Original Paper

Developmental Neuroscience

Dev Neurosci 2001;23:31–40

Received: June 28, 1999
Accepted: September 27, 1999

Effect of the Noncompetitive NMDA Antagonists MK-801 and Ketamine on the *Spastic* Han-Wistar Mutant: A Rat Model of Excitotoxicity

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Key Words
Cerebellum · Hippocampus · Glutamate receptors · Neuroprotection

Abstract
The neuroprotective effects of the NMDA antagonists MK-801 and ketamine were analyzed in a mutant strain of Han-Wistar rats which develop neurodegeneration in the hippocampus and cerebellum. Previous experiments have shown that the progressive neuronal degeneration observed in this mutant may be the result of a dysfunctional glutamatergic system. For MK-801 studies, mutants were injected in a chronic paradigm with (+)MK-801 or its weaker acting isomer (–)MK-801 at a dose of 1 mg/kg. Ketamine studies consisted of both acute (50 mg/kg once) and chronic (10 mg/kg multiple times) injection paradigms. MK-801-treated mutants exhibited longer life spans (8–23%) compared to saline-injected mutants. Ketamine-injected mutants in both paradigms also lived slightly longer (6–9%) than the saline mutants. Motor skill deterioration was monitored in an open-field test, and after 50 days of age the MK-801 and ketamine mutants displayed over 20% greater motor skill activity than the saline mutants. In the cerebellum, mutants treated with ketamine and both forms of MK-801 had 10–20% more Purkinje cells surviving at 55 days than the saline mutants. Further, the density of CA3c pyramidal hippocampal neurons in ketamine and MK-801-treated mutants as compared to saline mutants appeared to be greater upon qualitative analysis. This study shows that these mutants derive some protective effects from the NMDA antagonists MK-801 and ketamine, confirming glutamate-induced excitotoxicity as a possible cause of neuronal degeneration in this mutant strain of rat.

Introduction

The term excitotoxicity refers to the ability of glutamate and structurally related amino acids to destroy neurons [Olney, 1986]. This mechanism has been implicated as a causal factor in neurodegenerative brain disorders, such as ischemia, epilepsy, and Alzheimer disease [Choi, 1988a; Beal, 1992]. Glutamate-induced excitotoxic cell death is mediated by activation of ionotropic membrane receptors that are divided into the following three sub-

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types based on their selective agonists: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, N-methyl-*D*-aspartate (NMDA), and kainate [Monaghan et al., 1989; Young and Fagg, 1990]. Exposing mammalian central nervous system neurons to glutamate or other excitotoxins such as kainate can result in selective patterns of neurodegeneration [Olney et al., 1980; Schwob et al., 1980]. Some of these susceptible regions, such as the hippocampus, are consistent with affected regions in human neurodegenerative disorders, such as epilepsy [Margerison and Corsellis, 1966; Choi, 1988b].

The glutamate receptor of primary interest in this study is the NMDA receptor which has been shown to play a major role in triggering neuronal degeneration and cell death [Moriyoshi et al., 1991]. The NMDA receptor, which has the characteristic properties of controlling a cation channel of high conductance (50 pS) and being double gated by glutamate and extracellular magnesium [Kandel et al., 1991], regulates a channel that is permeable to sodium, potassium, and most importantly calcium. This receptor is expressed in neuronal cells throughout the mammalian brain, including the hippocampus, cerebral cortex, and cerebellum [Moriyoshi et al., 1991]. The NMDA receptor is involved in many other key functions throughout the central nervous system, including induction of long-term potentiation which is involved in learning and memory [Lynch et al., 1983].

