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Donepezil markedly potentiates memantine neurotoxicity in the adult rat brain

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Abstract

The NMDA antagonist, memantine (Namenda), and the cholinesterase inhibitor, donepezil (Aricept), are currently being used widely, either individually or in combination, for treatment of Alzheimer's disease (AD). NMDA antagonists have both neuroprotective and neurotoxic properties; the latter is augmented by drugs, such as pilocarpine, that increase cholinergic activity. Whether donepezil, by increasing cholinergic activity, might augment memantine's neurotoxic potential has not been investigated. In the present study, we determined that a dose of memantine (20 mg/kg ip), considered to be in the therapeutic (neuroprotective) range for rats, causes a mild neurotoxic reaction in the adult rat brain. Co-administration of memantine (20 or 30 mg/kg) with donepezil (2.5 to 10mg/kg) markedly potentiated this neurotoxic reaction, causing neuronal injury at lower doses of memantine, and causing the toxic reaction to become disseminated and lethal to neurons throughout many brain regions. These findings raise questions about using this drug combination in AD, especially in the absence of evidence that the combination is beneficial, or that either drug arrests or reverses the disease process.

Keywords

memantine; donepezil; tacrine; neurotoxicity; vacuoles; NMDA antagonist; cholinergic; glutamatergic; cell killing; excitotoxic; dendrotoxic

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JWO and NBF have a US patent that pertains to AD ((#6,391,871). The authors, and others consulted during the review process, believe that neither this patent nor Dr. Farber's consultancy with Pfizer Inc., represents a conflict of interest with respect to this report. However, given the current heightened sensitivity about conflict of interest issues, the authors, with the approval of the Editor-in Chief, have chosen to make these disclosures in order to remain above reproach.

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1. Introduction

In acute brain injury conditions, such as hypoxia/ischemia and head trauma, glutamate accumulates in excitotoxic concentrations at NMDA receptors, and blocking these receptors can be neuroprotective [6,34,46,58]. Reasoning from this line of evidence, it has been hypothesized that glutamate, by chronic low-grade overstimulation of NMDA receptors, may contribute to the neuropathology of Alzheimer's disease (AD), and that drugs that block NMDA receptors might be neuroprotective in AD [11].

In addition to its direct excitatory actions in the brain, there is evidence that glutamate is a major regulator of inhibitory tone [44,45,50,51]. By tonically stimulating NMDA receptors on GABAergic inhibitory neurons that modulate both glutamatergic and cholinergic excitatory pathways, glutamate exerts an inhibitory restraining influence over these excitatory pathways. Blocking NMDA receptors in this circuitry abolishes GABA's inhibitory action, thereby disinhibiting the glutamatergic and cholinergic excitatory pathways and causing excessive excitatory (excitotoxic) activity that injures or kills neurons that they innervate [44,49,50]. Thus, any condition leading to impairment or hypofunction of NMDA receptors might be conducive to disinhibition of these excitatory pathways. There is considerable evidence in several animal species [21,37,60,63] that the NMDA transmitter system becomes progressively less functional with increasing age, and it has been reported [62] that NMDA receptor hypofunction is even more extreme in the brains of AD patients than in age-matched controls. Based on these and related lines of evidence, NMDA receptor hypofunction and related disinhibition of excitatory pathways has been postulated as a disease mechanism that might contribute to the neuropathology of AD [46,51]. According to this proposal, it would be hypofunction of NMDA receptors that generates conditions leading to excitotoxic neuropathology in AD, whereas the competing hypothesis mentioned above postulates overstimulation (hyperfunction) of NMDA receptors leading to excitotoxic neuropathology in AD. It is important to recognize that these are conceptually opposite proposals, because for therapeutic intervention in AD one hypothesis calls for decreased NMDA receptor activity, and the other hypothesis calls for increased receptor activity.

The hope that NMDA antagonist drugs might be used therapeutically to prevent excitotoxic neurodegeneration has been nurtured for nearly two decades, but it has been difficult to bring the concept to fruition, the most significant problem being that at doses that exert neuroprotective effects, NMDA antagonists cause adverse side effects ranging from memory dysfunction and psychotic reactions in humans [22,25,32] to acute injury and/or death of neurons in animal brain [1,15,23,49,50,69]. Memory dysfunction arises at least in part from a direct interference in the memory functions sub-served by NMDA receptors [29,38,41,43, 68], but the disinhibition mechanism mentioned above is thought to be responsible for the other side effects.

An important feature of the disinhibition-mediated neurotoxic syndrome is that it involves excessive release of both glutamate and acetylcholine, which are the two main excitatory transmitter systems in the brain. NMDA receptor blockade leading to suppressed GABAergic inhibition and excess excitation of both glutamate (non-NMDA) receptors and cholinergic muscarinic receptors constitutes a latent pathological state of increased excitatory tone. Any additional destabilizing influence favoring increased excitation can have serious neurotoxic consequences. Accordingly, it has been found that drugs such as pilocarpine that increase cholinergic activity, markedly potentiate the neurotoxicity of NMDA antagonist drugs [9,69]. The potentiated neurotoxic syndrome is mediated by excessive release of glutamate at non-NMDA receptors, excessive release of acetylcholine at muscarinic receptors, and additional increased stimulation of muscarinic receptors by pilocarpine. The excitotoxic neuronal injury induced by treatment with an NMDA antagonist alone is potentially reversible and regionally

confined, or may be irreversible and disseminated, depending on the duration of NMDA receptor blockade [50,15]. Drugs that inhibit muscarinic cholinergic activity prevent NMDA antagonist neurotoxicity [50], whereas drugs that increase cholinergic activity cause the neurotoxic reaction to become more widespread and more lethal to neurons, and they cause the neurotoxic manifestations to be triggered by low doses of the NMDA antagonist that would not by themselves be neurotoxic [9,69].

