memory loss in humans. Doses of domoic acid below that which causes seizure can have dramatic influence on behavior. These are presumably a direct result of domoic acid induced glutamatergic cell death in the hippocampus or orthologous structures. Behavioral effects are consistent across diverse groups of vertebrates and include locomotor behaviors, uncontrolled repetitive behaviors, and learning and memory

deficits. Tasker et al. [93] produced a semi-quantitative toxicity index for mice in which animals were scored for hypoactivity, sedation, rigidity, stereotypy, loss of postural control, and tremors which respectively represent increasing severity of domoic acid effects on the nervous system. Using this index, the acute effects of domoic acid at low doses can be quantified regardless of whether the domoic acid was from PN extracts, contaminated mussel extracts, or purified domoic acid [93].

At higher doses, domoic acid causes problems with locomotion in fishes and sea lions. Erratic swimming behaviors have been described in zebrafish and Coho salmon as circle swimming and spiral swimming [47,94]. Circle swimming has also been described in sea lions that received large doses of domoic acid [84]. Domoic acid does not cause problems with general locomotion at lower concentrations; however, hypoactivity is one of the most common endpoints described in the literature [93,95-99]. Goldstein et al. [84] describe lethargy as one symptom of domoic acid in sea lions. In rats, Levin et al. [96] describe a reduction in activity of nearly 20% that is attributed to rapid habituation of exploration of the novel environment in a figure-8 maze.

Tasker et al. [93] originally described stereotypic behaviors in mice following acute exposure to domoic acid from *Pseudo-nitzschia* extracts, contaminated mussel extracts, and purified domoic acid. Other rodent studies have described animals exhibiting stereotypic scratching, circling, head weaving and repetitive flexion-extension of the hindlimbs directed toward the head and neck as a result of developmental exposure to domoic acid [100] or acute PI exposure [98]. Zebrafish embryos and larvae exposed to domoic acid exhibit constant non-locomotory pectoral fin movements [101].

Hippocampal degeneration from domoic acid exposure results in both learning and memory deficits in exposed humans and experimental animals [86,102-105]. The severity of deficit

appears to be dose-dependent but integrating complex data across the several species and numerous learning and memory tasks does not lend itself to a quantitative mechanistic evaluation. Levin and Rose [97] described learning and memory deficits using a radial arm maze and spontaneous alternation T-maze tests, reflecting some of the long-term memory and spatial memory problems seen in humans [86]. Developmental exposure to low levels of domoic acid results in low grade seizures in response to novel environments during spatial cognition tasks [106,107]. Developmental exposure to domoic acid also alters nicotine-induced position place preference resulting in substantially more time being spent in the area associated with the chemical [95,108]. These studies support the idea that early, low-level activation of kainate receptors during a critical period of development results in alterations in behaviors that are related to the functional integrity of the mesocorticolimbic dopamine pathway [108]. If these behavioral phenotypes associated with low dose domoic acid exposure extend to wildlife species, one could imagine a significant impact on fitness.

A suggested approach for identifying neurochemical markers that can be compared between in vitro systems and field samples is as follows. First, candidate neurochemical biomarkers in tissues of wild-caught animals should be assayed in case control studies (controls versus animals killed by algal blooms) and associative/ecologic approaches should be used to correlate neurochemical biomarkers with tissue/brain levels of domoic acid. Second, these results should be amalgamated with results from the same assays carried out with in vitro models, lab animals, and wild animals of other species. The use of ecological animal studies of other species will be further considered below.

A complementary approach to identifying and validating neurochemical biomarkers that are meaningful in vitro and in field samples is to conduct laboratory studies of key sentinel species.