Pharmacological studies of antagonists to the NMDA subclass of glutamate receptors have shown that this class of drugs has the ability to attenuate glutamate-induced neuronal cell loss [Choi et al., 1988]. Specifically, both MK-801 {(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate)} and ketamine {2-(2-chlorophenyl)-2-(methylamino)-cyclohexanone hydrochloride} have been shown to antagonize the excitatory [Lodge et al., 1982; Davies et al., 1987; Woodruff et al., 1987] and neurotoxic [Olney et al., 1987; Foster et al., 1987] properties of NMDA receptors. These findings are consistent with the hypothesis that NMDA receptors mediate most of the neurotoxicity caused by glutamate.

The *spastic* Han-Wistar (HW), first described in 1976, is a mutant strain of rats that exhibits hyperactivity, spastic paresis, fore limb tremor, and hind limb rigidity, which manifest in a progressive manner [Pitterman et al., 1976]. The first symptoms, which include hyperactivity and tremors, are diagnosable around 25–30 days postnatally. Eventually, the rats lose all motor control around 60–65 days of age and become unable to feed with death ensuing around this time possibly due to starvation or respiratory difficulties. In addition, this mutant undergoes neurodegeneration in several brain regions (hippocampus and cerebellum) that are consistent with areas susceptible to glutamate-induced neurotoxicity [Wagemann et al., 1991; Levine et al., 1992]. Thorough anatomical investigations have revealed that the primary affected cells are the CA3c pyramidal cells in the hippocampus and the Purkinje cells in the cerebellum [Wagemann et al., 1991; Levine et al., 1992]. Previous studies of this rat strain have shown the neurological disturbance to be the result of an autosomal recessive mutation [Pitterman et al., 1976]. Additionally, it has been documented that there are both physiological and molecular alterations in the glutamatergic systems of the cerebellum and hippocampus [Cohen et al., 1991]. Specifically, *Xenopus* oocytes injected with poly (A)+ mRNA isolated from affected rats produced peak glutamate currents that were 2–3 times larger than the peak currents exhibited by oocytes injected with mRNA from unaffected littermates. Thus, evidence suggests that this animal may be at risk for glutamate-induced excitotoxicity.

To further investigate the role of the glutamatergic system, and specifically, the involvement of the NMDA receptor in the *spastic* HW disturbance, the antagonists, MK-801 and ketamine, were administered to mutant rats. Neuroprotective effectiveness was determined by assessing the animals behaviorally and histologically.

Materials and Methods

Han-Wistar Rats. The current HW colony at the California State University at Northridge has been reared from heterozygote breeding pairs originally obtained from the University of California, Los Angeles, in 1994. Rats were maintained in United States Department of Agriculture (USDA)/Institutional Animal Care and Use Committee (IACUC) approved animal facilities. Animals were allowed free access to food (Purina rat chow) and water, and were maintained on a 12/12-hour light/dark cycle. A total of 80 mutant and 55 control rats were used in these studies. Control gender-matched littermates were either wild types or heterozygotes, since behaviorally, physiologically, and morphologically heterozygotes cannot be discerned from wild-type animals.

Injections. All injections were performed intramuscularly, because these compounds readily cross the blood brain barrier. The injections were initialized 28–32 days postnatally. The reason for starting injections at this time were twofold: (1) Positive identification of mutants, based on behavioral phenotype, is not possible until this age. (2) Administration of MK-801 has been shown to induce toxicity prior to 30 days of age [Ikonomidou et al., 1999]. For MK-801 studies, animals were injected once a week for 3 weeks (three injections total) with the noncompetitive NMDA antagonist (+)MK-801 or its weaker acting enantiomer (–)MK-801 each at a dose of 1 mg/kg [Wong et al., 1986]. For acute ketamine studies, rats were injected once with an anesthetic dose of 50 mg/kg of ketamine. For

chronic ketamine studies, rats were injected either once a week for 3 weeks (total of three injections) or twice a week for 3 weeks (six injections total) with 10 mg/kg of ketamine. The injection schedule varied due to the effectiveness of each antagonist used as determined by preliminary studies. All drugs were dissolved in 0.9% saline, which was used as the vehicle for control animals injected at the appropriate times for each paradigm. Ketamine, MK-801 and its isomer were obtained from Research Biochemicals International (Natick, Mass., USA).