Proponents of the hypothesis that a chronic low-grade over-stimulation of NMDA receptors may underlie the neurodegenerative process in AD have developed a specific NMDA antagonist drug, memantine, as an anti-excitotoxic neuroprotective therapy for AD patients [11,52]. They have described memantine as a unique NMDA antagonist with special receptor binding kinetics that allow it to block NMDA receptors without neurotoxic consequences [5, 11,52]. Memantine has been evaluated in human clinical trials and reportedly is beneficial for AD patients at doses that are free from neurotoxic side effects [55,61]. Prior to the introduction of memantine, cholinesterase inhibitors were the only drugs approved for treatment of AD patients and they were approved specifically for patients with mild to moderate AD. When memantine was approved by the FDA as a treatment for patients with moderate to severe AD, no precautions were stipulated regarding a potential adverse interaction between memantine and cholinesterase inhibitors. In fact, some of the research intended to establish the safety and efficacy of memantine in AD involved administration of memantine to patients already receiving cholinesterase inhibitor therapy [24,61]. While no rigorous data are available, the clinical reality is that many AD patients, regardless of the stage of illness, are currently receiving combined treatment with memantine and a cholinesterase inhibitor.

As mentioned above, there is ample evidence from animal studies that combined administration of an NMDA antagonist with drugs that increase cholinergic activity can have serious neurotoxic consequences. Cholinesterase inhibitors nonspecifically increase cholinergic activity by prolonging the action of acetylcholine at all of its receptors. We searched for, and could not find any published toxicological studies appropriately designed to evaluate the safety of memantine/cholinesterase inhibitor drug combinations and, therefore, undertook the present study in which adult rats were treated with memantine alone, or together with tacrine or donepezil, and the brains were examined 2 to 48 hrs later for evidence of either acute and potentially reversible injury or irreversible neurodegeneration.

2. Methods

2.1 Animals and Drugs

Harlan Sprague-Dawley female retired breeders (6 to 8 months old) were used because sensitivity to the neurotoxic effects of NMDA antagonists is influenced both by age and gender - female and fully adult rats are more sensitive than male or immature rats [14,17,28,49]. Memantine hydrochloride and 9-Amino-1,2,3,4-tetrahydroacridine (tacrine) were purchased from Sigma-Aldrich (St. Louis, MO). Both drugs were dissolved in distilled water and adjusted with NaOH to a pH of 7.4. Repeated efforts to obtain a supply of donepezil from Eisai, the company that manufactures the product, were unsuccessful. Therefore, we obtained 10 mg Aricept® (donepezil HCl) pills from the local pharmacy, solubilized the ingredients in acidified distilled water, sedimented the insoluble ingredients by centrifugation, collected the supernatant, adjusted its pH to 7.4, and used this as our drug preparation.

2.2 Dosing Regimens

Animals were treated with memantine, tacrine, donepezil or combinations of memantine plus tacrine or donepezil ($n \geq 6$ for all treatment groups). All drugs were administered intraperitoneally (ip). To test for early acute signs of neurotoxic injury, rats were sacrificed 2

or 4 h following treatment with saline, memantine alone (10, 15, 20, 30 or 50 mg/kg), tacrine alone (5 mg/kg), donepezil alone (5 or 10 mg/kg), memantine (10, 15, 20, or 30 mg/kg) plus tacrine (5 mg/kg), or memantine (10, 15, 20, or 30 mg/kg) plus donepezil (5 or 10 mg/kg). To test for irreversible neuronal degeneration, rats were sacrificed 24 or 48 h following treatment with saline, memantine alone (20, 30 or 50 mg/kg), donepezil alone (2.5, 5 or 10 mg/kg), or memantine (20 or 30 mg/kg) + donepezil (2.5, 5 or 10 mg/kg). Memantine was tested in a dose range from 10 to 50 mg/kg because the dose of memantine reportedly required to exert an anti-excitotoxic neuroprotective effect is 20 mg/kg [4,35,58]. Donepezil was tested at doses from 2.5 to 10 mg/kg because this dose range is reportedly well tolerated and reversibly inhibits cholinesterase activity in the adult rat brain by about 20% to 70% [31]. In addition to the above experiments, we administered memantine (30 mg/kg) + donepezil (10 mg/kg) and examined the brains at 2 and 48 h by electron microscopy to document that neurons affected by this treatment regimen showed acute pathological changes as early as 2 h posttreatment and were in a state of irreversible degeneration at 48 h posttreatment.

2.3 Procedures

2.3.1 Behavioral observations—Recently we conducted a study [10] in which a battery of behavioral tests was used to evaluate the effects of memantine on various behavioral parameters in adult female rats. In the present study we did not conduct formal behavioral testing but observed the rats continuously for 8 h following treatment (unless sacrificed earlier), and periodically thereafter up to 48 hours. We noted abnormal increases or decreases in locomotor activity, evidence of stereotypic behaviors, signs indicative of possible seizure activity and signs of distress or disturbances in health and well-being.

2.3.2 Histological procedures—All animals at the scheduled time for sacrifice were deeply anesthetized with pentobarbital and perfused with an aldehyde fixative solution via the left cardiac ventricle and ascending aorta. For brains embedded in plastic for combined light and electron microscopy, the fixative consisted of 1% paraformaldehyde and 1.5% glutaraldehyde in 0.1% cacodylate buffer. For brains sectioned by vibratome and processed for silver staining, the fixative consisted of 4% paraformaldehyde in tris buffer. Following perfusion fixation, the brains were removed from the skull and processed as described below.

To evaluate brains for signs of acute neuronal injury or for late cytopathological changes indicative of cell death, the brains were perfused with fixative as described above for plastic embedding, then immersed in the perfusate solution and stored overnight at 4°C. Then the brains were sliced, using a dermatome blade, into 1 mm thick transverse slabs which were post-fixed in 1% osmium tetroxide for 12 h, dehydrated in graded ethanols, cleared in toluene and embedded flat in araldite. Sections 1 µm thick were cut with 1/2-inch wide glass knives on an ultramicrotome and stained with methylene blue/azure II for evaluation by light microscopy. For electron microscopy, areas of special interest from any given block were trimmed to a smaller size, ultrathin sections were cut and suspended over a formvar-coated slot grid (1×2mm opening), stained with uranyl acetate and lead citrate and viewed in a JEOL 100C transmission electron microscope. Slot grids were used because they permit a continuous viewing field (1 × 2 mm) uninterrupted by grid mesh bars.

