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Ontogeny of the N-Methyl-D-Aspartate (NMDA) Receptor System and Susceptibility to Neurotoxicity

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The NMDA receptor has been widely investigated in recent years as a target for the pharmacological management of seizures, pain and a variety of neurological disorders. Its role in normal central nervous system (CNS) activity and development, as well as in the development of CNS abnormalities and neurodegeneration has also been of interest. The NMDA receptor is one of three pharmacologically distinct subtypes of ionotropic receptor channels that are sensitive to the endogenous excitatory amino acid, L-glutamate. The ontogeny of the NMDA receptor, a multiple tetrameric and heteromeric channel complex with at least six known subunits, is controlled by three gene families and varies in developmental profile with species and regional brain area. NMDA receptors play a role in excitatory synaptic transmission, in the activity-dependent synaptic plasticity underlying learning and memory, and in pre- and postnatal CNS development, including brain cell differentiation, axonal growth and degeneration of unused neurons. The results of recent studies suggest that sustained alteration of NMDA receptor activation during critical periods of development may have deleterious effects on normal CNS development and function. Neonatal rats administered the NMDA receptor antagonists 2-amino-5-phosphonovalerate (AP5) and MK-801 during the first two weeks of life develop abnormal axonal arborization in the retinal connections to the superior colliculus, interfering with normal visual responses. Results from monkey studies suggest that chronic developmental exposure to high doses of a NMDA antagonist, remacemide, has pronounced and long-lasting effects on learning. Recent findings indicate that if NMDA receptors are blocked during a specific period in neonatal life (first two weeks postnatally in the rat), massive apoptotic neurodegeneration results, due not to excitotoxic overstimulation of neurons but to deprivation of stimulation. These observations require further laboratory evidence and support in order to establish their relevance to drug-induced human neurodevelopmental concerns. It is necessary to investigate the relevance of these findings in other animal species in addition to the rat, most notably, nonhuman primates, where neuronal cytoarchitecture and development are significantly different than the rodent but more like the human.

Key Words: **N-methyl-D-aspartate; NMDA receptor; brain cell differentiation; CNS development; synaptic plasticity; apoptosis.**

Introduction

The N-methyl-D-aspartate (NMDA) receptor system is associated with many of the primary functions and developmental mechanisms of the nervous system. Memory is one such primary function. Long-lasting changes in the excitability of several associated neurons, as a result of repeated release of L-glutamate and activation of the NMDA receptor, are associated with the phenomenon of long-term potentiation (LTP) (Kato *et al.,* 1999). The involvement of the glutamate receptor system and LTP is strongly linked to new learning and memory in animal models (Scheetz and Constantine-Paton, 1994). The glutamate receptor system is thought to be a major signaling pathway for neuronal migration during brain development. As developmental inputs increase in strength and number, postsynaptic Ca^{++} ion influx through glutamate-activated NMDA receptors increases, and this Ca^{++} ion influx is postulated to trigger changes in neuronal metabolism and gene expression (Scheetz and Constantine-Paton, 1994).

Along with these central roles as "brain sculptor" and "memory maker," excessive activation or disruption of the NMDA receptor system also has the potential to mediate cellular damage. Overstimulation of this controlling receptor system can result in cell death. Via a cascade of events, excess glutamate release and receptor interaction can result in even more exaggerated glutamate release and receptor stimulation. Described by Choi as the "spiral of death," (1988, p. 623) this mechanism of cell death involves the influx of Ca^{++} and other ions with water. Subsequent cellular swelling and release of degradative

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enzymes results in cell death. Many neurotoxicants, including the excitotoxicants kainate and domoate, are thought to produce their adverse effects by overstimulating the fully mature glutamate receptor system.

During development in the rat, especially during postnatal days (PND) 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 as deriving from the increased expression of specific NMDA receptor subunits (Miyamoto *et al.*, 2001). 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 (Behar *et al.*, 1999). If stimulation of glutamate release reinforces neuronal connections, then blockade of that stimulation by NMDA antagonists may result in fewer or nonfunctional connections. Selected anticonvulsants and dissociative anesthetics are reported to produce their toxicity on the developing nervous system via antagonism of the NMDA receptor system (Ikonomidou *et al.*, 1999, 2001; Popke *et al.*, 2001a). The developmental toxicity of several agents, including methylmercury, lead, and ethanol (Guilarte, 1997; Guilarte and McGlothan, 1998; Ikonomidou *et al.*, 2000; Kumari and Ticku, 1998; Miyamoto *et al.*, 2001), is also thought to result from interaction with the NMDA receptor system.