Health Assessment. Progressive weight loss or weight gain of the experimental rats was recorded on a biweekly basis throughout the life span of the mutants. Longevity of saline-injected mutants compared to drug-injected mutants was monitored daily.

Open-Field Tests. Since one of the affected regions in this rat is the cerebellum, which functions in motor and balance coordination, motor skills were assessed using an open-field test to evaluate neuroprotection in this region. Motor activity was analyzed by placing the rats in a plastic rectangular cage (36 × 55 × 18.5 cm) that was divided into 15–10 × 10 cm squares and allowing them to move about freely for 2 min. The number of squares that were crossed, number of times they reared onto the side of the cage, and the number of times they reared in the center of the cage were tallied. The data were summed, and a total activity score was given per animal per test [Sagvolden et al., 1992]. The cage was cleaned between trials, and all tests were performed between 12 and 3 p.m. in a quiet environment. The initial test was performed at 28–32 days of age preceding the first injection. Subsequent tests were performed every 3–7 days after injection until 60–65 days of age when the saline-injected mutants typically died. Due to the heavy anesthetic effects of MK-801, the activity tests were conducted at a minimum of 3 days after injection.

Histology. For morphological analysis, 30 animals (5 mutant and 5 control rats for each of the three treatments) were sacrificed between 55 and 58 days of age. Animals were injected intraperitoneally with a ketamine overdose (200 mg/kg) and perfused transcardially with 0.9% saline followed by cold 4% paraformaldehyde fixative in 0.1 *M* phosphate buffer. The brains were removed, and the cerebellum and hippocampus were sectioned on a cryostat into 50-μm sagittal and coronal slices, respectively. To assess the cellular neuroprotective effects of ketamine and MK-801 in the cerebellum and hippocampus, the Nissl body stain cresyl echt violet was used to visualize the cerebellar Purkinje cells and the hippocampal CA3c pyramidal cells. Since the degeneration in the cerebellum has previously been documented to be scattered throughout both hemispheres of the cerebellum [Wagemann et al., 1991], only the left cerebellar hemisphere from each animal was sectioned and examined. The hippocampus was analyzed at the same stereotaxic level for all animals [from plate 20 to plate 23 in Paxinos and Watson, 1982] to account for differential rostral-caudal distribution of NMDA receptor subunits [Wedzony and Czyrak, 1997; Yeckel and Berger, 1998]. The CA3c pyramidal cells in the dorsal hippocampus have been previously shown to be susceptible to neurodegeneration in this mutant [Wagemann et al., 1991; Levine et al., 1992].

Cell Counts. Quantitative histological evaluations of cerebellar Purkinje cells were performed on paired drug- and saline-injected mutant and normal rats. Profile cell counts were performed using light microscopy on cerebellar sections chosen at random. We chose this method based on a previous study showing that nucleolar profile cell counts were more reliable than stereologic cell counts [Popken and Farel, 1997]. Each slide contained 15 sagittal cerebellar sections, and counters blind to the treatment of the tissue were assigned a unique group of 5 sections per slide to count. Within each designated section five linear Purkinje cell layer transects (350 μm) were randomly chosen from the cerebellar lobes, and the number of nucleoli was recorded [Coggeshall and Lekan, 1996]. The 350-μm transect measurement was selected because it represented the diameter of the lens system that all counters used (Olympus CH-2 microscope with a standard 40× objective). Nucleoli with similar diameters lying in the Purkinje cell layer were counted as viable cells regardless of their health. Thus, the counts of unaffected mutant Purkinje cells may have been overestimated. For each animal 5 sections (125 transects) were analyzed.