To determine the extent and pattern of disseminated cell death, the brains were perfused with fixative as described above for silver staining, then immersed in the perfusate solution and stored at 4°C for at least 4 days before sectioning. The brains were then bisected sagittally into two halves and cut on a vibratome into 50 µm thick serial sections, one half of the brain being cut in the coronal plane and the other half in the sagittal plane. The free floating sections were thoroughly washed in triple distilled water and stained by the method of DeOlmos et al. [13] as follows: Preincubate in cupric-silver solution for 1 hr @ 40°C (in dark); wash in acetone;

incubate in silver diamine solution for 35 min; reduce in formaldehyde/citric acid solution for 5 min; wash in triple distilled water; bleach with 0.3% $K_3Fe(CN)_6$ for 10 min; wash in triple distilled water; mount sections onto sub-gelatinized slides; dehydrate in a series of ethanols; clear in xylene and coverslip with DePex mounting medium.

2.3.3 Quantitative evaluation of histopathological changes—To quantify the acute vacuolar reaction in neurons of the retrosplenial cortex, we used thin (1 μ m) plastic sections to permit clear visualization of the intracytoplasmic vacuoles. To ensure sampling of each brain at the same rostrocaudal level, we used coronal sections cut at a level where the decussation of the corpus callosum begins to vanish (approximately -5.30 to -5.60 mm caudal to bregma) [53]. We have determined in prior studies [15,16] that the vacuolar reaction following treatment with NMDA antagonist drugs is maximally severe at this rostrocaudal level. Bilateral counts of vacuolated neuronal profiles were performed on a representative section randomly selected from within the defined rostrocaudal level by an observer blind to the treatment conditions. The mean number of vacuolated neurons per section for a given condition ($n = 6$ brains per condition) was calculated and the group means ($\pm$ SEM) were compared for statistically significant differences.

To evaluate the potential of a given treatment protocol to cause a disseminated cell death reaction, a blind observer examined silver-stained sections cut serially in the coronal plane (one half brain) and in the sagittal plane (other half) and recorded the number (percentage) of animals showing disseminated patches of silver-stained neurons following exposure to a given treatment regimen. To clarify whether the neurodegenerative response could be explained in terms of an action of memantine alone, donepezil alone or required the two drugs combined, we recorded and compared the patterns of degeneration associated with each treatment condition, with an aim toward determining whether the pattern associated with a given condition was distinctively different from that associated with each other condition.

3. Results

3.1 Behavioral Observations

Recently, we reported [10] that when adult female rats are treated with memantine in the dose range from 2.5 to 20 mg/kg, they display the same kind of behavioral manifestations that are seen following administration of other NMDA antagonists such as phencyclidine and MK-801. The response is dose dependent with mild behavioral signs at 2.5 to 5 mg/kg and increasingly more obvious signs in the 10 to 20 mg/kg range. The fully developed syndrome includes horizontal head wagging, truncal and limb ataxia and a wavering gait. Initially the animals appeared dazed and were hypoactive, but gradually they became hyperactive, and in the 2 to 4 h post-treatment interval they incessantly circled around the cage with frequent turning, tail whipping, and occasional rearing. At the highest dose tested (50 mg/kg) these behaviors became very extreme and some animals displayed behaviors suggestive of seizure activity, including vertical head tremor, eye blinking, arching of the back, and body jerking.

Donepezil by itself caused fine total-body tremors, splaying of the hind limbs, and an ataxic gait. The tremors were inconspicuous at rest and became exaggerated upon movement.

Combining donepezil with memantine, especially at the lower doses, resulted in a modification of the behaviors that would be expected from either drug alone. The locomotor stereotypies and hyperactivity characteristically produced by memantine were reduced to a minimum, and the donepezil-specific fine tremors were also decreased. At the highest doses (memantine 30 mg/kg + donepezil 10 mg/kg), new abnormal behaviors emerged. Within about 10 min after the high dose regimen, the rats exhibited a peculiar backward locomotion with their abdomens resting on the floor of the cage while they pushed themselves backwards with their forelimbs

and held their hind limbs rigidly extended rostrally in a splayed and very wide-based posture. After displaying this abnormal motor behavior for 30 to 60 min the animals ceased trying to walk and assumed a prone posture or rolled over on their side and remained motionless, except for a fine head tremor, occasional episodes of rapid eye blinking and periodic body jerks. All of the animals survived until termination of the experiments, including up to 48 h, but they appeared lethargic and hyporesponsive to stimuli throughout the 24 to 48 h interval.

It was difficult to assess whether any of the treatments caused either periodic or status seizure activity. Various behaviors possibly indicative of seizure activity were detected, (occasional rearing and falling, body jerking, facial twitching, eye blinking, tremors of the head and forepaws, wet dog shakes) especially in some animals treated at high doses, but in many animals these behaviors were absent or infrequent and most of the severely affected animals tended to display the above described motionless syndrome featuring only infrequent motor manifestations.

3.2 Neuropathological changes

3.2.1 Retrosplenial Vacuole Reaction—A single treatment of an adult rat with an NMDA antagonist at a neuroprotective dose typically causes an intra-cytoplasmic vacuole reaction affecting specific pyramidal neurons in the retrosplenial cortex. This retrosplenial vacuole reaction (RVR) is potentially reversible, depending on duration of NMDA receptor blockade. Memantine, at a non-neuroprotective dose (10 mg/kg), produced no evidence of a RVR, but at a neuroprotective dose (20 mg/kg) and all higher doses, memantine caused a dose-dependent RVR that was very mild at 20 mg/kg and quite severe at 50 mg/kg (Figs 1 and 2). A significant effect of memantine dose was confirmed by a one-way ANOVA [$F(4,33) = 15.23, p < 0.0005$]. A subsequent pairwise comparison showed that the 50 mg/kg dose produced significantly greater numbers of injured neurons compared to the 10 mg/kg dose [$F(1,33) = 35.16, p < 0.0005$].