NMDA Receptor System Function and Anatomy

The NMDA receptor has been widely investigated in recent years as a target for the pharmacological management of pain and a variety of neurological disorders, for its function in normal central nervous system (CNS) activity and development, and for its role in the development of CNS abnormalities and degeneration. Abnormalities in glutamate transmission, particularly involving excessive or insufficient activation of NMDA receptors, have been implicated in aberrations of normal CNS development (McDonald and Johnston, 1990), in the development of epilepsy, in the neurodegeneration associated with Parkinson's, Alzheimer's, and Huntington's diseases, and with amyotrophic lateral sclerosis (ALS). Excitotoxic neuronal death observed after head injury, ischemic events, hypoxia, and hypoglycemia have been attributed to excessive NMDA receptor activation (Choi, 1988).

The NMDA receptor is 1 of 3 pharmacologically distinct subtypes of ionotropic receptor channels that are sensitive to the endogenous excitatory amino acid, L-glutamate. The non-NMDA glutamate-receptor subtypes are pharmacologically sensitive to α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate, as described in the review by Dingledine *et al.* (1999). NMDA, AMPA, and kainate-receptor subunits are encoded by at least 6 gene families, as defined by sequence homology. There are 3 families for NMDA receptors, 2 for kainate, and 1 for AMPA. NMDA receptors play a role in nearly all excitatory synaptic transmission, in activity-dependent synaptic plasticity underlying learning and memory, and in pre- and postnatal CNS development, including brain cell differentiation, axonal growth, and degeneration of unused neurons (Lalonde and Joyal, 1993).

NMDA receptors are distributed ubiquitously throughout the CNS. Most are located on postsynaptic dendrites and dendritic spines in membranal structures named as postsynaptic densities (PSD; reviewed in Sheng, 2001). However, NMDA receptors have also been found on cortical astrocytes, and presynaptically as autoreceptors and heteroreceptors (reviewed in Conti, 1997).

NMDA receptors consist of multiple components that recognize and bind glutamate, coagonist, modulatory molecules (such as glycine and polyamines), dissociative anesthetics (such as phencyclidine and ketamine), redox agents, steroids, histamine, zinc, and Mg^{++}, and a transmembrane channel that is selective for specific cations (i.e., Na^{+}, Ca^{++}, K^{+}) with particular permeability to Ca^{++} (reviewed in Danysz and Parsons, 1998; McBain and Mayer, 1994). The Mg^{++}-binding site within the cation channel blocks ion permeability in a voltage- and use-dependent manner.

There is evidence that the NMDA receptor can exist in 2 different functional states, depending on the level of maturity of the rat, and based on pharmacological responses to the noncompetitive NMDA receptor antagonist dizocilpine (MK-801, Sircar, 2000). The functional states involving different channel conductances, mean open times, and sensitivities to Mg^{++} blockade appear to reflect differential combinations of NMDA receptor subunits (reviewed in Feldmeyer and Cull-Candy, 1996).

Molecular cloning technology has identified multiple tetrameric and heteromeric subunits of the NMDA receptor including NR1 (zeta), NR2A-D (epsilon), and NR3 forms (reviewed in Cull-Candy *et al.*, 2001; Danysz and Parsons, 1998; Dingledine *et al.*, 1999; McBain and Mayer, 1994; see Danysz and Parsons, 1998 and Dingledine *et al.*, 1999 for diagrams of the NMDA receptor complex). The receptor appears to have 3 transmembrane domains and a cytoplasm-facing re-entrant membrane loop. This membrane loop is postulated to control important aspects of ion channel function (Dingledine *et al.*, 1999). The subunits are expressed in varying combinations throughout the CNS and are responsible for conferring distinct pharmacological properties and functional diversity to the receptors. There are 8 known isoforms of the NR1 subunits (Hollmann *et al.*, 1993; Sugihara *et al.*, 1992), the glycine recognition sites that are likely present in all NMDA receptors throughout the CNS. The NR2A-NR2D subunits are the glutamate recognition sites found in varying combinations with NR1 (reviewed in Conti, 1997; Stone, 1993). In the adult, the NR2A subunit appears predominantly in the forebrain, hippocampus, and cerebellum, and the NR2B subunit is highly represented in the olfactory tubercle, hippocampus, olfactory bulb, and cerebral cortex, with intermediate expression in the striatum and midbrain. The adult cerebellum has the greatest