Statistics. All statistical analyses were performed using GB-STAT (Dynamic Microsystems, Silver Spring, Md., USA). Between-group differences were determined for activity and weight analysis using a two-way ANOVA containing two within-subject variables: treatment (MK-801 vs. saline for normal and mutant animals) and developmental time. A one-way ANOVA was used to detect differences among groups for cell counts and life span analyses. Significance level was set at $p \leq 0.05$ for all tests, and means ± SEM were reported. When significant differences were detected for cell counts or life span results, the post-hoc test, Tukey's multiple comparison, was used to identify significant differences among the means. For cell count analysis, the Purkinje cell number per transect per animal was obtained by averaging all counts per animal. All counts within an experiment were subjected to a two-way ANOVA (without replication) to test for homogeneity among the cohorts. Next, a one-way ANOVA was used to test for differences among groups.

Results

Administration of NMDA Antagonists Increases Spastic HW Rat Longevity

The effect of administration of the NMDA antagonists MK-801 and ketamine on average longevity was observed in HW mutants (fig. 1). Longevity data were pooled and expressed only as percent increase from paired saline-injected mutant littermates. Administration of both enantiomers of MK-801 resulted in a statistically significant increase (one-way ANOVA; $p < 0.05$) in life span as compared to the saline-injected counterparts. As expected, the mutants injected with the more potent isoform (+)MK-801 (n = 13) showed the largest increase (23%) in longevity surviving 82.1 ± 5.1 days as compared to the saline-injected mutants (n = 15) that survived an average of 66.5 ± 0.8 days. In addition, mutants injected with the less effective isomer (–)MK–801 (n = 10) survived an average of 72.0 ± 3.5 days, which was also significantly longer (8% increase) than the saline-injected mutants. There was also a significant difference between the mutants injected with (+)MK-801 that lived 12% longer than mutants injected with (–)MK-801 (Tukey's; $p < 0.05$). No normal animals died throughout the experimental period.

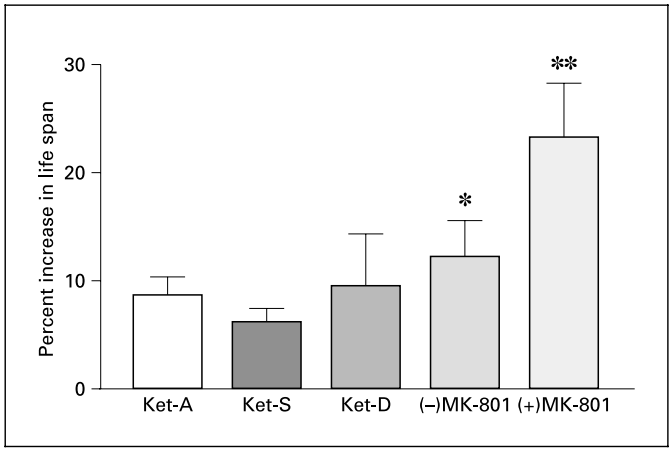

Fig. 1. Mean longevity was recorded among HW mutants treated with NMDA antagonists. Values represent percent increase in life spans of mutants treated with (+)MK-801 (1 mg/kg; n = 13), (–)MK-801 (1 mg/kg; n = 10), or ketamine compared to saline-injected mutant littermates. The three different ketamine injection paradigms were: Ket-A = Mutants injected with ketamine at an acute dose of 50 mg/kg once (n = 17); Ket-S = injected with ketamine (10 mg/kg) once a week for 3 weeks (n = 4); Ket-D = injected (10 mg/kg) twice a week for 3 weeks (n = 6). * $p < 0.05$ vs. saline mutant; ** $p < 0.05$ vs. (–)MK-801 mutant (ANOVA and Tukey's multiple comparison analysis). All values are given as means ± SEM.