In this case, fish (e.g., zebrafish, trout, goldfish, and fathead minnows), birds (e.g., chickens, quail, and zebrafinch), and mammals (mink) would be fed controlled levels of domoic acid in the laboratory. Tissues would be obtained and preserved in an optimal manner so that molecular-level events (e.g., mRNA levels, protein levels, and DNA) might be further examined. Laboratory studies have already been performed in captive bony fish and one shark species for domoic acid toxicity, but the inclusion of additional species may enable ecotoxicologists to better predict species sensitivity. It may be particularly helpful to characterize kainic acid receptor binding and function in response to domoic acid in a series of organisms in order to explore species sensitivity, based on the assumption that kainic acid receptor binding is the key initiating event in domoic acid toxicity. Several ecotoxicology cases in which species sensitivity has been explored by studying differences in receptor ligand binding [109] or changes in receptor amino acid composition [110] provide a roadmap and rationale for this type of research.

DISCUSSION

In the preceding section, we have identified many challenges and needs faced by a predictive ecotoxicology paradigm using domoic acid as a case study. The NRC's [1] strategy for predictive toxicology recommends four phases of research: toxicity pathway elucidation; in vitro assay development; assay relevance; data assembly and validation. Extension of this strategy for predictive ecotoxicology requires AOP elucidation and the data to support development of quantitative AOP models as illustrated for domoic acid in **Figure 4**. Though we did not develop an actual computational model for domoic acid, we worked through the early steps in the process by reviewing the extant literature and developing conceptual models that help to identify data gaps which need to be filled prior to transforming a conceptual model to a computational one. In the following, we generalize what we have learned from the domoic acid case study to

environmental toxins and toxicants.

Mining the extant literature for relevant information

The traditional approach to mining the scientific literature for information relies upon manual searching of centralized literature repositories such as PubMed or Web of Science to identify relevant documents. The process of searching for information can be arbitrarily divided into at least three separate phases: exploratory, targeted, and manual evaluation [111,112]. Exploratory searching is initially used to acquire some perspective on the topic and perhaps identify a few review papers that summarize and interpret previous publications. For the initial development of the domoic acid AOP, exploratory searching quickly identified the Pulido [48] and Bejarano et al. [37] papers as the two most useful publications for developing the initial conceptual model. Once the initial model was developed, more focused questions could be asked for targeted searching on specific topics. For example, extending the domoic acid TP to an AOP required information on how hippocampal cell death leads to changes in behavior, which was obtained through a supervised literature search. Finally, most searches return numerous results and required time consuming manual evaluation of the publications to determine relevancy and usefulness in developing the AOP.

An alternative to manual searching is to use automated literature mining tools. There are now many approaches to automated searching such as Arrowsmith (http://arrowsmith.psych.uic.edu/arrowsmith_uic/index.html), which primarily searches titles for meaningful links between two different papers to the development of more advanced tools that can analyze grammatical structure of sentences [113, (http://biomedicalcomputationreview.org/4/3/6.pdf)]. We tried several automated literature searching tools including Arrowsmith, carrot2 (http://www.carrot2.org/) and FACTA

(http://text0.mib.man.ac.uk/software/facta/) for comparison with the manual approach. However, we found that manual searching was still the most practical approach in developing an AOP.

Although automated search tools did not provide advantages for domoic acid, there is a clear need for their application in the future. Our experience in developing the domoic acid AOP suggests the most difficult challenge in literature mining will be to identify sources that contain valuable information for extending cellular pathway models to models of tissue function and whole organism health. Here, the data mining requirements become significantly broader and thus more demanding. The potential sources of useful information may draw upon more diverse scientific disciplines than those encountered at the cellular level. For example, studies of disease or environmental stress and nutrition may provide useful information on compromised tissue function, behavior and fitness. A relatively narrow search strategy that focuses only on the ecotoxicology / toxicology literature might overlook useful information. Thus, some type of automated or semi-automated text mining tool would be helpful during the initial literature interrogation to identify useful sources of information that might otherwise be ignored and also to reduce the number of sources that need manual evaluation. However, it was apparent that a hindrance to the use of automated searching is the complexity of data types encountered and the relative lack of uniform or structured terminology across scientific disciplines. This problem has been acknowledged in ecology, where the need for the development of ontologies associated with ecological processes has been advocated to help establish a set of well defined terms and more formal descriptions of how they interrelate [114,115]. This would seem to be equally important for ecotoxicology and should be encouraged. In the interim, approaches developed for phenotype clustering (phenoclustering) based on automated literature searching using semantic

(text) clustering tools [116] may have some value for assisting in AOP development.