concentration of the NR2C subunit (Stone, 1993), and the NR2D subunit is expressed weakly in the adult thalamus, brainstem, olfactory bulb, and spinal cord (Monyer *et al.*, 1994; Watanabe *et al.*, 1994a).

Functional NMDA receptors require the presence of the NR1 subunit (Lynch *et al.*, 1994) in addition to variable combinations of the NR2 subunits. The 2 isoforms of the NR3 subunit may play a modulatory role in NMDA receptor function, reducing channel open time and conductance (Das *et al.*, 1998; Perez-Otano *et al.*, 2001). The differential distribution of the various combinations of NMDA receptor subunits throughout the CNS confers diversity of pharmacological sensitivity to different agonists and antagonists (reviewed in Monaghan *et al.*, 1998).

Ontogeny

The NMDA receptor system has been shown to play a major role in the normal development of the CNS; this development occurs through many stages of neurogenesis, migration, proliferation, and death of neurons, axonal outgrowth, and synapse formation and elimination. Glutamate was identified as one of the more than 36 trophic factors involved in modulating each of these phases of neuronal differentiation. The results of recent studies suggest that sustained alteration of NMDA receptor activation during critical periods of development may have deleterious effects on normal CNS development and function (Ikonomidou *et al.*, 1999, 2001).

During early cortical development, NMDA and other amino acid receptors (e.g., GABA) are found on cycling neuroblasts, prior to their development into functioning neurons (Dammerman and Kriegstein, 2000). However, the NMDA receptor does not appear to be functionally mature in the rodent until several weeks after birth. For example, a recent study by Zhu and Barr (2001) showed that pretreatment with MK801 failed to attenuate the development of morphine dependence and the behavioral signs of acute opiate withdrawal in 7-day-old rat pups. However, it did reduce the development of morphine dependence in 14-day-old and 21-day-old pups and in adult rats, and decreased the expression of morphine withdrawal in 14-day-old pups.

Cortical neurogenesis and neuronal migration is complete by the first week of postnatal life in the rat. Thereafter, neocortical development proceeds by progressive and variable strengthening or elimination of synapses and neuronal death. Animal studies have demonstrated specific patterns of transient increases and decreases in the expression of NMDA receptors and receptor subunits, and in glutamate regulation of prenatal and postnatal CNS development via NMDA receptor activity in many regions, including the cerebellum (Burgoyne *et al.*, 1993), visual cortex (Quinlan *et al.*, 1999a,b), superior colliculus (Simon *et al.*, 1992), forebrain (Watanabe *et al.*, 1992), hippocampus (Guilarte and McGlothan, 1998), striatum (Hurst *et al.*, 2001), and neostriatum (Colwell *et al.*, 1998). In the neonatal rat striatum, NMDA receptor maturation occurred later than kainate and AMPA receptor expression (Colwell *et al.*, 1998; Nansen *et al.*, 2000).

In a study by Rao *et al.* (1997), the use of *in vitro* receptor autoradiography showed increased global and NMDA receptor-specific glutamate binding from birth to PND 9, and decreased global, but not NMDA receptor-specific binding thereafter to PND 30 in the rat nucleus tractus solitarii and ventrolateral medulla (brainstem regions involved in the regulation of several autonomic functions including breathing, blood pressure regulation, and swallowing). In the rat neostriatum, NMDA-receptor responses, measured using infrared videomicroscopy and whole-cell patch-clamp analysis, were absent at age 3 days, and showed increasing response strength at age 7 days to a maximum at age 14 days, then an attenuation in response-strength on days 21 and 28 (Colwell *et al.*, 1998). The results of that study showed increased MK-801 binding in rat neostriatal tissue homogenates from PND 3 to 7 and 14 to 21, a peak at PND 28, and then a decline in binding thereafter to adult levels by PND 60, with no changes in MK-801 binding affinity throughout the study.

