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REGULATORY RESEARCH PERSPECTIVES

Impact on Public Health

Application of a Systems Biology/Systems Toxicology Approach to Developmental Neurotoxicology

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Abstract: Systems biology/systems toxicology can be applied to enhance the understanding of complex biological processes such as apoptosis in the developing brain. Systems biology, as applied to toxicology, provides a structure to arrange information in the form of a biological model. The approach allows for the subsequent and iterative perturbation of the initial model, with the use of toxicants, and the comparison of the resulting data against the proposed biological model. It is postulated that the exposure of the developing rat to NMDA antagonists, e.g., ketamine or phencyclidine (PCP), causes a compensatory up-regulation of NMDA receptors, thereby making cells bearing these receptors more vulnerable to excitotoxic effects of endogenous glutamate. Although comprehensive gene expression/proteomic studies and mathematical modeling remain to be accomplished, a biological model has been established and perturbed in an iterative manner to allow confirmation of the biological pathway for NMDA antagonist-induced brain cell death in the developing rat.

Introduction

Systems biology has been defined as the iterative and integrative study of biological systems as systems respond to perturbations [1]. As adapted for toxicology and referred to as systems toxicology, it involves the study of perturbations by chemicals and stressors by monitoring alterations in gene and protein expressions that are linked firmly to toxicological outcome in an iterative and integrative manner [2]. This process requires the understanding of functional interactions between key components of cells, organs and systems and how these interactions change with toxicant exposure or disease [3]. To exploit the virtues of systems biology, the toxicological community must move beyond the collection and compilation of genomic and proteomic data that has improved functional annotations of individual genes, of protein-protein interactions and of components of specific modules. The goal of systems biology is to predict functional outcome of component-component relationships with computational models that allow the directional and quantitative description of the complete organism in response to environmental perturbations [2].

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High throughput molecular biology approaches including genomics, proteomics and metabonomics are providing the fundamental data necessary for the building blocks of systems biology. As these databases grow and become linked together as integrated modules, they will provide the intermediate components necessary for the systems biology approach. It is the appropriate placement of these biological modules into a wiring diagram, allowing the development of a holistic computational model, which remains to be routinely accomplished [1]. It is at this level that connectivity of the system determines its state, and the whole becomes greater than the sum of its parts [4]. For toxicology, it is essential that quantitative correlations of exposure, dose and outcome be integrated into the computational model [5]. In addition to knowledge of the proximate toxicant and its mechanism of action, the primary toxicological effect or phenotypic anchor must be utilized [6]. At this systems biology level, quantitative simulations can be conducted, and predictions of the model output can be tested. The quantitative outcome of these iterative experiments is systematically incorporated back into the model to improve its design and refine its predictive capabilities.

The success of the systems biology/systems toxicology approach to solve toxicological problems lies in the establishment of cross-disciplinary teams of scientists including toxicologists, molecular biologists, mathematicians, modelers, and risk assessors. The integration of the rapidly growing biological databases, including models of cells, tissues and organs, with the use of powerful computing systems and algorithms is necessary [3]. These interdisciplinary scientists should conduct systematic experiments allowing for small variations of a large number of model components to determine the overall functioning of the biological system [1]. Although the global interactive processes are generally depicted in graphic form, mathematically based models are essential for the full potential of systems biology to be achieved. Powerful mathematical approaches have been used to quantitatively describe physiologically based pharmacokinetic models with pharmacodynamic components, but these existing frameworks, based on simultaneously solved differential equations, have not been routinely applied to systems biology [7]. Examples of emerging systems biology applications that have been reported include galactose utilization in yeast [8] and sea urchin development [9].

Because of the complexity and temporal features of the manifestations of developmental neurotoxicity, no area of toxicology could benefit more from the systematic application of the systems biology/ systems toxicology approach. Neurotoxicity may be defined as any adverse effect on the structure or function of the central and/or peripheral nervous system by a biological, chemical, or physical agent that diminishes the ability of an organism to survive, reproduce or adapt to its environment. Neurotoxic effects may be permanent or reversible, produced by neuropharmacological or neurodegenerative properties of a toxicant, or the result of direct or indirect actions on the nervous system [10]. These effects can often be measured by neurobiological, neurophysiological, neuropathological, or behavioral techniques. Extrapolation across species is feasible but must take into account the relative ontogeny of the nervous system among species. Insults to the nervous system may take various forms and may be quite subtle [11]. Although its manifestations may change with age, neurotoxicity may occur at any time in the life cycle from gestation through senescence. The developing nervous system may be more or less susceptible to neurotoxic insult depending on the stage of development.

One recently described developmental neurotoxicity involves the apoptotic cell death of neurons in several brain regions of the developing rat pup following postnatal exposure to the dissociative anesthetic and noncompetitive, N-methyl-D-aspartate (NMDA) receptor antagonist, ketamine [12, 13]. When ketamine is administered to the neonatal rat pup, a rapid and significant increase in apoptosis in several brain regions is observed. The apoptosis was quantified by counting degenerating neurons and by caspase-3 immunocytochemistry, 24 hours after agent exposure. The window of vulnerability to this effect of ketamine is restricted to the period of rapid synaptogenesis (also known as the brain growth spurt), which occurs immediately after neurons have differentiated and migrated to their final destinations. It is postulated that excessive suppression of neuronal activity by ketamine during the brain growth spurt triggers neurons to commit "suicide" by apoptosis.

During development in the rat, especially during postnatal days 7-14, the central nervous system (CNS) exhibits enhanced susceptibility to the toxic effects of modulation of the NMDA receptor system. This enhanced susceptibility has been suggested to be derived from the increased expression of specific NMDA receptor subunits [14]. Because of the critical role of the NMDA receptor system in brain development, antagonism of this system can have profound, long-lasting and detrimental effects [15]. If stimulation of glutamate release reinforces neuronal connections, then blockade of that stimulation by NMDA antagonists may result in fewer and/or nonfunctional connections.

Although the mechanisms were not fully elucidated, a comparable insult was noted by Hubel and Wiesel [16, 17]. They established that visual experience during critical periods of development was necessary for the normal anatomical and functional development of the visual

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system. When deprived of light stimulation, normally preserved neural pathways atrophied and aberrant pathways were maintained. Glutamate is widely considered to be a major and ubiquitous excitatory neurotransmitter [18, 19, 20]. Therefore, it is not surprising that the visual system plasticity explored by Hubel and Wiesel, in response to light deprivation, may have its roots in reduced glutamate neurotransmission. In fact, evidence for NMDA receptor involvement in long-term potentiation [21, 22, 23] and neuronal plasticity of the visual system [24, 25, 26, 27, 28] has been reported.

Thus, NMDA receptor antagonists may block neurotransmission mediated by glutamate, just as deprivation of light prevents the propagation of glutamate-driven action potentials in visual system pathways. The original observations that CNS apoptosis is associated with the noncompetitive NMDA antagonists MK-801 and ketamine [12] are consistent with a mechanism involving blockade of NMDA receptor mediated neurotransmission. More recently, Ikonomidou, Olney and colleagues have replicated and expanded their observations to include nitrous oxide, isoflurane, propofol, midazolam, halothane, barbiturates, benzodiazepines, and ethanol as suspect apoptotic agents, either alone or in combination when administered to neonatal rodents [29, 30]. Taken together, these data raise the concern that most anesthetic agents may cause similar apoptotic effects because they block the NMDA receptor and or stimulate the GABA receptor [29, 30]. Several other independent groups of investigators have also reported that MK-801 or ketamine increases apoptosis either *in vivo* [13, 31, 32], in motor neurons of a chick embryo preparation [33], or in cultured neurons [34].

The goal of this report is to outline the application of the systems biology/systems toxicology approach to the problem of developmental neurotoxicity produced by ketamine, phencyclidine (PCP) and related NMDA antagonists. The four steps of the systems biology approach reported by Hood's group [1, 8] were followed. These steps are: 1) Define all components of the system. Use this information to formulate an initial model. 2) Systematically perturb and monitor components of the system. As in step one, an approach in which all components are perturbed and globally monitored (defining all genes, all mRNAs and protein expressed in a particular condition) is favored. 3) Reconcile the experimentally observed responses to perturbation with those predicted by the model. When predictions and observations disagree, alter initial model by proposing alternative hypotheses. 4) Design and perform additional perturbations experiments to distinguish between competing model hypotheses. Select a new perturbation and repeat steps two through four to refine the model over successive iterations. The overall goal is for the data to be integrated and graphically displayed and ultimately modeled mathematically. Thus systems biology provides the framework to match global observations against model predictions in an iterative fashion. With this approach one can formulate new models, new predictions and new experiments to test them. The aim is to use these mathematical models to predict the structure and behavior of the informational pathway [1, 8].