Strategy for constructing a conceptual framework

The development of a conceptual framework for an AOP is an iterative process that results in two important outcomes. The first outcome is a critically reviewed pathway from exposure to adverse outcome that is stressor specific. The second is the identification of a key cellular process (or processes) that is not chemical specific and may be the nucleus for the development of testing methods (described earlier in the present study). Critically important to success is the inclusion of an interdisciplinary team of researchers who span the breadth of science from exposure to adverse outcome. The process begins with a clear statement of a problem. The next step is developing a list of possible AOPs based on rough correlations between exposure and outcome. In the case of domoic acid, both behavioral and reproductive effects were considered as important AOPs. However, after a review of the extant literature, we determined that domoic acid effects upon the hypothalamus and subsequent reproductive impairment were subordinate to effects upon the hippocampus and behavioral changes. We recommend that when more than one AOP is identified, each should be analyzed separately with an objective to identify the key rate-limiting events in the pathway from exposure to adverse outcome. The goal is to establish confidence in the AOP based on a weight-of-evidence for causality. Use of the Bradford Hill Criteria for causality can be especially useful [117-119].

Our proposed AOP facilitates discussion between risk assessors, basic ecologists and applied ecotoxicologists. Discussions lead to important outcomes, first of which is the identification of data gaps in the causative links of the AOP that allow prioritization of targeted research in critical areas. New information, as it becomes available through discussion or research, can easily be added to this process to improve the analysis. Secondarily, this approach identifies the key

cellular process(es) that when perturbed lead to adverse outcomes in the individual (Figure 4). This latter step is critically important to development of a new paradigm for toxicity testing [1].

Strategy for developing a dynamic, computational model

In general, construction of a conceptual TP or AOP will involve extensive review of the extant literature and in the process a suitable computational model may be found. Further, once a TP or AOP has been defined, the actual biological processes that require computational modeling may have been modeled by scientists in a discipline that had not been reviewed for the construction of the TP or AOP. In the case of domoic acid, a key process in the TP is Ca^{2+} regulation in excitatory neurons. Once we identified this key process, a literature search for computational models of calcium regulation revealed models that had been developed for normal Ca^{2+} signaling in various types of cells [67,68,120]. To adapt an existing computational model or to develop one from a conceptual model requires data that may be obtained from the extant literature, or measured experimentally (see *Predictive in vitro assays for excitatory neurotoxins*).

Transforming a conceptual AOP to a quantitative model is likely to be less mechanistic than a TP model because of data limitations. For domoic acid, a literature review produced numerous studies that measured changes in behavior, learning, and memory loss due to hippocampal cell damage (see Supplemental **Table S1**). These data allow establishment of causative relationships between the key events in the AOP [119]. However, few studies contained data that could be used to develop a quantitative model. Furthermore, only a relatively small number of species are studied, thus interspecies extrapolations [121] may be needed depending on the species of interest (e.g., mammal to fish). To develop a quantitative AOP model based upon the extant literature, we recommend statistical models based upon regression analysis. Ideally, these models will enable predictions of an adverse impact (e.g., upon individual fitness, reproduction,

or mortality) that can be used as input into a population dynamic model [122].

Evaluating model predictive capabilities

Testing or validating the TP/AOP model will require a multi-step approach. The model is likely to be built using data from studies of only a few mammalian model species, and much of the data may come from in vitro assays. Data from non-mammalian vertebrates are likely to be rare and data from ecologically relevant species may be non-existent. The model's quantifiable attributes from the molecular initiating events to cellular level dysregulation, organ system dysfunction, and behavioral abnormalities will need to be compared to measured data in order to evaluate its goodness-of-fit.