Tacrine, by itself, at up to 10 mg/kg did not produce a RVR. However, administering 5 mg/kg tacrine together with memantine resulted in a marked potentiation of memantine's RVR neurotoxic action. Whereas memantine alone was only mildly neurotoxic at 20 and 30 mg/kg, the tacrine/memantine combination triggered a neurotoxic reaction that progressed from mild to moderate to extreme severity at memantine doses of 10, 15 and 20 mg/kg, respectively. An ANOVA yielded significant main effects of drug treatment (memantine vs. memantine + tacrine) and memantine dose (10, 15, 20, and 30 mg/kg) [$F(1,56) = 53.95; F(3,56) = 8.79, p < 0.0005$, respectively]. There was also a significant drug treatment by memantine dose interaction [$F(3,56) = 7.38, p < 0.0005$]. In both drug treatment groups, numbers of injured neurons increased as a function of progressively higher doses of memantine, although the pattern of increase in neuronal injury was different for each type of drug treatment. Subsequent pairwise comparisons showed that tacrine significantly increased the number of injured neurons induced by memantine beginning at a 15 mg/kg dose of memantine [$F(1,56) = 7.33, p = 0.009$]. Thus, at doses lower than or only barely within the neuroprotective range, memantine rapidly became severely neurotoxic when combined with 5 mg/kg tacrine (Fig 2A).

Donepezil, by itself, at up to 10 mg/kg did not produce a RVR, but like tacrine, it markedly potentiated the neurotoxicity of memantine. For example, combining memantine with a 5 mg/kg dose of donepezil clearly augmented the RVR, resulting in a neurotoxic reaction that progressed in severity from mild to moderate to statistically significant at memantine doses of 15, 20, and 30 mg/kg, respectively (Fig 2B). An ANOVA on the three drug treatment groups (memantine alone, memantine + donepezil 5 mg/kg, and memantine + donepezil 10 mg/kg) involving 10, 15, 20, and 30 mg/kg doses of memantine revealed significant main effects of drug treatment and memantine dose [$F(2,68) = 13.66, p < 0.0005; F(3,68) = 5.18, p < 0.003$, respectively]. Subsequent contrasts showed that memantine + donepezil (5 mg/kg) injured

significantly more neurons compared to memantine alone [$F(1,68) = 5.64, p = 0.02$], as did memantine + donepezil (10 mg/kg) [$F(1,68) = 27.28, p < 0.0005$]. Additional pairwise comparisons demonstrated that the group treated with memantine (30 mg/kg) + donepezil (10 mg/kg) had significantly more injured neurons than those treated with memantine alone (30 mg/kg) [$F(1,68) = 7.82, p = 0.007$]. However, this dose of donepezil (5 mg/kg) added to lower doses of memantine did not significantly increase the numbers of injured neurons. In contrast, adding 10 mg/kg of donepezil to memantine resulted in a significant increase in numbers of neurons, beginning at 15 mg/kg of memantine, compared to levels observed in the memantine-alone group [$F(1,68) = 16.13, p < 0.0005$].

One-way ANOVAs were also conducted to compare the levels of neuronal injury in the highest memantine-alone (50 mg/kg) group with the memantine + tacrine and memantine + donepezil groups that showed significant increases in neuronal injury compared to their respective memantine-alone groups. The results of these analyses showed no significant differences, indicating that cholinesterase inhibition, regardless of whether it is produced by tacrine or donepezil, interacts with memantine at a dose of 15–20 mg/kg, to induce a neurotoxic reaction in the retrosplenial cortex that is as strong as that caused by a 50 mg/kg dose of memantine alone.

3.2.2 Disseminated Dendrotoxicity—In the course of examining thin plastic sections to assess vacuole toxicity in retrosplenial neurons, we observed that in many of these brains there were signs of an acute pathological reaction in the adjacent temporoparietal and occipital cortices. This reaction included pathological changes in the cytoplasmic compartment of pyramidal neurons, but the most conspicuous feature was a laminar display of spongioform changes in superficial layers (II and III) and deeper layers (V and VI). These changes were localized to the neuropil, and consisted of membrane-bound dilated processes that were spherical in shape and vacuous in content (Fig. 3A & B). In previous studies we have observed similar acute changes associated with status epilepticus syndromes [47, 48], and in most cases have concluded that the dilated processes are edematous dendritic spines that are undergoing excitotoxic injury due to excessive activation of glutamate excitatory receptors. We examined these dilated structures by electron microscopy and found that they receive synaptic contact from presynaptic axon terminals, which corroborates that they are dendritic structures (Fig 3C). To clarify the time course of these spongioform changes we treated a group of animals with memantine 30 mg/kg + donepezil 10 mg/kg and examined the brains at 2 h following treatment. We found that already at 2 h following treatment these dendrotoxic changes were clearly evident in many regions of the cerebral cortex and had the same laminar distribution that we observed at 4 h.

3.2.3 Disseminated Cell-Death Reaction—In previous studies, we found that combining a neuroprotective dose of an NMDA antagonist (e.g. MK801 or phencyclidine) with the cholinergic agonist, pilocarpine, caused an irreversible neurotoxic reaction resulting in death of neurons [9,69]. This cell killing action was detectable 24 to 72 h posttreatment using the DeOlmos cupric silver method [13], which reliably stains neurons that are in an advanced stage of cell death. In these prior studies, cell killing was observed in the RSC and in several other regions of the rat brain. It was not accompanied by definite seizure activity, and was not as fulminating and widespread as reactions we have observed following treatment of adult rats with very high doses of pilocarpine, or with pilocarpine plus lithium [7]. The latter reactions are excitotoxic in nature, are invariably accompanied by prolonged seizure activity, and selectively kill neuronal populations throughout specific neural networks that have received the brunt of the seizure discharge activity [48].