In the fetal human brain, NMDA receptor binding sites were demonstrated in the hippocampus, thalamus, and subthalamic nucleus by gestational day (GD) 115 (Lee and Choi, 1992). The numbers of ionotropic, glutamate receptor binding sites in those regions increased until GD 140–150, and then decreased in number by GD 168 or 182.

Examination of the NMDA-receptor subunit mRNA expression and binding sites has revealed differential expression at varying stages of development in different regions of the CNS (reviewed in Constantine-Paton, 1994; Cull-Candy *et al.*, 2001; Dunah *et al.*, 1999; Ritter *et al.*, 2001; Scheetz and Vallano, 1998). High levels of NMDA receptor activity (Hestrin, 1992) and synaptic plasticity (Lund and Lund, 1976) were observed in the superior colliculus shortly after birth, and declined over the subsequent two weeks of neonatal life in rats. During that time, the expression of the NR1 subunits showed an increase from PND 6 to a peak at PND 19, and then declined thereafter to levels observed in adult rats (Hofer *et al.*, 1994, as reviewed in Scheetz and Constantine-Paton, 1994). In contrast, expression of the NR2B subunit decreased during the early neonatal weeks from its highest observed level at birth in rodents (Hofer *et al.*, 1994; Watanabe *et al.*, 1992). The pattern was different in the rat striatum than in other brain regions, showing low levels of NMDA receptor responses in the early postnatal period and increases in NMDA receptor sensitivity in the second and third postnatal weeks (Hurst *et al.*, 2001).

In the rat cerebellum, the NR1 subunit is expressed in fetal, postnatal, and adult stages; the NR2B subunit is expressed early in the postnatal period and decreases as the animal matures; and the NR2A subunit increases to its highest levels in the adult (Takahashi *et al.*, 1996). After PND 10, the expression of the NR2C subunit also increases to high levels in the adult rat cerebellar granule cells (Watanabe *et al.*, 1994b).

The NR2D subunit is expressed in Purkinje cells for the first 8 days of postnatal life (Akazawa *et al.*, 1994).

Autoradiography and *in situ* hybridization demonstrated the presence of NMDA receptor subunit mRNA in human fetal brains obtained at gestational ages 57–140 days, with transient increases in gestation weeks 11, 13, and 19 (Ritter *et al.*, 2001). The predominant subunits represented in the human fetal brains were the NR1, NR2B, and NR2D types similar to observations in neonatal rat brain (Ritter *et al.*, 2001). The NR2A and NR2C subunits were detected in the human fetal cortex, but were not expressed until after birth in the rodent (Watanabe *et al.*, 1992). The high level of expression of the NR2D subunit in prenatal and neonatal brain compared to that in the adult CNS suggests a strong role in brain development (reviewed in Dunah *et al.*, 1999). NR2A subunit mRNA was found in most regions of human neonatal brain, gradually replacing the NR2B function throughout the CNS. This was followed by an increase in NR2C receptor subunits, particularly in the cerebellum (see reviews by Akazawa *et al.*, 1994; Cull-Candy *et al.*, 2001; and Monyer *et al.*, 1994).

CNS development can be modified by sustained exposure to specific NMDA-receptor agonists and antagonists and by factors such as sensory deprivation and isolation, chronic pain, and maternal separation during critical periods of CNS development. Neonatal rats administered the NMDA receptor antagonists 2-amino-5-phosphonovalerate (AP5) and MK-801 during the first 2 weeks of life developed abnormal axonal arborization in the retinal connections to the superior colliculus, which interfered with normal visual responses (Simon *et al.*, 1992). Stress-induced circulating glucocorticoids can alter normal hippocampal neurogenesis indirectly by interaction with NMDA receptor-dependent activity (Gould and Tanapat, 1999). It has been hypothesized that aberrant behavior in adult rats subjected to sensory isolation, chronic pain, or maternal separation during the critical neonate period of CNS development resulted from excessive or insufficient NMDA-receptor activity and subsequent increased or decreased apoptosis in specific areas of the developing brain (Anand and Scalzo, 2000).