To test a TP model, a suite of neurochemical and molecular markers from the published literature needs to be identified that span the molecular to cellular level effects. Primary cultures have been established in a number of ecotoxicology model animals and a series of in vitro assays as described earlier can be used to validate the conservation of these markers across multiple species (fishes: zebrafish, trout; birds: quail, zebrafinch; mammals: mink, common vole). The first test of the TP/AOP model will be made at the molecular and cellular level using primary cultures from ecotoxicology's model organisms and the suite of identified neurochemical and molecular markers.

To date, in vitro neurotoxicology has been useful for understanding major and alternative mechanisms of toxicity, but it must refine its focus on outcomes relevant to AOPs in order to be of value to predictive ecotoxiology. Decades of in vitro neurotoxicity research support certain common principles for neurotoxicity testing: test the active compound/metabolite(s), test over ranges of concentration that include toxicologically relevant concentrations, use cell models that possess appropriate toxicant targets (if known), test functional endpoints in addition to

cytotoxicity, and calibrate or validate results with in vivo results. Furthermore, in vitro systems have taught us that neurotoxicants may act through multiple mechanisms, cell types demonstrate different functional sensitivities to toxicants, and cells may be direct or secondary targets for functional impairment. Established goals of in vitro neurotoxicity testing are to develop model systems that respond in a toxicologically relevant manner to exposure, identify and elucidate mechanisms that underlie the adverse neurotoxic outcome and serve as rapid and discriminating systems for screening the potential toxicity of new or unknown potential neurotoxicants [63].

These broader criteria are important in a general manner; the test assay for a TP does have a single clearly definable goal - to create cellular based assays that measure perturbations of the TP and to support development of an interpretive, biologically based computational model to calculate expected degree of perturbation as a function of the toxicant concentration. As discussed previously, it is important that assays and outputs measured in the assay system are carefully designed to be amenable to mechanistic, predictive, quantitative dose-response modeling that will eventually serve as one of the cornerstones of predictive ecotoxicology.

The complete AOP model must also be evaluated for its ability to predict effects at the whole organism level. The most striking effects of domoic acid are those that influence neurological function and behavior. Standard neurobehavioral assays have been developed in birds and mammals and similar assays can be developed for other organisms. Hypoactivity assays are fairly routine using new software programs that track movements during a defined trial period and this approach has been used successfully in a number of different terrestrial and aquatic species. Repetitive movements can also be assayed using the same software systems. Learning and memory can be assessed by modifying many of the assays developed for small laboratory birds and mammals. A modified t-maze has been used successfully in fishes to assess learning

and memory [123].

The next level of validation involves a comparison between the neurochemical and molecular markers in laboratory organisms and tissues of wild-caught animals both unexposed and exposed to the chemical of interest (domoic acid in algal blooms). Associative/ecologic approaches should be used to correlate neurochemical biomarkers with tissue/brain levels of domoic acid.

The predictive capabilities of the model can also be assessed by studying other environmental chemicals that act on the key event in the TP/AOP. Data may be obtained using the in vitro strategies discussed earlier or from data mining exercises. While the TP/AOP described here is highly specific for domoic acid there are several chemicals that may interact with its key initiating event. For example, tributyltin causes hippocampal loss, but since this may not be realized via the NMDA receptor [124] it may be used to assess the latter portion of the AOP. In contrast, one of the mechanisms by which methylmercury causes neurotoxicity is via glutamate-induced excitotoxicity, which has been observed in several types of animals (e.g., terrestrial mammals, fish-eating birds, marine mammals) as compensatory decreases in NMDA receptor binding [73,74,76]. Although our TP/AOP has focused on excitotoxicity, many chemicals specifically disrupt inhibitory pathways which will have consequences for the TP/AOP. For example, Babot et al. [69] found that reduction of GABA(A) receptor function by dieldrin, an organochlorine insecticide, was followed by a decrease in NMDA receptor function as a compensatory response; and RDX, an explosive compound that causes seizures, has been shown to disrupt GABAergic signaling [125]. Application of the TP/AOP will not only enable us to better resolve the pathway/mechanisms/risks for domoic acid, but similar assessments can be carried out for other chemicals that impact different points in the TP.