In the present study, we administered memantine at a dose of 20 or 30 mg/kg + donepezil at 2.5, 5, or 10 mg/kg and sacrificed the rats at 24 or 48 h following treatment. We used the

DeOlmos silver stain to evaluate the pattern and extent of disseminated cell death (DCD) by light microscopy and also examined the brains of a separate group of rats by electron microscopy to clarify the nature of the DCD reaction at 48 h following treatment.

When we combined a neuroprotective dose of memantine (20 mg/kg) with donepezil at 2.5 mg/kg we did not find evidence for a DCD reaction, but combining 20 mg/kg memantine with 5 or 10 mg/kg donepezil induced a DCD reaction in 17% and 67% of treated rats, respectively (n = 6 per group). Combining a higher dose of memantine (30 mg/kg) with donepezil at 2.5, 5 or 10 mg/kg, caused a DCD reaction in 50%, 83% and 100% of treated rats, respectively (n = 6 per group). Severity of the neurotoxic reaction was dose related with respect to the contribution of each drug. For example, at memantine 30 mg/kg, every dose of donepezil was substantially more toxic than at 20 mg/kg memantine, and at either dose of memantine, the DCD reaction became increasingly more widespread and more severe with each step up in the dose of donepezil. The DCD reaction was detectable by the DeOlmos silver stain in a fully developed pattern at either 24 or 48 h, the only difference being that the silver-positive neuronal profiles appeared to be in a more advanced stage of condensation and deterioration at 48 h.

The cell killing reaction, especially at higher doses, was widely disseminated and severe, more so than in rats treated in previous studies with MK801 and pilocarpine [69]. The same regional pattern of degeneration was observed in all affected brains, and the reaction was consistently confined to highly specific cell populations within each of the general brain regions involved. For example, almost the entire neocortical mantle (frontal, parietal, temporal, occipital) was affected, but the reaction was primarily confined to the deeper layers, especially layers IV/V, with only mild cell killing in the more superficial layers (Fig 4A, 4C, 5 and 6A). In the rostral pole of the brain the ventral anterior olfactory area was densely affected. In the frontal and prefrontal regions, the cell killing was localized to specific layers of the insular (Fig 4A & B), orbital, primary (Fig 4A & C) and secondary motor cortices and to the claustrum. In the amygdala, the reaction was confined to the lateral, basolateral (Fig 5) and cortical nuclei and to the amygdalopiriform transition area. In the thalamus, the reticular (Fig 5 & 6C), ventromedial (Fig 5 and 6D), pretectal and posterior nuclei were selectively affected. Concentrated cell killing was also observed in the posterior cingulate, retrosplenial, ventral auditory, ectorhinal and perirhinal (Fig 5 & 6B) cortices and inconsistently in the pars reticulata of the substantia nigra. Notably, some major brain regions were totally spared, including the caudate-putamen and hippocampus.

In order to evaluate to what extent the DCD reaction induced by the drug combination can be explained in terms of the neurotoxic properties of the individual drugs adding to one another, as opposed to a synergistic reaction that exceeds in quality and/or quantity their additive potential, we administered memantine alone (20, 30 and 50 mg/kg), or donepezil alone (2.5, 5 and 10 mg/kg), or saline, and evaluated the brains at 48 h by cupric silver staining.

In the animals treated with memantine we found silver-positive neurons in the cingulate, retrosplenial and entorhinal cortices in moderately large numbers at 50 mg/kg, but at 20 and 30 mg/kg, no silver-positive neurons were detected. Therefore, the neurotoxic potential of memantine itself cannot explain more than a tiny component of the DCD reaction caused by the drug combination.

In rats treated with donepezil alone, we were surprised to find that in the majority of animals treated at 5 and 10 mg/kg (n = 6 per group) there was a cell killing reaction affecting CA-1 hippocampal pyramidal neurons. This cell killing reaction was not very robust (about 5 silver-positive profiles per microscopic section) but it was consistently found in these donepezil rats and there were no silver-positive profiles in the hippocampus or elsewhere in the brains of the saline controls (n = 6). Since we found that the hippocampus is a region that is not affected by

memantine/donepezil combined treatments, this signifies that NMDA receptor blockade by memantine protects CA-1 hippocampal neurons against the neurotoxicity of donepezil, but in many other brain regions the two drugs exert a synergistic neurotoxic action that cannot be explained by the neurotoxic potential of either individual drug.

In rats sacrificed 48 h following treatment with memantine 30 mg/kg and donepezil 10 mg/kg, and studied by electron microscopy, we found ample evidence that the neurons showing silver positivity at 24 and 48 h are neurons that have been lethally injured and are in various stages of relatively advanced cell death (Fig. 7 and 8). In addition, we found that in cortical regions where dilated dendritic spines were abundantly present at 2 and 4 h following treatment, the same dilated profiles were still present, albeit in smaller numbers, at 48 h (Fig 9). This signifies that the dendritic pathology detected at 2 and 4 h was not a rapidly reversible phenomenon but rather was the initial manifestation of a toxic process that is still ongoing as a degenerative process 48 h post-treatment. A puzzling feature of the neuropathological process is that at 48 h, the degenerating neuronal cell bodies and their dendritic arbors are condensed and shrunken, but lie side by side with edematous and massively dilated dendritic spines that do not seem to be connected to the condensed cell bodies or dendrites. In fact, they do not appear to be connected to any other structure, but rather present as independent membrane-bound spherical or ovoid dendritic fragments that apparently became detached from the parent dendrite during the acute injury process. Except for numerous degenerating neurons that were clearly in an advanced stage of cell death, and the dilated dendritic spines, the brain regions where these pathological changes were evident, had an essentially normal appearance at 48 h. This signifies that the pathological process is quite selective for specific neural elements and leaves surrounding brain tissue relatively undisturbed.