Considerably more is known about the timing and sequence of NMDA receptor-subunit maturation in the rodent than in the human. The general pattern of NMDA-subunit expression appears to be similar in rats and humans, with ubiquitous NR1 subunit expression throughout development and adulthood, and high levels of NR2B and NR2D early in development, which decrease, while NR2A and NR2C subunit expression increases into adulthood. The NR3A subunit expression is initiated around the time of birth in rodents. Observed interspecies differences in subunit expression reflect varying temporal patterns related to CNS maturation. The maturity of rat CNS at PND 6–9 was suggested as corresponding with that in human infants at birth when full-term, based in part on measures of thermal pain thresholds, peak NMDA receptor density, and peak rates of brain growth and synaptogenesis (Anand and Scalzo, 2000).

Behavioral Effects of NMDA Receptor Blockade

Given the important role of the NMDA receptor systems in both normal development and learning processes, the functional consequences of exposure to agents that affect these systems during development are of great interest. While little is known about the ontogeny of specific NMDA receptors in nonhuman primate species, it is logical to presume that in these animals, they continue to play a critical role throughout development and in learning processes. Data from human studies also support the presumption that NMDA receptor populations continue to evolve with age, since there are significant differences in amino acid-binding sites across development from the neonatal period into old age (Court *et al.*, 1993; D'Souza *et al.*, 1992; Johnson *et al.*, 1993; Slater *et al.*, 1993). Recent studies in juvenile rhesus monkeys (Popke *et al.*, 2001a,b) examined the effects of chronic (18 months) daily exposure to remacemide or MK-801 (dizocilpine) on the acquisition of several cognitive function tasks designed to model learning, color and position discrimination, short-term memory, and motivation.

Remacemide acts as a relatively low-affinity, noncompetitive antagonist of NMDA receptors and as a relatively high-affinity blocker of fast sodium channels. MK-801 is the classical high-affinity, selective, noncompetitive NMDA receptor antagonist. Treatment during this protracted period of development was thought to provide sufficient exposure to allow for the assessment of effects of NMDA-receptor blockade during continuing brain maturation. It is unknown whether NMDA-receptor blockade during this stage of development in the nonhuman primate will cause either the classical "Olney lesion" (see below) or changes in the pattern of apoptosis as noted in neonatal rats (see also below), or neither. Low or high doses of both drugs were administered orally and daily for 18 months to separate groups of young monkeys, beginning at about 9 months of age. Their ability to learn how to perform several complex tasks was measured throughout treatment. The dosing and testing procedures that were utilized served to minimize the role that acute drug effects played in the results and enabled analyses to focus on the long-term effects of chronic treatment. Because both compounds are known to inhibit the function of NMDA receptors, it was hypothesized that both remacemide and MK-801 would disrupt the acquisition of behaviors.

The data showed that chronic developmental exposure to a relatively high dose of remacemide (50 mg/kg/day) delayed acquisition of simple discriminations (e.g., color and position discrimination; Popke *et al.*, 2001b) and the acquisition of tasks requiring new learning. This latter effect was both striking and long lasting: affected subjects showed no evidence of recovery after exposure was either decreased or eliminated for 6 months (Popke *et al.*, 2001a). Alternatively, a lower dose of

remacemide (20 mg/kg/day) had no discernible effects on these same tasks, and there was no effect of either dose of remacemide on motivation (Popke *et al.*, 2001a) or short-term memory (Popke *et al.*, 2001b). There were also no effects of either dose of remacemide on clinical chemistry, hematology, or ophthalmic parameters, or on general comportment; thus, the noted behavioral effects appeared targeted to very specific aspects of brain function. Chronic treatment with MK-801 manifested only as a delay in the acquisition of simple discriminations, and this effect was only noted at the high dose (1.0 mg/kg/day; Popke *et al.*, 2001b). Given the differential effects of these drugs and their somewhat different mechanisms of action, it is likely that the long-lasting effects of remacemide on learning resulted either from its ancillary activity at fast sodium channels or from its ability to block NMDA receptors and sodium channels concurrently.