Testing of the AOP may lead to the realization that the published data used to build the model

may not be sufficiently predicative in all cases. After all, the vast majority of published scientific literature is on a very small number of mammalian species and ecotoxicology must deal with a large number of species that may have dramatically different sensitivities to perturbation of a given TP/AOP. Regulatory agencies that will use these TP/AOP models must be willing to adopt a very nimble and focused approach to generating information that will fill whatever data gaps are weakening the model. This may require a very different, though complementary, extramural funding mechanism than what is currently being employed.

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FIGURE CAPTIONS

Figure 1: Glutamate neurotransmitter system and excitatory neurotoxicity.

Figure 2: Flow chart for developing a domoic acid adverse outcome pathway (AOP) model.

Figure 3: Normal Ca^{2+} signaling adapted from Fayazi et al. [67]. Arrows represent flows of Ca^{2+} between different pools which can vary in magnitude.

Figure 4: The Exposure-Dose-Response Continuum Perspective for Domoic Acid. The toxicity pathway focuses on maintenance of normal neuronal function, which is balanced by excitatory glutmate (GLU) and inhibitory γ-aminobutyric acid (GABA) inputs – excess excitation leads to prolonged intracellular calcium, cell injury, apoptosis / cell death. Excessive cell death leads to tissue damage and eventual morbidity / mortality.