In the application of the systems biology/systems toxicology approach to the problem of developmental neurotoxicity produced by ketamine, phencyclidine (PCP) and related NMDA antagonists, the four steps set forth by Hood's group were followed to the extent possible. First, available information on the biological system from *in vitro* and *in vivo* rodent experiments was gathered, and a preliminary model of how it functions in descriptive and graphical terms was formulated, and, where possible, the genes and proteins expressed in the described pathways were defined. Second, the system was perturbed with chemical agents (PCP and ketamine) and kinetic experiments providing information across developmental time spans were conducted. Third, the model was improved by testing the initial hypothesis and, based on the experimental data, incorporating the new information into the model. And fourth, additional perturbations were performed to refine the model by repeating steps two through four in an iterative manner.

The process of apoptosis

Apoptosis or programmed cell death is a physiological process in which intrinsic molecular programs are activated to cause cellular destruction. Apoptosis is vital for all multi-cellular organisms, occurs in a variety of physiological conditions and is crucial for normal development, organ morphogenesis and tissue homeostasis [35]. Apoptosis is also a defense mechanism against infection or cellular damage [36]. When cell injury is moderate to severe, the best and most efficient solution is to eliminate the damaged cell by apoptosis. However, excessive apoptosis may lead to conditions such as immunodeficiency and neurodegenerative diseases [37]. The machinery of cell suicide is maintained at a critical threshold between off/on by positive and negative regulatory factors. Many apoptotic factors have a double identity with a quite different, sometimes even opposite, function in different contexts and cell types (e.g. NF-kB). The dynamic balance of these positive, negative and often double-faced factors determine if a cell commits suicide in response to endogenous and exogenous stimuli and thus comprise the comprehensive and interactive molecular pathways of apoptosis. The key

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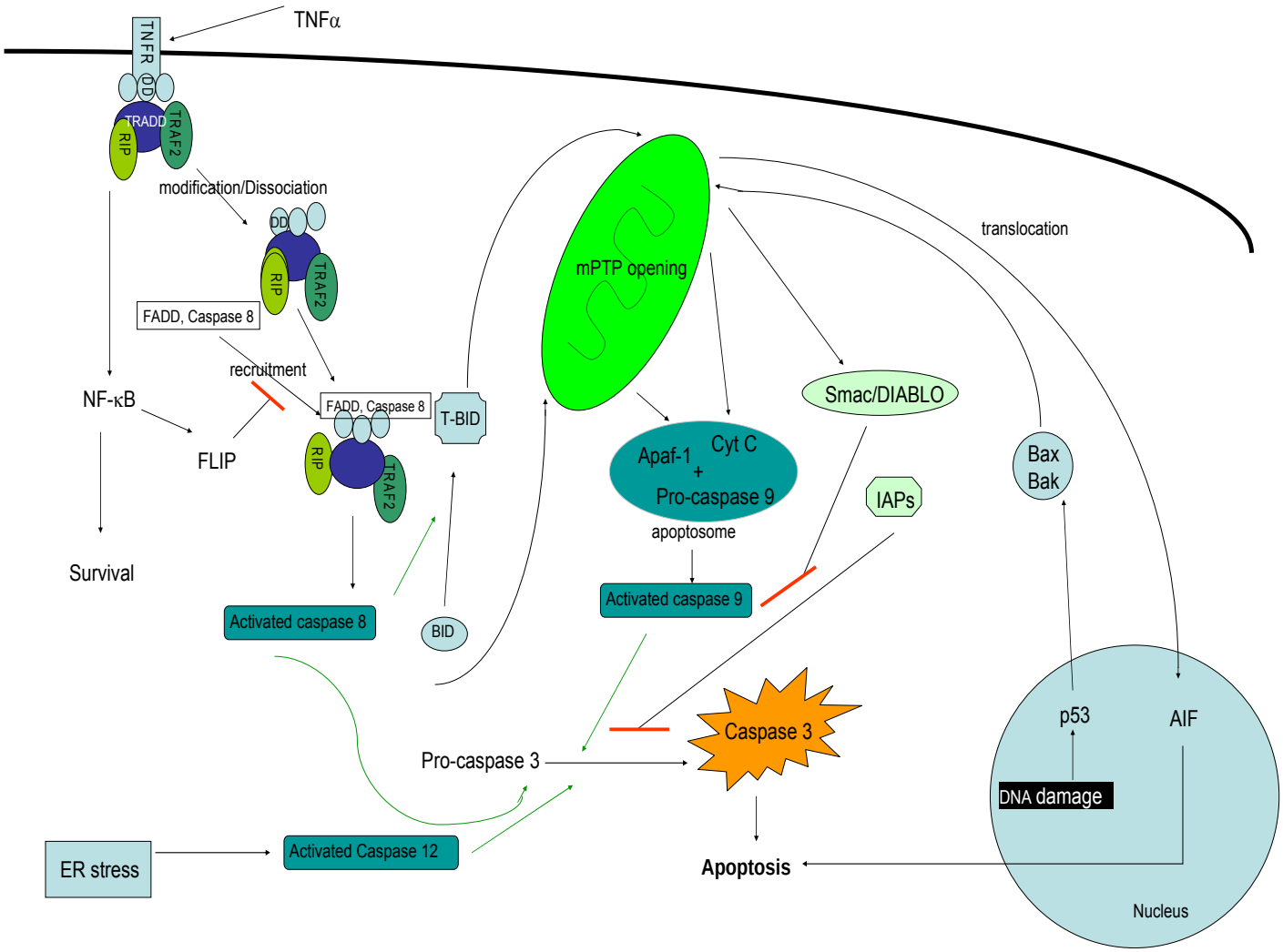

Figure 1. A simplified scheme of apoptotic molecular pathways. Death signals (TNFα) binding to death receptors (TNFR), initiates plasma membrane bound complex (TRADD, RIP and TRAF2), and signals activation of NF-kB. The complex dissociates with TNFR and recruits FADD and pro-caspase-8 forming a second complex, resulting in the activation of caspase-8 and apoptosis. If NF-kB is activated, it promotes the expression of caspase-8 inhibitor FLIP and thus sequestered apoptosis. Apoptotic signals (increased intracellular Ca^{2+}, DNA damage, tBID, etc.) induce opening of the mitochondria permeability transition pore and recruit cytoplasmic apoptotic members of Bcl-2 family and other proteins, causing release of apoptotic executors (cytochrome c, Apaf-1 and pro-caspase-9) to the cytoplasm and formation of apoptosome, which activates caspase-9. Extrusion of Smac/DIABLO into the cytoplasm blocks the inhibition of caspase activation by inhibitors of apoptosis (IAPs), and the translocation of apoptosis inducing factor (AIF) to nucleus initiates caspase-independent apoptosis. Stress in the endoplasmic reticulum (ER), such as disruption of calcium homeostasis, induces the activation of caspase-12 that subsequently activates caspase-3, representing a novel apoptotic pathway.

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mediators of apoptosis are the proteolytic enzymes (caspases). The activation of these enzymes is responsible for apoptotic events such as mitochondrial damage, nuclear membrane breakdown, DNA fragmentation, chromatin condensation, and the eventual consequence of cell death. However, caspase-independent pathways have been identified recently [38]. The Bcl-2 family of proteins, which are involved in the regulation of apoptosis, include anti-apoptotic (such as bcl-2, bcl-X_L) and pro-apoptotic (such as Bax, Bak) members. Balance shifting of these proteins in cells determines the initiation of apoptosis or cell survival. Classically, two distinct pathways of apoptotic signaling have been identified: an *extrinsic* apoptotic pathway, mediated by cell membrane receptors, and an *intrinsic* pathway, controlled by mitochondria (*mitochondria-centric*) [39]. Stress in the endoplasmic reticulum (ER) can also result in apoptosis through activa-

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tion of caspase-12, which has attracted considerable attention in the mechanistic studies of neurotoxicology and neurodegeneration [40, 41]. A simplified scheme is used here to attempt to summarize and distinguish the different apoptotic pathways (Figure 1).