While a role for sodium-channel blockade in the effects of remacemide seems likely, it is also possible that the effects of remacemide resulted in part from the action of its primary metabolite. Shortly after oral administration, remacemide is desglycinated to an active metabolite, which has an even greater affinity for the NMDA receptor than does remacemide (Palmer *et al.*, 1992). Thus, although the time course of MK-801 and remacemide in blood may be similar (Hucker *et al.*, 1983; Vezzani *et al.*, 1989), the persistence of the active remacemide metabolite (up to 24 h after high-dose administration) may result in a somewhat longer inactivation of NMDA receptors. This, in turn, may result in a longer duration of NMDA-receptor blockade (and a more prolonged blocking of the laying down of memory) after remacemide treatment than after MK-801 treatment.

Although the effects of remacemide are indeed noteworthy, so is the fact that chronic treatment with MK-801 had minimal effects. Previous experiments in monkeys indicate that acute treatment with MK-801 can have pronounced effects on the performance of the same behavioral tasks in adults (Buffalo *et al.*, 1994; Paule, 1994). Yet, in the Popke studies, daily administration of MK-801 (1.0 mg/kg) during development, starting at 9 months of age, was largely without effect. This result appears remarkable, given the substantial literature suggesting that the excitatory amino acids, and NMDA receptors in particular, play important roles regulating neuronal survival, axonal and dendritic structure, synaptogenesis, and plasticity (McDonald and Johnston, 1993). Developmental observations in humans indicate that marked differences exist with respect to excitatory amino acid binding sites from the neonatal period through the 10th decade of life (Court *et al.*, 1993; D'Souza *et al.*, 1992; Johnson *et al.*, 1993; Slater *et al.*, 1993). These observations suggest that the infant brain is differentially sensitive to agents that affect NMDA-receptor function relative to the adult (D'Souza *et al.*, 1992). This may help to explain why the juveniles in the monkey study were relatively insensitive to the effects of MK-801. It is important to emphasize that this experiment did not include subjects that began treatment and testing as adults. As a result, it is impossible to discern whether the results reported here reflect a specific effect of these NMDA receptor antagonists during development or whether a similar pattern of results would emerge in adult animals that were chronically exposed in this way.

Another explanation for the lack of pronounced effects of chronic MK-801 treatment is that subjects could have become tolerant to its cognitive-behavioral effects. Hasselink *et al.* (1999) reported that acute injections of MK-801 resulted in impaired passive-avoidance performance in rats, but these effects disappeared after 14 days of chronic treatment. In the monkey experiment, MK-801 produced significant increases in response rates on a motivation task that emerged about 5 months after treatment began, but this effect disappeared within the next 5 months. In ongoing rodent studies (Paule *et al.*, unpublished), however, it is clear that chronic treatment with the same dose of MK-801 (1.0 mg/kg/day, per os) during development (from weaning well into adulthood) stunts growth, profoundly disrupts the ability of subjects to learn simple discriminations, and decreases subjects' ability to demonstrate new learning. These findings in the rat are in stark contrast to those observed in the monkey study, where little effect of chronic exposure to MK-801 was observed, and suggest that there may be dramatic species differences with respect to the effects of chronic NMDA receptor blockade and brain function.

In summary, the results of the monkey study suggest that chronic developmental exposure to high doses of remacemide has pronounced effects on learning, whereas similar exposure to MK-801 does not. These effects occurred in the absence of reductions in motivation or reductions in the ability of subjects to perform the motor requirements of the tasks. The effects of remacemide on learning persisted for months, even after treatment ceased, suggesting an enduring effect of blocking NMDA receptors and fast sodium channels.