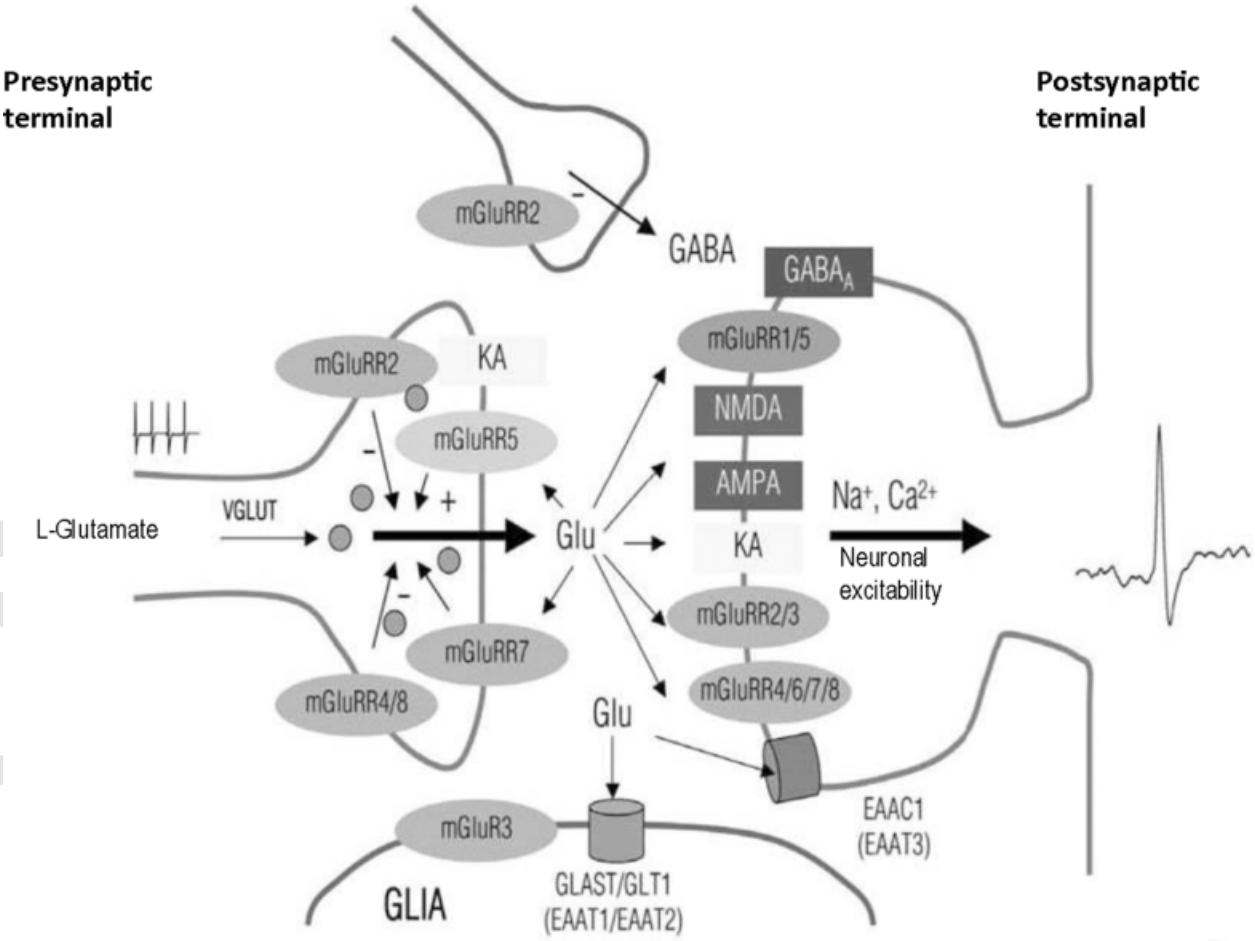

Figure 1