The extrinsic apoptotic pathway. The major players of the initiation of phase of the apoptotic events at the plasma membrane are the death receptors, which include the TNF or Fas (Apo-1/CD-95) receptor families [42, 43]. Binding of TNFα to TNF receptors activates various TNF receptor-associated factors [44], leading to the formation of an early plasma membrane bound complex, which activates NF-kB. The recruitment of pro-caspase-8 to the second complex activates this enzyme and leads to apoptosis [45]. In cases where NF-kB activation has been achieved, the second complex will recruit the caspase-8 inhibitor FLIP, which blocks apoptosis [46]. Activation of caspase-8 can further activate caspase-3 and trigger mitochondria-centric apoptosis through cleavage of BID [47].

The intrinsic apoptotic pathway. Mitochondria are at the crossroads of the life and death signaling pathways and can also collect apoptotic signals from the plasma membrane truncated BID (t-BID) and nucleus (DNA damage-activated p53) [48, 49,50]. When the balance of the bcl protein families shifts from mitochondrial anti-apoptotic (bcl-2, bcl-W, bcl-G, bcl-X_L) to pro-apoptotic (Bax, Bak, Bad, BID) members, a high-conductance nonselective mitochondrial channel, the mitochondrial permeability transition pore (mPTP) is opened, and, subsequently, a variety of apoptotic molecules are released into the cytoplasm (AIF, Apaf-1, cytochrome c, and Smac/Diablo) [51, 52, 53]. Apaf-1, cytochrome c and procaspase-9 comprise apoptosome that activates the caspase-9 cascade. After the release from mitochondria of the flavoprotein AIF, it mediates caspase-independent cell death [38, 54]. With the mitochondrial alterations in response to apoptotic signals, AIF induces cell apoptosis by translocation from mitochondria to the nucleus, inducing nuclear condensation and DNA fragmentation [55, 56]. Stress in the endoplasmic reticulum (ER), including the disruption of calcium homeostasis and accumulation of unfolded proteins in the ER, can also result in apoptosis through the activation of caspase-12 [57]. Pro-caspase-12 is predominantly localized in the ER. The disturbance to intracellular calcium stores causes activation of caspase and release from the ER to the cytoplasm possible through the activation of a cytosolic protein, calpain [58]. Active caspase-12 can, in turn, activate caspase-9/caspase-3 and elicits cell apoptosis [39].

The roles of glutamatergic transmission and NMDA receptor dysfunction in anesthetic-induced neurodegeneration

Glutamate promotes certain aspects of neuronal development including migration, differentiation and plasticity during development and throughout life [59]. Malfunctions of the glutamate system affect neuroplasticity and can cause neuronal toxicity. In anesthetic-induced neurotoxicity, many glutamate-regulated processes seem to be perturbed. Abnormal neuronal development, abnormal synaptic plasticity and neurodegeneration have been proposed to be causal or contributing factors in anesthetic-induced neurotoxicity. Glutamate receptors in the vertebrate central nervous system were classified into three families on the basis of pharmacology defined using the agonist α-amino-3-hydroxy-5-methylisoxazolepropionic acid (AMPA), kainic acid and NMDA. It is becoming clear that some of the most important functions of the nervous system, such as synaptic plasticity and synapse formation, critically depend on the behavior of NMDA receptor channels, and that neurological damage caused by a variety of pathological states can result from exaggerated activation of NMDA receptor channels [60, 61]. Here, we discuss how the molecular aspects of NMDA glutamate receptor malfunction can explain some of the neuropathology observed in anesthetic-induced neurotoxicity and how the available neuroprotective treatments can intervene by interacting with the glutamate receptor system.

NMDA glutamate receptors are widely distributed in the mammalian central nervous system. The NMDA receptor is a ligand-activated ion channel primarily composed of two families of NMDA receptor subunits: NR1 with 8 splice variants and NR2 (A-D) [62, 63, 64, 65, 66, 67, 68]. The NR1 subunit is essential for receptor function. The functional properties of the NMDA receptor vary throughout the central nervous system**,** and the binding affinities for various ligands of recombinant NMDA receptors depend on subunit composition [69]. A novel family of NMDA receptor subunits, consisting of NR3A and NR3B, has been described recently [70, 71]. NR3A has at least two splice variants [72]. NR3 subunits form functional channels with NR1, and, unlike other subunits, work in a dominant-negative fashion, i.e., they assemble with NR1 and suppress glutamate-induced currents [70, 71].

In rats, during the first two weeks of life, the NMDA subtype of glutamate receptors undergoes a period of hypersensitivity, in which neurons bearing NMDA receptors are rendered highly sensitive to excitotoxic degeneration [73]. Paradoxically, it has been reported that NMDA antagonists were most effective in triggering apoptosis in the rat forebrain on PND 7 [12, 13]. This toxic effect of antagonists could be due to either a pathological upregulation of NMDA receptors or to a deprivation of critical NMDA-mediated signals. During this period, NMDA receptors are the primary mediators of glutamatergic fast excitatory neurotransmission in the brain [74]. It has been suggested from the clinical

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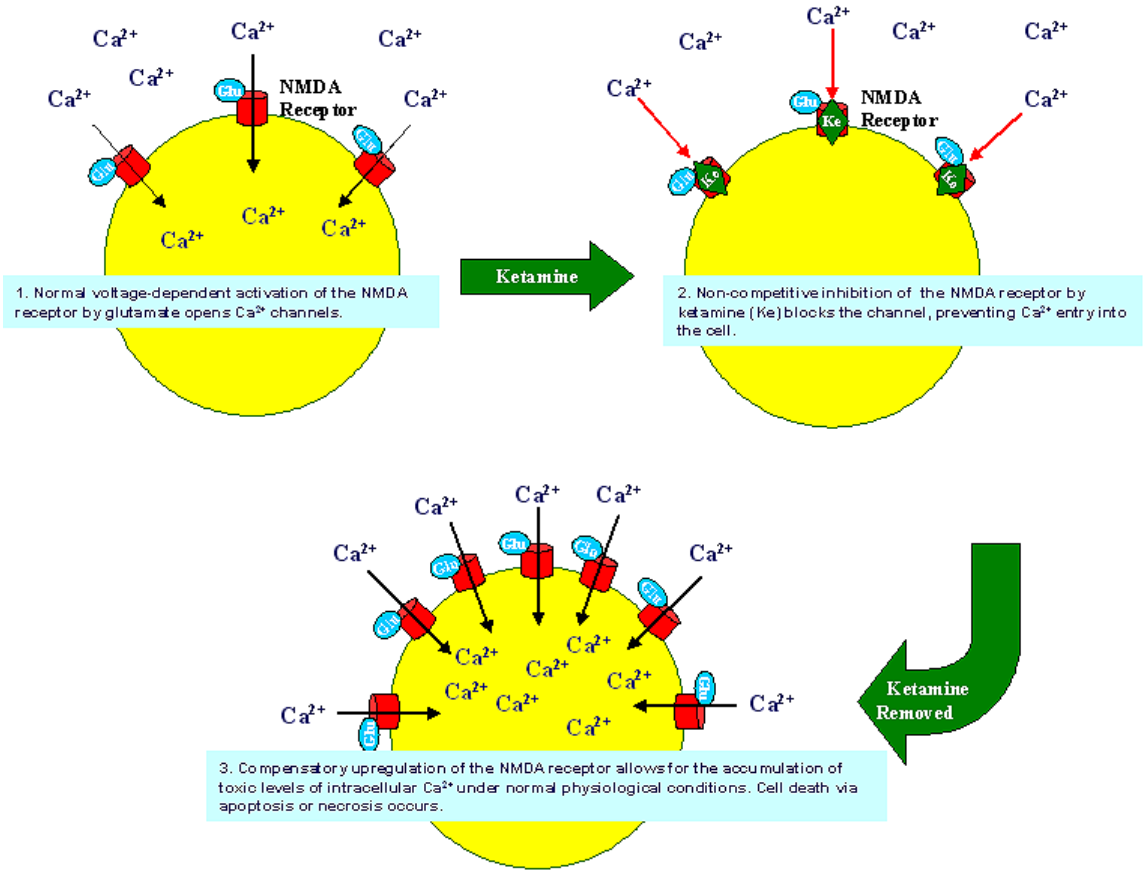

Figure 2. Working model of the effects of NMDA antagonists. This figure shows that the exposure of developing brains to NMDA antagonists (such as ketamine or phencyclidine) causes a compensatory upregulation of NMDA receptors, making cells bearing these receptors more vulnerable to the excitotoxic effects of glutamate.