4. DISCUSSION

Here we report that administering memantine to adult rats at 20 mg/kg ip, which is the dose required for neuroprotection in the rat brain [5,10], induced mild neurotoxic injury regionally confined to the retrosplenial cortex. The cholinesterase inhibitors, tacrine and donepezil, in a dose range that inhibits cholinesterase activity by 20% to 70%, were relatively non-toxic by themselves, but when combined with non-toxic or barely toxic doses of memantine (10 to 30 mg/kg), a potentiated neurotoxic reaction occurred that killed neurons throughout many regions of the brain. This reaction, with one notable exception, displayed the typical characteristics of an excitotoxic seizure-related brain damage syndrome, closely resembling other such syndromes that have been described as a manifestation of unbridled glutamatergic and/or cholinergic excitatory activity [48]. The exception is that behavioral manifestations of seizure activity were often either absent or only equivocally present. Although all severely affected animals survived the acute neurotoxic process, 24 h later their brains were riddled with dead or dying neurons distributed in a pattern characteristic of excitotoxic seizure pathology.

It will be important in future studies to clarify the possible role of subclinical seizure activity in the neuropathological syndrome induced by memantine/donepezil treatment. We consider it likely that electrophysiological recordings will show that animals thus treated, including those that remain relatively motionless with minimal behavioral signs of seizure activity, are experiencing non-convulsive seizure discharges that increase synaptic glutamate concentrations, but provide little behavioral evidence that seizure activity is occurring. By blocking NMDA receptors, memantine may suppress propagation of seizure activity through NMDA receptors [8], but NMDA antagonists are known to release both acetylcholine and glutamate in various regions of the cerebral cortex [19,20,30,40]. This would leave cerebrocortical neurons in a hyperexcitable state, a state that could readily be converted to status epilepticus by donepezil, a cholinesterase inhibitor that prolongs the action of acetylcholine at all cerebrocortical cholinergic receptors. Although memantine likely

suppresses propagation of seizure activity through NMDA receptors, it may promote seizure activity generalized through other transmitter pathways, especially those responsive to cholinergic excitation. By blocking NMDA receptors, memantine may alter the seizure syndrome such that the motor manifestations that would otherwise accompany a cholinergically-induced seizure state are less evident. This provides a plausible explanation for the relatively motionless behavioral state of rats that may be in status epilepticus following treatment with memantine and donepezil. The finding in rats that this drug combination can have such devastating consequences by an apparent excitotoxic mechanism, without producing behavioral signs that clearly reflect the severity of the neuropathological reaction occurring in the brain, is potentially problematic because the treatment of AD patients with this drug combination has become widespread, and guidelines for using the combination are non-existent. In fact, if FDA guidelines were being followed, no patients would be receiving the combination, because donepezil is approved only for early stages of AD and memantine only for later stages of AD.

In a separate recent study [10], we have demonstrated in adult rats that memantine, at a dose (5 mg/kg) well below the neuroprotective dose, disrupts memory processes, and at higher doses tested (10 and 20 mg/kg) produces readily demonstrable locomotor disturbances, including stereotypic behaviors that other NMDA antagonist drugs are known to cause. Thus, our prior and present findings collectively support the conclusion that memantine produces in adult rats the same type of behavioral and neurotoxic side effects that other NMDA antagonist drugs are known to produce and, like other NMDA antagonists, its threshold for beginning to produce these side effects is well below its threshold for providing neuroprotection in hypoxia/ischemia models.

Our findings appear to contradict reports in the published memantine literature that have consistently characterized this drug as having little or no potential for triggering neurotoxic reactions in either the adult rat or human CNS. In rats, it reportedly exerts a protective effect against hypoxic/ischemic neurodegeneration beginning at a dose of 20 mg/kg, and at this dose no neurotoxic side effects were described [5]. Many human adults in Europe, and more recently in the United States, have been treated with memantine at doses in the range of 20 - 30 mg per day without serious side effects being reported, except for occasional case reports of psychotic reactions or seizures [27,56,57]. Several clinical trials conducted in recent years have provided evidence that at 20 mg/day memantine is well tolerated by AD patients and may have beneficial effects, which have been interpreted variously as improvement in cognitive function and various behavioral measures. Some authors have characterized memantine as a safe drug that can provide anti-excitotoxic protection against neurodegeneration in AD [11,52], AIDS dementia [35] and glaucoma [36], and as a promising therapy for neuropathic pain and drug addiction [52]. Not only is memantine assumed to be safe as monotherapy in AD, it reportedly is well tolerated when administered in combination with cholinesterase inhibitors to AD patients [61].

How can our present findings be reconciled with prior reports describing memantine as a non-toxic agent in rats, and as a safe and beneficial therapy for AD and other neurological disorders? Regarding the previously reported rodent data, the contradiction may be more apparent than real. Chen et al. [5] reported that memantine did not produce a neurotoxic reaction at 20 mg/kg, but this is the highest dose they tested. We believe a more complete dose-response evaluation would have produced evidence for a neurotoxic reaction at all doses above 20 mg/kg, in which case equivocal findings at 20 mg/kg, the dose required for neuroprotection, does not attest to the safety of this agent as a neuroprotective drug. As we have discussed previously [10], lack of evidence in earlier studies for memory and locomotor disturbances at neuroprotective doses, can be explained by features of the research design that were not optimal for detecting such adverse effects. To the best of our knowledge, no previous studies have been

conducted to evaluate the ability of cholinesterase inhibitors to potentiate the neurotoxicity of memantine in any animal species. The only animal study purporting to evaluate this drug combination is one in which it is reported that memantine does not interfere with the pharmacokinetics of donepezil [65]. This finding led the authors to suggest that concomitant use of the two drugs may provide greater benefit than donepezil alone, but the possibility of greater toxicity was not addressed. Thus, previous animal studies do not contradict our findings. Rather, these studies did not describe the findings we are describing because they were not designed to evaluate the same parameters we have examined.