Neuroanatomical Effects of NMDA Blockade

The discovery that NMDA receptor antagonists such as MK-801 cause dose-dependent reversible or irreversible degeneration in cerebrocortical neurons in adult rats was made over 10 years ago (Olney *et al.*, 1989) and has since been referred to as the "Olney lesion." The localization of the lesion was restricted to a very specific region of the retrosplenial cortex, just distal to the transition zone between the hippocampal subiculum and the cortex. The findings indicate that the adult neurotoxic effect occurs when an NMDA antagonist blocks the endogenous glutamatergic excitation of an inhibitory innervation of the retrosplenial cortex. When disinhibited, the retrosplenial cortex becomes sensitive enough to sustain "excitotoxic" damage from its excitatory glutamatergic and cholinergic input (Olney *et al.*, 1995, 1997).

This adult-type neurotoxicity cannot be induced in the developing brain until the animals reach almost full adult age

(Farber *et al.*, 1995). However, If NMDA receptors are blocked during a specific period in neonatal life (the first 2 weeks postnatally in the rat), it leads to massive apoptotic (not excitotoxic) neurodegeneration, due not to excitotoxic overstimulation of neurons but to deprivation of stimulation. There is a period between the first 2 postnatal weeks and adolescence during which blockade of NMDA receptors in the rat has not been shown to produce either apoptosis or disinhibition-mediated excitotoxicity (Ikonomidou *et al.*, 1999, 2001; Ishimaru *et al.*, 1999).

In a recent report, Olney and coworkers made the following statement: "Programmed cell death (apoptosis) occurs during normal development of the central nervous system. However, the mechanisms that determine which neurons will succumb are poorly understood. Blockade of N-methyl-D-aspartate (NMDA) glutamate receptors for only a few hours during the late fetal or early neonatal life triggered widespread apoptotic neuron degeneration in the developing rat brain, suggesting that the excitatory neurotransmitter glutamate, acting at NMDA receptors, controls neuronal survival. These findings may have relevance to human neurodevelopmental disorders . . ." (Ikonomidou *et al.*, 1999, p. 70).

In 2000, Olney *et al.* proposed that a variety of agents, including several NMDA antagonists, ". . . have the potential to delete large numbers of neurons from the developing brain by a newly discovered mechanism involving the interference in the action of neurotransmitters (glutamate and gamma amino butyric acid, GABA) at NMDA and $GABA_A$ receptors during the synaptogenesis period, also known as the brain growth-spurt period. Transient interference (lasting $\geq$ 4 h) in the activity of these transmitters during the synaptogenesis period (the last trimester of pregnancy and the first several years after birth in humans) causes millions of developing neurons to commit suicide (die by apoptosis)" (Olney *et al.*, 2000, p. 383).

These very recent findings are the first to describe and establish the significant difference of the so-called "Olney lesion" involving brain neurodegeneration and vacuolation seen in adult rats following high doses of NMDA receptor antagonists, with the pathology consisting of apoptosis in multiple brain regions observed in neonates (PND 7 in the rat). PND 7 coincides with the greatest period of hypersensitivity of NMDA receptors and blockade of these receptors during this period results in deletion of large numbers of neuronal cells and significantly diminished cell density in various brain regions. The decrements in cell density vary from 3- to 39-fold, depending on the NMDA blocker administered and the area of the brain measured. These changes were dose-dependent and neuron specific, and they did not occur when blockers of other receptor types (Ca^{++} channel, muscarinic, non-NMDA glutamatergic, or dopaminergic) were administered. PND 7 represents the age when the rat forebrain is most vulnerable to the apoptotic effects of the NMDA antagonists, indicating the dependence of this cell type on glutamatergic input for survival. This period also corresponds to the greatest expression of NR1, as well as the peak of the brain growth spurt. The data indicated that the NMDA receptor must be blocked for at least 4–6 h at a threshold dose of 0.25 mg/kg (+) MK-801. This phenomenon is stereospecific, in that the same dose of (-) MK-801 showed a much weaker effect.