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perspective that in some anesthetic-induced or NMDA antagonist-induced psychiatric states, the primary pathological insult may occur during the pre- or perinatal period, [75, 76, 77] though the functional consequences of this insult do not become evident until after puberty, a time at which the affected neural networks reach maturity [78].

Our previous studies have shown that chronic administration of the NMDA antagonist, phencyclidine (PCP), in adult rats results in a substantial loss of cells in the olfactory, cingulate and dorsolateral cortices [79, 80]. This loss involves an apoptotic mechanism associated with increased NMDA receptor synthesis and altered function [79, 81]. It also results in the development of a robust locomotor sensitization with a complex pharmacology [80, 82]. It is possible that up-regulation of NMDA receptors is responsible for a more heightened sensitivity to excitotoxic insult. As alluded to previously, perinatal treatment of rat pups with PCP results in alterations in baseline prepulse inhibition, enhanced locomotor sensitization to PCP challenge (2 mg/kg) and delayed acquisition tasks with both learning and memory components. These effects might be preceded by increases in NR1 mRNA, as well as several markers for apoptotic pathways. Therefore, we hypothesize that anesthetic (such as ketamine)-induced behavioral changes are the result of a neurodevelopmental disturbance that occurs subsequent to a loss of neurons in the frontal cortex, which results from an up-regulation of NMDA receptor function.

For the purpose of identifying mechanisms that could link neurodegenerative abnormalities to altered NMDA receptor expression, two cartoons depicting a working model of NMDA antagonist-induced apoptosis are provided (Figures 2 and 3). It is postulated in Figure 2 that the exposure of developing brains to the individual anesthetics (such as ketamine or PCP) causes a compensatory up-regulation of NMDA receptors, making cells bearing these receptors (after ketamine/PCP washout) more vulnerable to the excitotoxic effects of glutamate. To define the relationship between altered NMDA receptors and neurodegeneration, *in situ* hybridization studies have been performed on organotypic cultures using a 35S-labeled oligonucleotide probe that specifically targets NMDA receptor NR1 subunit. Figure 4 indicates that, in the frontal cortex (A) and nucleus accumbens (C), NMDA receptor NR1 subunit mRNA is prominent. In addition, the autoradiograph grain density for NR1 subunit mRNA is up-regulated in PCP-treated cultures in frontal cortex (B) and nucleus accumbens (D) compared with control (A and C). PCP-induced apoptosis is further confirmed by the examination at the electron microscopic level. Figure 5 shows representative electron micrographs of frontal cortex neurons from control (A) and PCP-

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treated organotypic culture (B). Panel A shows a normal neuron with intact cytoplasmic and nuclear membrane from a control culture. Panel B shows typical nuclear condensation that may indicate an advanced apoptotic neurodegeneration. Although these observations were not quantitated, it is our impression that neurons with apoptotic morphology were much more numerous in PCP-treated slices.

It is also postulated that the excitotoxic effects of glutamate are largely mediated by increased Ca^{2+} influx through activated NMDA receptors [83, 84]. Associated with Ca^{2+} influx (Figure 3) is an increase in reactive oxygen species (ROS) that appears to originate in the mitochondria [85]. This Ca^{2+} overloading (glutamate-induced) by the mitochondria beyond its buffering capacity reduces the membrane potential and disrupts electron transport, resulting in the increased production of the reactive free radical superoxide anion ($O^{\cdot -}_2$) [86]. Several experiments have shown that inhibition of the electron transport chain with rotenone (complex I), antimycin A (complex III), and oligomycin (complex V) prevents

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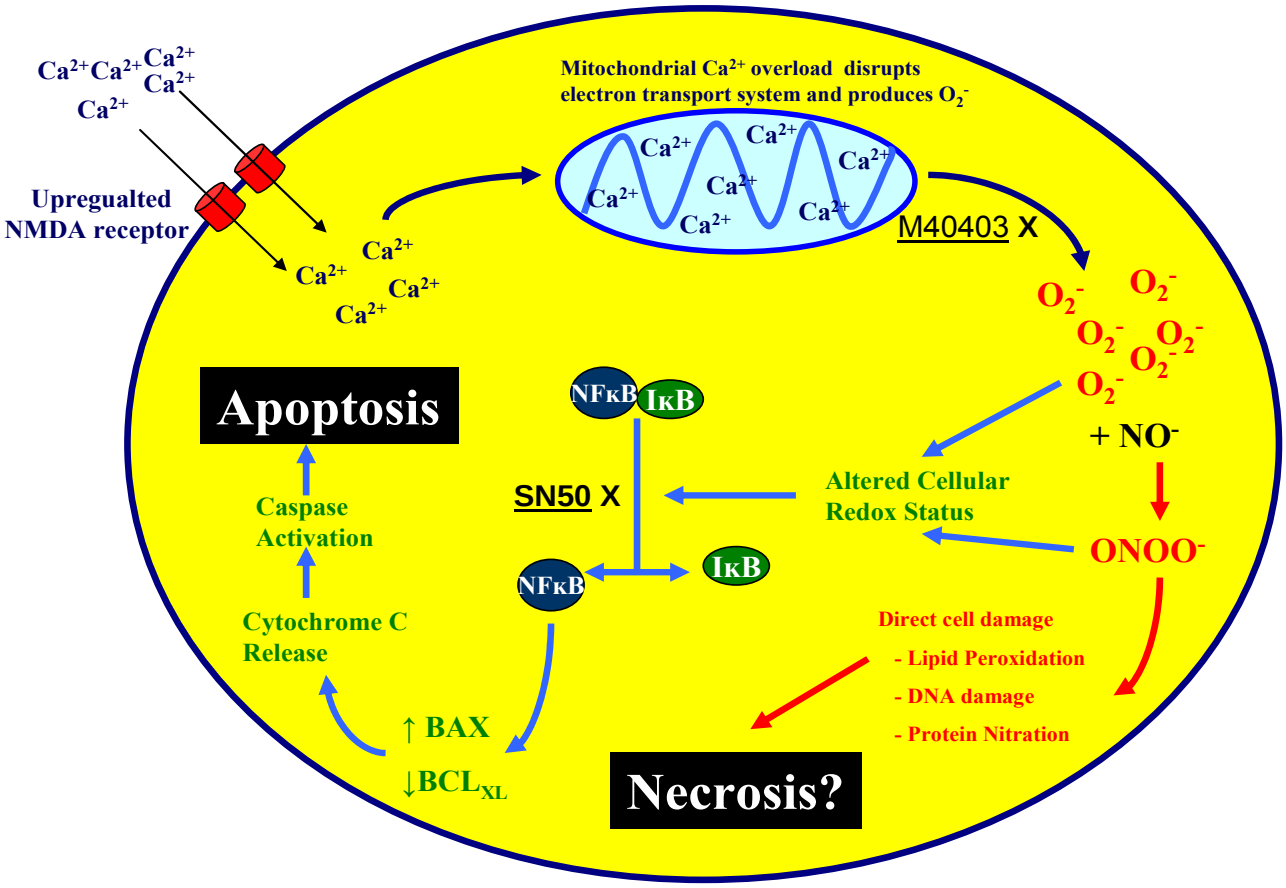

Figure 3. Proposed mechanism of NMDA antagonists. This figure depicts a working model of NMDA antagonist-induced neuroapoptosis. Prolonged activation of NMDA receptors results in an overload of intracellular Ca^{2+} that exceeds the buffering capacity of the mitochondrion and interferes with electron transport in a manner that results in the production of superoxide anion ($O^{\cdot}_2$). The increase in $O^{\cdot}_2$ turns on I-kB kinases, resulting in the phosphorylation of I-kB serine residues, the dissociation of NF-kB proteins and their transport into the nucleus. In the nucleus, these transcription factors bind to several known genes including p53 and Bcl-X_L. The consequence of this binding is not completely certain, but the transcription of p53 and a subsequent increase in Bax is enhanced in several systems. With decreased Bcl-X_L, increased Bax diminishes the formation of antiapoptotic Bax/Bcl-X_L heterodimers in favor of pro-apoptotic Bax/Bax homodimers. These homodimers are thought to alter the permeability of the mitochondrial membrane through which cytochrome c can leak into the cytoplasm, where it can activate caspases that play a critical role in the ultimate demise of the cell. Several reactive oxygen species, including nitric oxide and superoxide anion ($O_2^{\cdot -}$), have been implicated in glutamate-induced neuronal death. Little is known about the signaling pathway that mediates the postulated roles of peroxynitrite ($ONOO^-$). Virtually any protein containing one or more tyrosyl residues can undergo nitrosation *in vitro*. Thus, it is important to determine whether nitosation of a protein alters its biologic function. Much attention and debate has been devoted to determining the precise reactive nitrogen species responsible for protein nitrosation (not the focus of this review). Further identification of the specific targets of endogenous nitrosation will be essential to provide clues to the pathological mechanisms.