Reconciling our findings with the reported safety profile of memantine in humans is more difficult. Species differences between rats and humans would be a logical consideration, but it is well known that humans are quite sensitive to the toxic effects of NMDA antagonist drugs (for example, hallucinations and psychotic symptoms induced by phencyclidine and ketamine, both of which act at the same receptor site as memantine); and cholinesterase inhibition, if prolonged or irreversible, is the mechanism underlying nerve gases that are among the most deadly human neurotoxins in the world. It seems unlikely that rats would be exquisitely sensitive and humans totally insensitive to the toxic reaction induced by this drug combination. Interestingly, prior memantine researchers have not attempted to explain the safety profile of memantine in terms of species specificity. Rather, they have argued that memantine in both rats and humans has kinetics of binding to the NMDA receptor that are different from any other NMDA antagonist, and this putatively confers the unique ability to protect neurons against excitotoxic injury at doses that do not have adverse neurotoxic consequences in either rats or humans [5,11,52]. It is unknown whether humans are more or less sensitive than rats to donepezil potentiation of memantine neurotoxicity, but a factor that could unfavorably influence the equation is that the half-life of memantine is reportedly much longer in humans (60-80 h) than in rats (3-5 h) [11,18,52,66,67]. Also relevant is the clinical observation that patients with AD have a 6-fold increase in risk for seizures [26], which suggests that this patient population may have a lowered seizure threshold and an increased sensitivity to drugs that have convulsant potential. Seizures have been reported as an adverse effect in AD patients treated with tacrine [33], donepezil [3], and memantine [54]. These lines of evidence are consistent with the view that humans may be at least as sensitive as rats to memantine/donepezil neurotoxicity. Thus, they do nothing to resolve, but rather tend to deepen the enigma.

It has been argued [5] that the dose of memantine being administered to humans (20 mg/day) produces brain concentrations in the range of 1-10 μ M, and that a neuroprotective dose of memantine (20 mg/kg) produces similar memantine concentrations in rat brain, from which it is reasoned that the human dose can be considered neuroprotective in neurological disorders such as AD. We believe this reasoning may be incorrect. The human data rely on one case report [67] in which the concentration of memantine in brain was measured at autopsy in a single individual who died while receiving a daily dose (20 mg) of memantine. In this individual, the brain level measured was 1.3 μ M, but in rats a dose of 20 mg/kg produces brain levels ranging from 35-40 μ M (not 1-10 μ M as mentioned above)[39,64,66]. Thus, although a measurement in one person does not establish, with any degree of confidence, what concentrations of memantine are produced in the human brain by the dosing regimen currently being administered to AD patients, it may be as little as one fortieth the concentration required for neuroprotection in rat brain. It is fortunate that memantine, when administered to human AD patients, has been relatively free from adverse side effects, but we propose that the most logical explanation is that the dose of memantine being used in AD patients may not create brain levels high enough to appreciably interact with NMDA receptors.

The above interpretation is consistent with data from human clinical trials of memantine as a drug for relieving neuropathic pain. It has been clearly shown in animal studies that various NMDA antagonists [4,12], including memantine [42], if administered in a manner that blocks

NMDA receptors, can relieve neuropathic pain. In a clinical trial [59] in which memantine was administered to neuropathic pain patients for 9 weeks at doses titrated to the individual patient's level of tolerance, the maximum tolerated dose was 55 mg/day. This was the dose at which subjectively-determined disagreeable side effects began to occur, but at this dose there was no relief of neuropathic pain. This indicates that in human patients memantine may begin to produce intolerable side effects at a dose lower than is required to provide a therapeutic benefit that clearly depends on blockade of NMDA receptors. It should be noted that the disagreeable side effects were gastrointestinal and not the more severe type of side effects (e.g., hallucinations and agitation) that are associated with effective NMDA receptor blockade. Thus, both animal and human data support the interpretation that the reason memantine does not produce intolerable side effects in human AD patients is that it is being used at doses that are well below the threshold for interacting with NMDA receptors. Obviously, if this interpretation is correct, it is untenable to maintain that memantine arrests neurodegeneration in AD, or has any other beneficial effect in AD, by blocking NMDA receptors.

In summary, on the basis of the present findings and analysis of other relevant data, we consider it a possible misconception that memantine, by blocking NMDA receptors, arrests neurodegeneration and thereby confers a beneficial effect in AD. When memantine is administered to adult rats in a neuroprotective dose (i.e., a dose that effectively blocks NMDA receptors) typical NMDA antagonist type of neurotoxic side effects are readily demonstrable, despite the unique binding kinetics described for this agent in rat brain. Therefore, we propose that: 1) if the doses of memantine used in AD patients were neuroprotective by virtue of blocking NMDA receptors, typical NMDA antagonist type of side effects, including memory impairment, locomotor disturbances and psychotic reactions, would be encountered; 2) absence of such side effects may indicate that memantine, at these doses, does not block NMDA receptors; 3) this interpretation is more compelling than the unique binding kinetics interpretation for explaining memantine's favorable safety profile in humans; 4) the unique binding kinetics interpretation is problematic because it may mislead physicians into thinking that it is safe to treat refractory AD patients with escalating doses of memantine, together with donepezil, and this could have unfortunate consequences; 5) if continuing clinical experience with memantine proves that it is definitely beneficial in AD, and there continues to be no evidence that the benefit in humans is mediated by NMDA receptor blockade, it would be wise to determine the real mechanistic basis for this beneficial effect (for example, memantine reportedly [2] binds with greater affinity to the α -7 nicotinic receptor than to the NMDA receptor), and develop new drugs that are safer and more effective in acting by this beneficial mechanism; 6) for safe therapy of AD, NMDA receptor blockade should be avoided, especially in the presence of cholinesterase inhibitors; 7) for developing a rational therapy of AD, it should be remembered that there is no evidence that NMDA receptors are hyperactive in AD or that NMDA receptor-mediated excitotoxicity plays a role in the neuropathology of AD, but there is ample basis for believing that NMDA receptors are hypoactive in the AD brain and that NMDA receptor hypoactivity can trigger neurodegenerative reactions in the normal adult mammalian brain, reactions that can be markedly potentiated by drugs that inhibit cholinesterase activity.