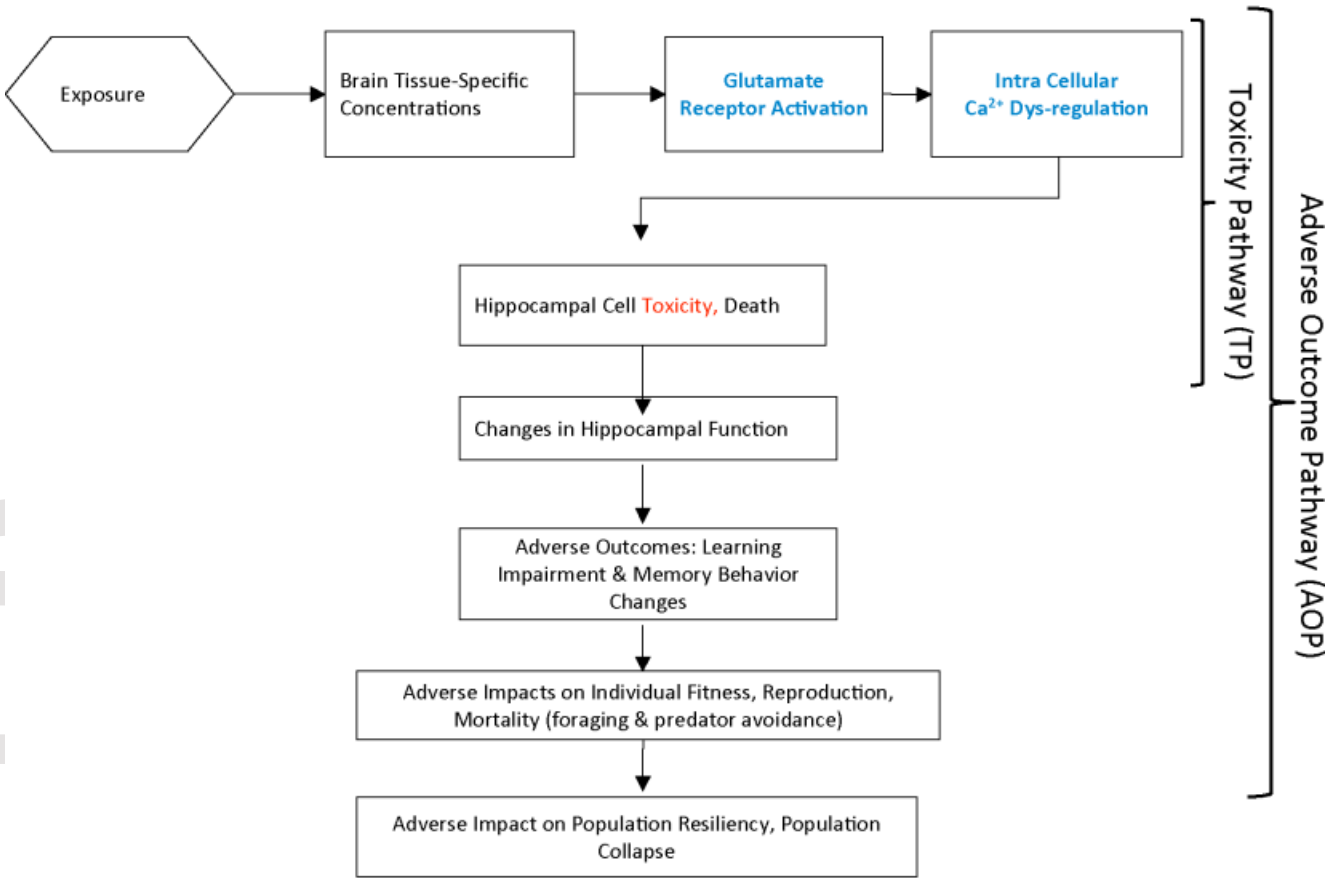

Figure 2

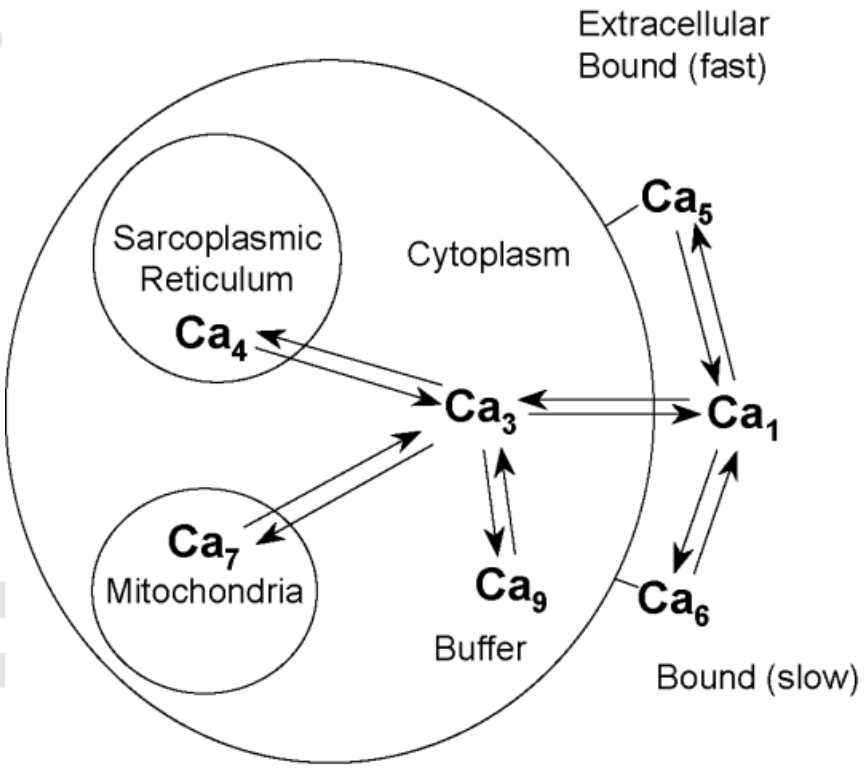