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ROS formation and, in some cases, can be neuroprotective [86].

Although apoptosis can be the final result of an excitotoxic insult, the pathways leading from mitochondrial dysfunction and ROS generation are not completely understood. The use of metalloporphyrins such as manganese tetrakis (4-benzoyl acid) porphyrin has implicated $O\cdot^{-}_{2}$ in glutamate exicitotoxicity [86, 87]. However, relative to M40403, a selective nonpeptidyl superoxide dismutase mimetic, these reagents lack specificity for $O\cdot^{-}_{2}$ [87, 88] and are much less potent. Recently, our studies [89, 90] demonstrated that this nonpeptidyl superoxide dismutase mimic significantly blunts both NMDA agonist-induced, as well as NMDA antagonist-induced, cell death over a range of about 10-fold (0.3-2.5 μM), whereas the maximal effect of manganese tetrakis (4-benzoyl acid) porphyrin was observed at 150 to 200 μM [87]. Thus, the use of M40403 appears to be a valuable tool for exploring the specific role of $O\cdot^{-}_{2}$ in NMDA antagonist-induced apoptosis. The ability of M40403 to prevent NMDA receptor antagonist-induced apoptosis relatively early in this cascade suggests its potential therapeutic utility in central nervous system diseases such as stroke, hypoxia, ischemia, and alcohol addiction that are associated with NMDA receptor mediated production of superoxide anion ($O_2^{\cdot-}$).

One consequence of increased oxidative stress is the activation and inactivation of redox-sensitive proteins [91]. The transcription factor NF-kB is known to respond to changes in the redox state of cytoplasm and has been shown to translocate in response to NMDA/NMDA antagonist-induced cellular stress [92]. NF-kB is normally sequestered in the cytoplasm, bound to the regulatory protein IkB. In response to a wide range of stimuli including oxidative stress, infection, hypoxia, extracellular signals, and inflammation, IkB is phosphorylated on serine residues Ser-32 and Ser-36 by the enzyme IkB kinase. This targets the IkB protein for ubiquination and subsequent degradation by the 26S proteasome [93]. The net result is the release of the NF-kB dimer, which is then free to translocate into the nucleus. The ability of M40403 to prevent NMDA/NMDA antagonist-induced neurotoxicity and nuclear translocation of NF-kB strongly suggests that $O\cdot^{-}_{2}$ is a key reactive oxygen species in this pathway.

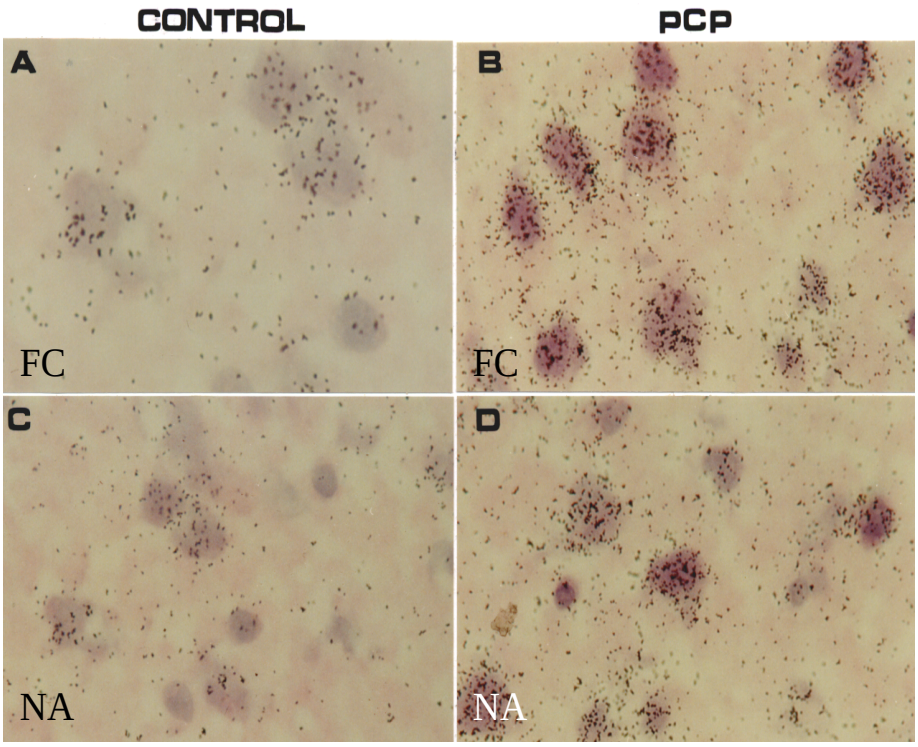

Figure 4. NR1 subunit abundance in organotypic cell culture. *In situ* hybridization was performed on organotypic cultures using a 35S-labeled oligonucleotide probe specific for NMDA receptor NR1 subunit. This slide shows that in the frontal cortex (A) and nucleus accumbens (C), NMDA receptor NR1 subunit mRNA is prominent. The autoradiograph grain density for NR1 subunit mRNA is up-regulated in PCP-treated cultures in frontal cortex (B) and nucleus accumbens (D) compared with control (A and C).

Activation of NMDA receptors is known to kill neurons via a necrotic mechanism characterized by excessive sodium and calcium entry, accompanied by chloride and water entry that leads to cell swelling and death [94]. More recently, it has been realized that NMDA receptor activation can also lead to apoptotic cell death [90, 95, 96]. The characteristics of an excitotoxic insult that leads to necrosis or apoptosis are not clear-cut and may depend on the concentration of glutamate agonist, the duration of the treatment, the receptor subtype activated, and the cell type and its stage of development or maturity [97, 98]. In general, it is thought that a mild insult, given sufficient time, will result in apoptosis, whereas a more severe insult will lead to necrotic cell death. However, it is becoming apparent that glutamate-mediated cell death is often not exclusively either necrosis or apoptosis, but presents with features characteristic of both [96, 99, 100]. Although it was not the intent of our studies to absolutely distinguish between necrosis and apoptosis, (i.e., the TUNEL assay is not an unambiguous marker of

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apoptosis), our Western blot assessment of increased Bax expression strongly supports the correlation between positive TUNEL staining and enhanced apoptosis. In addition, the concentration-dependent decrease in mitochondrial function assessed by MTT metabolism occurs at the same level of applied ketamine as the increase in internucleosomal DNA fragments, an index of apoptosis [101]. Thus, most of the loss of cell viability appears to be due to an apoptotic mechanism. We suggest that modest activation of up-regulated NMDA receptors (ketamine concentration-related), over a relatively long time period, produces an increase in neuronal apoptosis, and that this is a valid model for the study of apoptotic cell death.

Previous research has shown a very complex and often contradictory role for NF-kB in neuronal apoptosis. Studies examining hypoxia/reoxygenation, serum withdrawal and extracellular signaling proteins have generally found a protective role for NF-kB [102]. On the other hand, NF-kB translocation appears to be a necessary step in apoptosis induced by cyanide and excitotoxic stimuli [103]. The mechanism by which NF-kB translocation induces apoptosis is not completely clear, but it is assumed that this mechanism involves the regulation of one or more genes known to play a role in apoptosis. However, because NF-kB is known to regulate both anti-apoptotic and pro-apoptotic proteins, depending on the cell type and the nature of the stimulus [93, 102, 103], it is possible that the ultimate effect will be the sum of several downstream regulators. In neurons, astrocytes and glia these regulators include the anti-apoptotic Bcl family members, Bcl-X and Bcl-2 [102, 105], the important antioxidant, manganese superoxide dismutase, and the potentially detrimental proteins p53, inducible nitric oxide synthase and cyclooxygenase-2 [106].