Of course other subcellular mechanisms—probably mechanisms associated with apoptosis—may be involved with the mechanism of the developmental neurotoxicity produced by NMDA antagonists or the adult neurotoxicity produced by NMDA agonists. For example, important pathways in the initiation of apoptosis involve the production of excessive oxidative stress within the mitochondria, secondary to the operation of the tricarboxylic acid (TCA) cycle (Benzi *et al.*, 1991; Bondy and Lee, 1993). If not compensated for by sufficient free-radical scavengers, the release of cytochrome-*c* (Luetjens *et al.*, 2000) through the mitochondrial membrane then leads to activation of cytoplasmic caspases (Glazner *et al.*, 2000; Tenneti and Lipton, 2000) and ultimately to apoptosis. Thus, any toxicants or environmental conditions, whether indirectly activating the NMDA receptor through afferents from another transmitter system or by bypassing the NMDA receptor to act intracellularly, and which promote oxidative phosphorylation through the TCA cycle, would lead to apoptosis. Moreover, other agents besides NMDA receptor antagonists, particularly those that block the actions of growth factors and steroid hormones (Toran-Allerand, 1996), would be expected to cause apoptosis.

Something Old, Something New: Additional Evidence Regarding the Induction of Apoptosis by NMDA Antagonists

Although the mechanisms were not fully elucidated, Nobel prize winning research by David Hubel and Torsten Wiesel (Hubel and Wiesel, 1970; Wiesel, 1999) established that visual experience during critical periods of development was necessary for the normal anatomical and functional development of the visual 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 (Brann, 1995; Hertz *et al.*, 1999; Trudeau and Castellucci, 1993).

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 (Altmann *et al.*, 2001; Kato *et al.*, 1999; Youssef *et al.*, 2000) and neuronal plasticity of the visual system (Bear and Rittenhouse, 1999; Philpot *et al.*, 2001; Quinlan *et al.*, 1999a,b; Udin and Grant, 1999) 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 agents such as MK-801 or ketamine (Ikonomidou *et al.*, 1999) 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 (Ikonomidou *et al.*, 2001; Pohl *et al.*, 1999). Several other independent groups of investigators have also reported that MK-801 increases apoptosis either *in vivo* (Hsu *et al.*, 2000), in motor neurons of a chick embryo preparation (Llado *et al.*, 1999), or in cultured neurons (Terro *et al.*, 2000).

Questions Left Unanswered

The neuropathology induced by glutamate agonists on various species of adult animals is well documented. The histopathologic structural changes identified by Olney and coworkers are identifiable by the neuronal intracellular vacuoles, resulting in a phenomenon known as "vacuolation." As discussed previously, recent reports from Olney's group have described the action of some of these glutamate antagonists in neonatal rats 7–10 days of age. The significant damage on neuronal cytoarchitecture is different in the neonatal versus adult models, perhaps because, in the developing rat, neurons are migrating and connections are being formed. This apoptosis that is triggered by exposure of the developing brain at a critical period to NMDA-receptor antagonists has the potential to disrupt vast developmental and proliferative processes that result in serious decrements in neuron numbers and synaptic density, as well as to produce profound functional and behavioral deficits (Ikonomidou *et al.*, 1999, 2001; Pohl *et al.*, 1999; Popke *et al.*, 2001a,b).

These observations require further laboratory evidence and support in order to establish the relevance of the observations of Olney and coworkers to drug-induced human neurodevelopmental concerns. Extension of the observations of Olney in the developing rat is a logical first step. Toxicokinetic information derived from rodent studies would be very useful for further investigations into other animal species, in which it will be necessary to investigate the relevance of these findings. Such studies would be most notable in nonhuman primates, where neuronal cytoarchitecture and development are significantly different from the rodent but more like the human. Such studies are much more complicated to perform than small animal studies, where issues such as timing, dosing, nutrition, temperature, and maternal nurturing may all have significant impact on the experimental outcome. The data indicate that for the rat, a critical relationship exists between dose, duration, and the developmental timing of exposures to NMDA receptor antagonists. The 7–10 day age window appears to be critical for even brief exposures of NMDA-receptor antagonists to result in CNS damage in the rat. Whether a similar critical window of susceptibility exists in primates has not been established, but should be explored. As suggested by Anand and Scalzo (2000), the developmental maturity of the NMDA receptor system in the primate brain at birth may most closely mimic that of the 7–10-day-old rat. Whether the observed neuropathology results in irreversible behavioral alterations that may have a logical corollary in the primate should also be investigated. The accumulation of such data could provide pivotal information for assessing the potential public health risk associated with the use of agents known to interact with the NMDA receptor system.

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