Figure 3

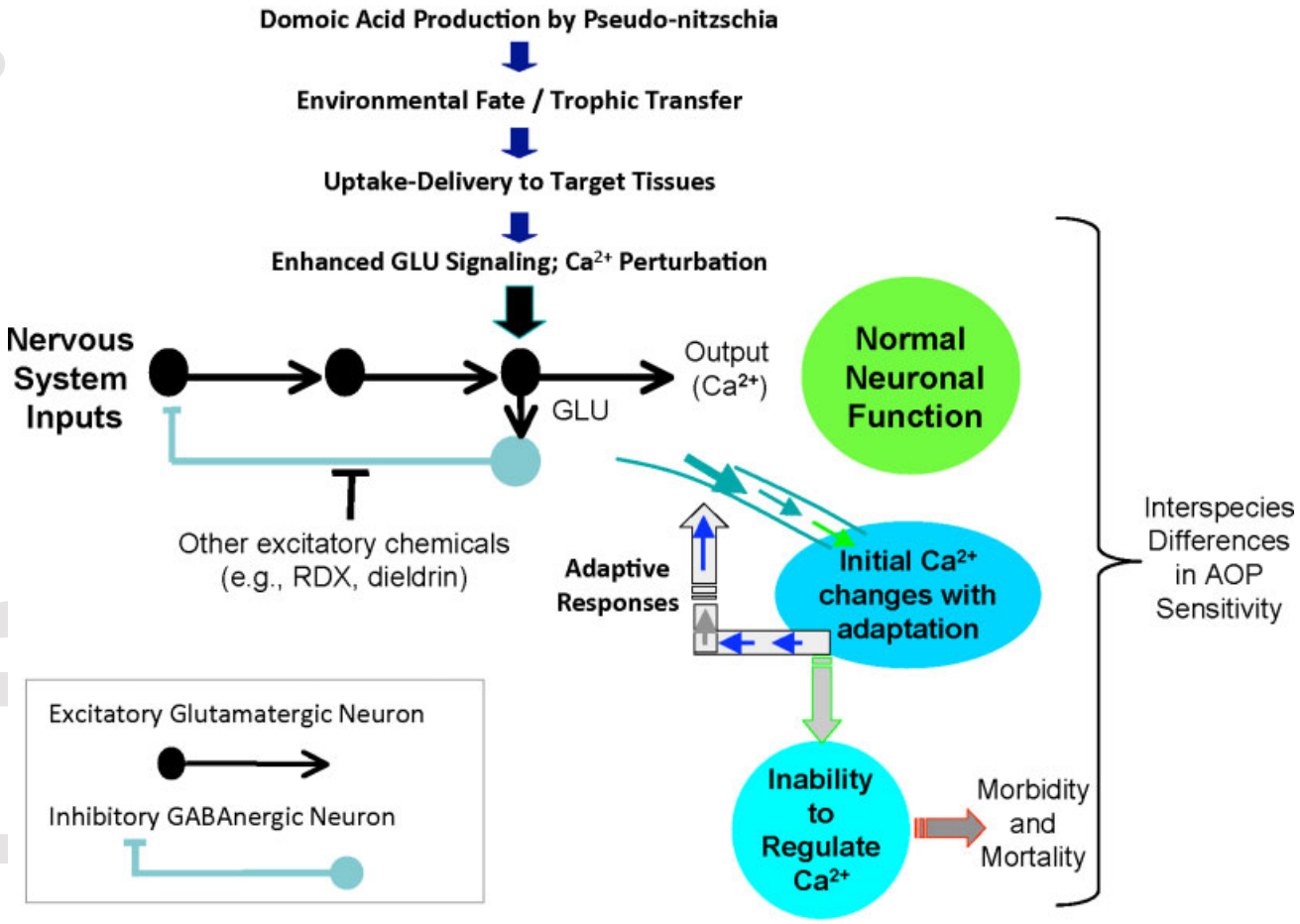

Figure 4