The prevention of NMDA/NMDA antagonist-induced cell death in this preparation by SN50, a peptide inhibitor of NF-kB translocation, clearly suggests that the overall effect of NF-kB translocation is pro-apoptotic [89]. Among the potential downstream regulators, we measured the effect of NMDA/NMDA antagonists on Bax and Bcl-X_L, pro- and anti-apoptotic members of the Bcl family [81, 89, 90, 107] that have been reported to be up- and down-regulated, respectively, in various models of apoptosis [108, 109, 110, 111, 112]. Bax is activated by p53 in neurons [113]. In hippocampal neurons cultured from p53 -/- and +/+ mice, it was recently demonstrated that an NMDA-induced increase in Bax was p53 dependent [114]. This study also demonstrated that NMDA-induced DNA fragmentation and TUNEL (terminal dUTP nick-end labeling) staining were found only in cultures from p53 +/+ mice. The ability of SN50 to prevent NMDA-induced apoptosis and increased expression of Bax in our recent study [89] demonstrated that NF-kB is crucial to this process and, when considered in light of the study on hippocampal neurons, suggests p53 may be an intermediate in this pathway. Furthermore, the antagonistic effect of M40403 on NMDA/NMDA antagonist-induced increases in NF-kB translocation and Bax strengthens the argument that increased NF-kB is critical in Bax up-regulation.

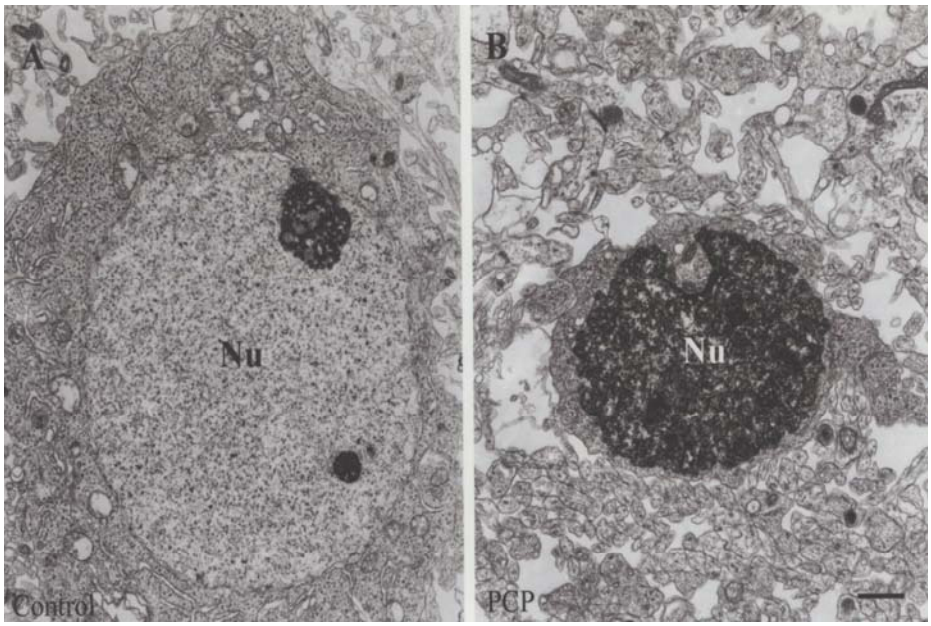

Figure 5. Electron micrographs of frontal cortex neurons from control and PCP-treated organotypic cultures (rat brain slices). Panel A shows a normal neuron with intact cytoplasmic and nuclear membrane from a control culture. Panel B shows a very condensed nucleus of an apoptotic neuron from PCP-treated culture.

Application of NMDA receptor antagonists (ketamine or PCP) to developing brain caused an increase in DNA fragmentation as measured by an ELISA specific for DNA-associated histone proteins (nucleosomes) and by an increase in TUNEL [90, 107]. This was blocked by M40403 at the optimally effective concentration. M40403 also prevented NMDA receptor antagonist-induced nuclear transport of NF-kB and increased expression of Bax relative to Bcl-X_L. Meanwhile, SN50, a peptide inhibitor of NF-kB translocation, was also able to block NMDA receptor antagonist-induced cell death as well as the increased Bax/Bcl-X_L expression ratio [107]. These data demonstrate that NMDA receptor antagonist-

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induced apoptosis requires the production of $O_2^{\cdot-}$, which serves as an upstream regulator of NF-kB translocation. NF-kB translocation appears to then increase Bax expression and/or to down-regulate Bcl-X_L, ultimately leading to apoptosis.

In summary, anesthetic (NMDA receptor antagonists)-induced cell death would be characterized by temporally distinct changes in superoxide anion ($O_2^{\cdot-}$), nitric oxide ($NO^{\cdot}$), phosphorylation of I-κBα, NF-κB nuclear translocation, Bcl-2 family protein, mitochondrial cytochrome-c release, and caspase-3 activation. The success of inhibiting any of these steps in preventing cell death would be time-dependent, and further pharmacological inhibition of $O_2^{\cdot-}$ and $NO^{\cdot}$ formation would prevent changes in proposed downstream events, including phosphorylation of I-κBα by I-κB kinase (IKKβ), NF-κB nuclear translocation, and increase in Bax, mitochondrial cytochrome c release and caspase-3 activation. On the other hand, blockade of NF-κB translocation and caspase-3 activity will have no effect on postulated upstream steps.

We have attempted to clarify the role of superoxide anion in NMDA receptor-mediated apoptosis and to delineate the downstream signaling pathways. It is hypothesized that superoxide generation via mitochondrial dysfunction and perhaps other Ca^{2+}-sensitive enzymatic pathways occur upstream of NF-kB activation and acts as an intracellular signal by changing the redox state of the cell. The resulting translocation of NF-kB is proposed to be pro-apoptotic, either directly or indirectly, leading to a relative change in the expression of the Bcl family proteins Bax (pro-apoptotic) and Bcl-X_L (anti-apoptotic).

Based on these data, several strategies to ameliorate the apoptoic effect of NMDA antagonists in the developing brain can be envisioned. In addition to M40403 (to mimic superoxide dismutase and block the effects of $O_2^{\cdot-}$) and SN50 to block nuclear translocation of NF-κB transcription factors, the following inhibitors could be added at various times relative to NMDA receptor antagonist treatment and removal to determine the contribution and temporal position of several elements of the proposed pathway leading to cell death. The nitric oxide syntase inhibitor, 7-nitroindazole can be used to block neuronal NOS. Likewise, PS-1145 can be used to block IKKβ and Z-DEVD-FMK can be used to block caspase-3. Therefore, these inhibitors could be selected to block early, middle and late events in the proposed pathway. Within the framework of the systems biology approach, these agents can be used to perturb the model in an iterative fashion in order to test specific hypotheses and enhance our understanding of the overall pathway.

Increased levels of oxidative damage to DNA, lipids and proteins can be detected by a range of assays. Mitochondria and mitochondrial DNA are major targets of free radical attack. How mitochondrial DNA mutations lead to impaired electron transport chain functioning, and how impaired electron transport, in turn, leads to decreased ATP production, increased formation of toxic free-radicals and altered calcium homeostasis are topics beyond the scope of this report.