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Fig. 1. Neurons in the retrosplenial cortex of the adult rat brain 4 hrs following treatment with 10 mg/kg donepezil (A) or 50 mg/kg memantine (B). The neurons have a normal morphological appearance following donepezil treatment but are heavily studded with cytoplasmic vacuoles following memantine. A and B same magnification; Scale bar = 20 μ m.

Fig. 2.

Quantitation of neuronal injury in the retrosplenial cortex four hours following administration of memantine alone or together with tacrine (A) or with donepezil (B). Tacrine alone or donepezil alone did not cause a vacuole reaction at a dose up to 10 mg/kg. Memantine alone did cause a vacuole reaction that increased in severity at a slow rate in the dose range from 15 to 30 mg/kg, but escalated more rapidly in the dose range between 30 and 50 mg/kg. Co-administration of tacrine (5 mg/kg) with memantine caused a significant neurotoxic response at a dose of memantine (15 mg/kg) that by itself caused little or no toxic reaction. When the same dose of tacrine was combined with memantine at 20 mg/kg, the neurotoxic reaction escalated to maximal severity ($\geq$ the response to 50 mg/kg memantine alone). Combining

donepezil with memantine caused a similar potentiation of the neurotoxic reaction. Donepezil at 5 mg/kg, caused a moderately robust potentiation which was dose-related with respect to the memantine dose, and the potentiation occurred at graduated doses of memantine that were non-toxic or only minimally toxic by themselves. Donepezil at 10 mg/kg caused a maximal neurotoxic reaction when combined with any dose of memantine (15, 20 30 mg/kg) higher than 10 mg/kg, despite the inability of these doses of memantine to cause a substantial neurotoxic reaction by themselves ($n \geq 6$ per treatment condition in each experiment).

Fig. 3.

Early dendrotoxic changes in the frontal cortex 4 hrs following combined treatment with memantine (20 mg/kg) and donepezil (5 mg/kg). The panel in A is presented at low magnification and shows spongioform changes distributed diffusely across several layers of the frontal motor cortex (Scale bar = 100 μ m). Layers I and II are relatively spared, but all deeper layers are severely affected. The tissue is riddled with small round to oval vacuous profiles, each of which represents a massively swollen small caliber dendrite that is in an acute stage of excitotoxic injury due to excessive excitatory transmitter (glutamate or acetylcholine) activity. The boxed region in A is shown at higher magnification in B (Scale bar = 100 μ m). In some cases the vacuous profiles can be identified as dendritic spines because they are lined up along apical dendrites of pyramidal neurons (arrow heads), and in other cases they probably originate from lateral branchings of apical dendrites, or from the basilar dendritic arbor. The panel in C is an electron microscopical view from the same scene illustrating one of the dilated vacuous processes, which can be identified as dendritic because it is post-synaptic to a pre-synaptic axon terminal (arrow). Scale bar = 2 μ m.

Fig. 4.

Cell killing reaction demonstrated in the prefrontal cortical region by the cupric silver stain 24 hrs following combined treatment with memantine (20 mg/kg) and donepezil (5 mg/kg). The boxed regions in A are shown at higher magnification in B and C. Silver-stained degenerating neurons in the insular cortex and claustrum are shown in B and in the primary motor cortex in C. Scale bar = 1,000 μm in A; 100 μm in B; 50 μm in C.

Fig. 5. Cell killing reaction at the mid-thalamic level as demonstrated by the cupric silver stain 24 hrs following combined treatment with memantine (20 mg/kg) and donepezil (5 mg/kg). The boxed regions are shown at higher magnification in Figures 6 A to D. Scale bar = 1 mm.

Fig. 6.

Detail views from the boxed regions in Fig 5. Depicted in Fig 6A are acutely degenerating neurons in layers IV and V of the parietal cortex. Each degenerating neuron is impregnated with silver throughout its cell body and dendritic arbor. Figs 6B, C and D illustrate the same selective degenerative phenomenon in the perirhinal cortex (B), reticular nucleus of the thalamus (C) and ventromedial nucleus of the thalamus (D). Scale bar = 170 μ m in A; 200 μ m in B; 220 μ m in C; 200 μ m in D.

Fig. 7.

Electron microscopic views from the parietal cortex 48 hrs following treatment with memantine 20 mg/kg and donepezil 5 mg/kg. In panel A there are two distinctly abnormal structures, the dark, condensed pyramidal shaped neuronal profile filling the middle of the photograph, and a dilated dendritic spine (asterisk) beneath it. These are the two most conspicuous pathological changes detectable 48 hrs following drug treatment. See Figure 8 for additional examples of neuronal cell bodies in an advanced stage of degeneration and Figure 9 for a more detailed evaluation of the dendrotoxic pathology. Panel B is from the same brain and same parietal cortical region as panel A and illustrates a normal appearing neuron that remains uninjured by

the pathological process that has killed many neighboring neurons. Magnification same in A and B; scale bar = 2 μ m.

Fig. 8.

Degenerating neuronal profiles from the frontal cortex 48 hrs following treatment with memantine 20 mg/kg and donepezil 10 mg/kg. All of these neurons are in a relatively advanced stage of cell death and display many features in common, including condensation of the entire cell body, vacuolization of the cytoplasm which sometimes extends into the nucleus, apparent dissolution of the nuclear membrane and irregular clumping of nuclear chromatin. Panel D displays a typical degenerating neuron at higher magnification. Some of the vacuoles appear to be bloated, vacuous mitochondria, but others are difficult to identify with any cellular organelle system. Scale bar = 6 μ m in A; 3 μ m in B and C; 1 μ m in D.

Fig. 9.

This electron micrograph, from the frontal cortex 48 hrs following treatment with memantine 20 mg/kg and donepezil 10 mg/kg, depicts a swollen edematous dendritic spine which is in synaptic contact with a presynaptic axon terminal (displayed at higher magnification in the inset). This abnormal structure is presumed to be dendritic because of its postsynaptic relationship to an axon terminal, (arrow) and it is thought to be a dendritic spine because it always appears in a spherical shape. If it were a swollen dendritic shaft some of the profiles would appear oblong in cross section. Scale bar = 1 μ m; inset = 0.3 μ m.