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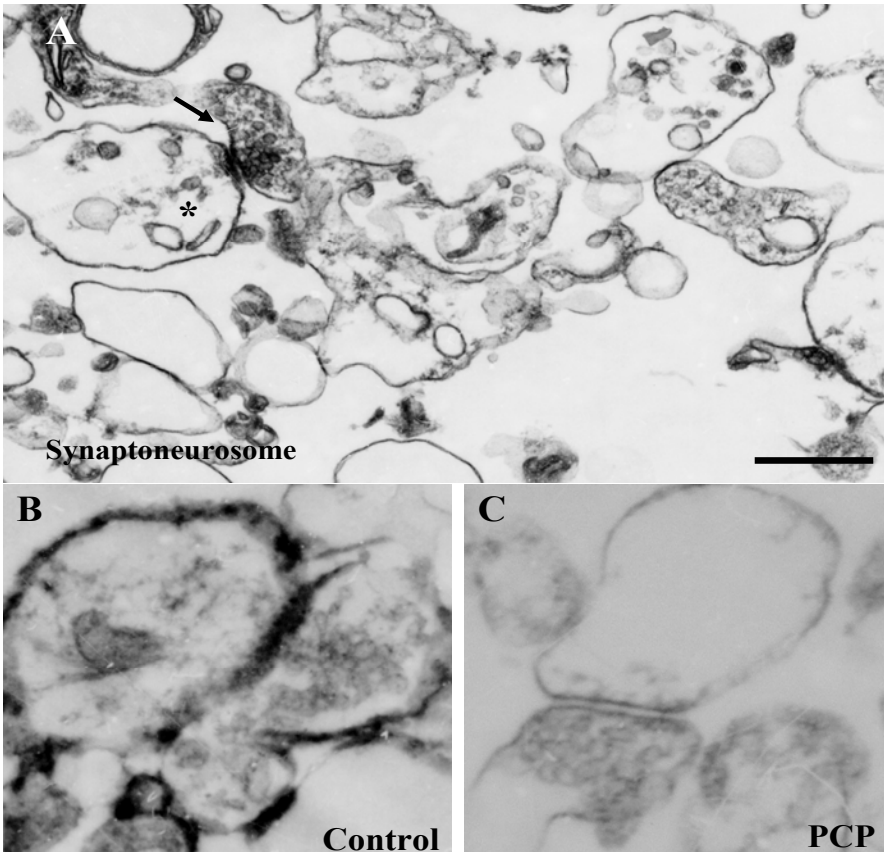

Figure 6. Electron micrograph of the synaptoneurosomes prepared from the striatum-nucleus accumbens complex of the organotypic culture. (A) General view of the synaptoneurosome. Synaptoneurosomes from control (B) and PCP-treated culture (C) were immunostained with anti-PSA-NCAM antibody.

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NMDA receptor dysfunction, neurodegeneration and synaptogenesis

Previous studies *in vivo* have demonstrated that perinatal PCP administration results in profound behavioral abnormalities in the adolescent rat that may be related to enhanced apoptotic cell death of neurons in the frontal cortex [107, 115]. These cortical deficits could have a significant impact on the function of subcortical structures, such as the nucleus accumbens, which serves as an important regulatory center by integrating the functions of the basal ganglia and the limbic system. The nucleus accumbens receives glutamatergic afferents from several brain regions, in particular the frontal cortex [116, 117, 118, 119]. Medium spiny neurons account for the majority of the neostriatal cell population and represent a major synaptic target of dopaminergic input to the striatum [120, 212, 122, 123]. Thus, alteration in the cortical input to these neurons during development could play a significant role in mediating the behavioral effects of perinatal ketamine/PCP treatment later in life.

In one of our recent studies, an organotypic slice culture model was used to examine the relationship between the neurotoxic effects of PCP in the frontal cortex and a postulated disturbed synaptogenesis in the striatum and nucleus accumbens [90]. The organotypic slice allows the investigation of the mechanisms of ketamine/PCP-induced cortical cell death in a simplified system that retains its original cytoarchitecture, including important connections between the cortex and striatum.

Polysialic acid neural cell adhesion molecule, PSA-NCAM, is formed by the enzymatic transfer of large, negatively charged carbohydrate polymers of α-2,8-linked sialic acid to the fifth immunoglobulin domain of the NCAM molecule. The presence of polysialic acid residues on NCAM is thought to inhibit homophilic, Ca^{2+}-dependent NCAM binding, thereby decreasing cell-cell adhesion [124, 125, 126, 127]. The sialylation state of NCAM is controlled by developmentally regulated Golgi sialyltransferase activity [128]. This transferase activity is Ca^{2+}-dependent [129], and this may account for its regulation by NMDA receptors [130, 131]. PSA-NCAM exhibits a highly regulated expression pattern. During embryonic development, its expression is closely correlated with axon path finding and targeting. Later in development, PSA-NCAM expression is more restricted, being primarily associated with regions capable of morphological or physiological plasticity, such as the hippocampus. The loss of PSA-NCAM in rats is postnatally regulated, and its timing is highly region-specific [132, 133, 134]. In rat striatum, immunostaining for polysialylated NCAM is still intense at PND 16 [133], but is not detected in adults [133, 134]. The regulation of PSA-NCAM expression by NMDAergic activity plays a critical role in neuroplasticity during development, particularly in NCAM-mediated cell-cell interactions and synapse formation [135].

In these organotypic experiments, treatment of corticostriatal slices with PCP caused a substantial increase in apoptosis as indicated by TUNEL-positive neurons in the cortex [90] along with a concomitant decrease in PSA-NCAM staining in the striatum. To confine the analysis of PSA-NCAM expression at synaptic sites, synaptoneurosomes were isolated from striatum-nucleus accumbens complex of organotypic cultures. Synaptoneu-

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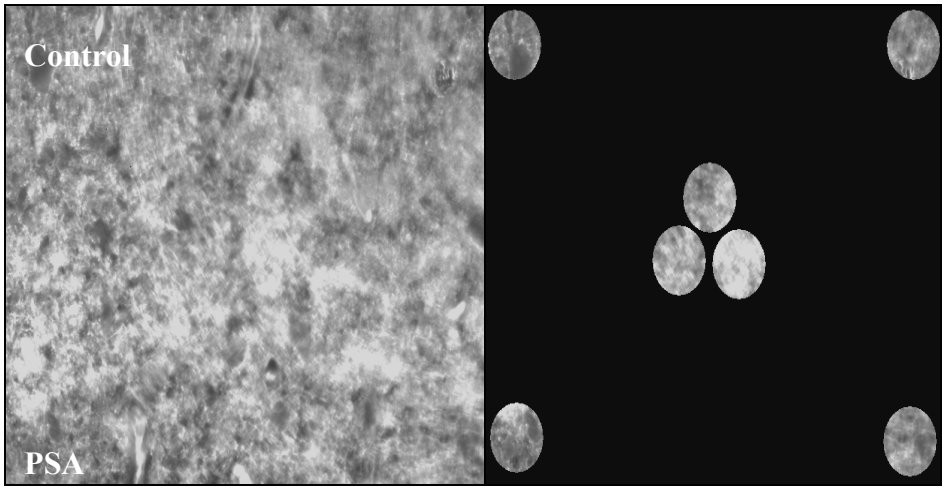

Figure 7. Quantitation of PSA-NCAM immunostaining. Images were acquired with a digital microscopy apparatus (SimplePCI, Cranberry Township, PA, USA) and saved as BMP-24-bit gray scale files. The regions of interest (ROI) were randomly selected from the ventral portion of the striatum. A threshold that segments the images into labeled immunostaining and background was determined interactively by consensus of two trained observers (one of whom was blind to the treatment) and then held constant for all ROI within the sections. The density of PSA-NCAM immunostaining in each 1.43 mm^2 ROI was estimated by measuring the density within seven 50-μm fixed diameter circles (three circles arranged in a triangle in the center and one circle in each of the four corners) that exceeded the threshold value. Two coronal cryostat sections (10 μm) were taken through the anterior striatum from each brain. The values were then averaged between sections to give a value for each animal. Three or four animals from each group were measured. One-way ANOVA with Tukey's post hoc test for individual treatment differences was used for statistical analysis.

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rosomes are obtained from a subcellular fraction enriched for synaptic protein. Figure 6A, at the electron micrograph (EM) level, shows a general view of the synaptoneurosome prepared from a portion of the striatum-nucleus accumbens of the organotypic culture. It reveals an enrichment of "button-shaped" profiles. The arrow indicates a representative synapse with intact presynaptic component (numerous clear vesicles). Also, at the EM level, synaptoneurosomes from control cultures (6B) and PCP-treated cultures (6C) were immunostained by a monoclonal antibody, PSA-NCAM, using a pre-embedding immunoperoxidase technique. In this preparation, final sections were not counter-stained with uranyl and lead. Intense PSA-NCAM immunostaining (electron dense peroxidase reaction product representing PSA-NCAM immunoreactivity) was observed on synaptic contact, and both pre- and post-synaptic membrane surface of the control culture (Figure 6B). In the pre-synaptic component, representative clear vesicles can still be identified. However, PSA-NCAM immunostaining is largely diminished from either pre- or post-synaptic components after PCP treatment at a concentration of 3 µM.

The decrease in PSA-NCAM corresponded to an approximate 50% decrease in PSA-NCAM immunoreactivity as assessed by immunocytochemical examination (Figures 7 & 8), and immunoblot assay of synaptoneurosomes compared with control [90]. This decrease could be the direct result of local NMDA receptor blockade (and subsequent reduction in Ca^{2+}-regulated polysialyl transferase activity) or the indirect result of cortical neurotoxicity and a subsequent decrease in NMDAergic input to the striatum. The fact that the superoxide dismutase mimetic, M40403, blocked cortical apoptosis, as well as the loss of PSA-NCAM immunoreactivity in the ventral striatum, supports the latter mechanism [85]. Regardless of the potential relationship between PSA-NCAM and synaptophysin, the reduction of both PSA-NCAM and synaptophysin by PCP treatment strongly suggests that PCP alters striatal synaptogenesis [90].

Taken together, these experiments demonstrate that PCP treatment results in significant cortical cell death as evidenced by increased DNA condensation and fragmentation. These data suggest that selective cortical apoptosis induced by PCP can be mimicked *in vitro*, and that cortical neurodegeneration negatively impacts synaptogenesis in the striatum. Although the mechanism is not completely understood, it could involve a partial loss of corticostriatal afferent neurons and/or the blockade of NMDA receptors in the striatum. It is also possible that the dysfunction of striatal synaptogenesis contributes to the behavioral abnormalities observed following perinatal PCP administration.

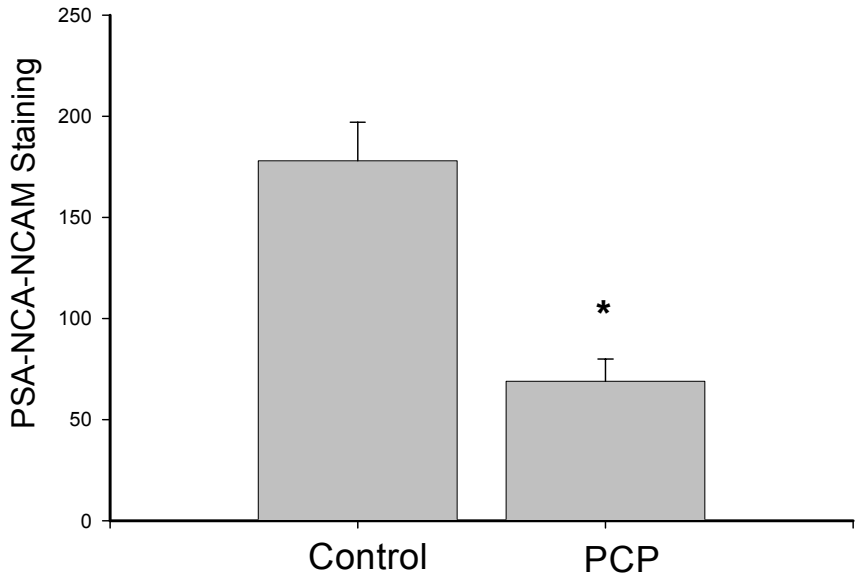

Figure 8. Quantitation of PSA-NCAM immunostaining following *in vivo* treatment of rat pups with saline (control) or PCP.

Conclusion

Although not fully delineated, the working model for NMDA/NMDA antagonist-induced neurodegeneration involves the modulation of the normally occurring brain sculpting mechanisms controlling central nervous system development. Exposure of the developing mammal to PCP, ketamine or other NMDA antagonists perturbs the endogenous NMDA receptor system and results in enhanced neuronal cell death [136]. Up-regulation of the NR1 subunit of the NMDA receptor by the presence of a NMDA antagonist (e.g., PCP), appears critical to the subsequent cell death. Blockade of this NMDA receptor up-regulation dramatically diminishes the apoptotic cascade. Another perturbation of the system, the addition of a superoxide dismutase mimetic (M40403), also blocks the apoptotic cascade by decreasing the abundance of the superoxide anion. With the use of a third perturbation, the inhibition of NF-kB

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translocation by SN50, NMDA/NMDA antagonist-induced neuronal cell death is also prevented. These results indicate that the overall effect of the NF-kB dimer translocation into the cell nucleus is pro-apoptotic. The iterative perturbations of the system allowed refinement of the general model and reinforced the selective pathways that represent the whole of the data.

Along with the aberrant neuronal apoptosis, synaptogenesis was also affected by NMDA/NMDA antagonists in this developing mammalian model. The importance of the NMDA receptor system to synaptogenesis was reinforced by perturbation of the system with the superoxide dismutase mimic, M40403. The resultant data support the hypothesis that PCP treatment reduces striatal synaptogenesis by producing cortical neuron apoptosis, resulting in a subsequent decrease in NMDAergic input into the striatum.

Major data gaps were defined during the application of the systems biology approach that serve to define research needs. For example, global genomic and proteomoic data sets are necessary and are scheduled to be developed. These global data sets will be integrated and compared against the model. Discrepancies will be identified and hypothesis-driven studies conducted to explain the discrepancies and to design additional perturbations to discriminate among alternative explanations for the disparity between the model and the data. Thus, the data generated with the iterative repetition of the third and fourth steps will be used to recast the model in light of the new experimental data. In addition, mathematical modeling is the ultimate goal of the systems biology/systems toxicology approach, and more work will be necessary to achieve this computational step with this data set.

Although many more studies will be necessary to achieve a quantitative model, a general pathway has been constructed and discretely perturbed in an iterative manner with carefully selected agents as defined by the systems biology/systems toxicology approach [137]. Precise developmental stage and dose-response experiments with the use of the phenotypic anchor, neuronal cell death, and global gene and protein expression assays remain to be completed. As these data become available for integrative and iterative evaluation, the model will be improved. A well described model will lead to a better understanding of the potential neurotoxicity of the NMDA antagonists; including many commonly used anesthetic agents, in the developing human.

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Glossary

Systems Biology/Systems Toxicology is a scientific approach that involves the study of perturbations by chemicals and/or stressors by monitoring alterations in gene and protein expressions that are linked firmly to toxicological outcome in an iterative and integrative manner.

Apoptosis, also known as programmed cell death, is a ubiquitous mode of cell death known to play an important role during embryogenesis, development and adult cellular homeostasis.

NMDA Receptor or N-methyl-D-aspartate receptor is linked to a calcium permeable cationic channel that is blocked by magnesium in a voltage-dependent manner. The receptor and channel comprise a receptor/channel complex that includes several regulatory sites. Multiple NMDA receptor subunits and splice variants have been identified.

Excitotoxicity is acute neuronal death in response to a variety of insults to the nervous system, including anoxia, hypoglycemia, seizure, and mechanical trauma.

Oxidative Stress is produced by free radicals or reactive oxygen species (ROS) and reactive nitrogen species (RNS) including superoxides, hydroxyl radicals, and hydrogen peroxide and for RNS, NO and peroxynitrite. The free radicals interact with cellular macromolecules resulting in damage to DNA, mitochondria, cell membranes and other cellular constituents.

Ketamine is a dissociative anesthetic, used in animals and humans, that induces sedation, amnesia and marked analgesia and is a noncompetitive blocker of the calcium permeable cationic channel of the NMDA receptor. Although it has been used in adults as an anesthetic, it is now primarily limited to pediatric use. As a drug of abuse it is known as Special K.

Phencyclidine (phenyl cyclohexyl piperidine) or PCP is a dissociative anesthetic that has fallen out of use because of the frequent occurrence of unpleasant hallucinations and psychological problems. It is a noncompetitive blocker of the calcium permeable cationic channel of the NMDA receptor. As a drug of abuse it is known as Angel Dust.

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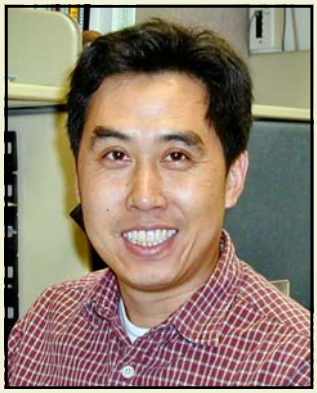

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