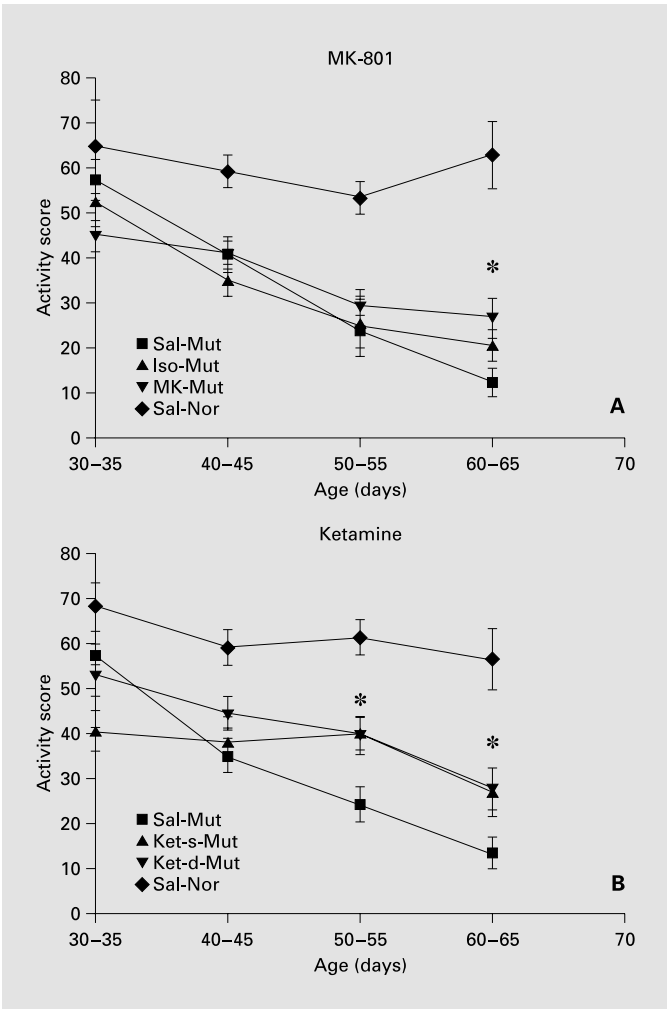

Fig. 2. Mean motor activity was analyzed using an open-field test. All mutant rats showed a significant reduction (two-way ANOVA; $p < 0.05$) in activity compared to the saline-injected normal rats (Sal-Nor, n = 9) after 30–35 days of age. **A** After 40–45 days of age, the (+)MK-801-injected mutants (MK-Mut; n = 6) sustained a higher activity level than (–)MK-801 (Iso-Mut; n = 5) or saline (Sal-Mut; n = 12) mutants. **B** Activity levels of chronic ketamine-injected mutants compared to their saline-injected sibling littermate controls (Sal-Nor, n = 11) are represented. After 50–55 days of age, mutants injected with both chronic ketamine paradigms (Ket-s-Mut, n = 4 for single and Ket-d-Mut; n = 6 for double) exhibited significantly higher activity levels ($p < 0.05$) than their saline-injected mutant siblings (Sal-Mut; n = 10). * $p < 0.05$ vs. saline mutant. All values are given as means ± SEM.

Mutants treated with the various ketamine paradigms showed a slight, but nonsignificant increase (one-way ANOVA; $p > 0.05$) in life span as compared to paired saline-injected mutants (fig. 1). Animals injected acutely (one injection of 50 mg/kg) with ketamine (n = 17) lived 8.6 ± 1.8% longer than their saline-injected littermate siblings, while ketamine-single-injected mutants (n = 4) lived 6.0 ± 1.3% and ketamine-double-injected mutants (n = 6) lived 9.4 ± 4.9% longer than their saline-injected siblings.

Effect of NMDA Antagonists on Motor Skills

The open-field test which was used to measure loss of motor activity, most likely due to cerebellar deterioration, indicated that all mutant rats regardless of treatment showed a slight decrease in activity at 30–35 days of age and a significant decrease (one-way ANOVA; $p < 0.005$) in activity starting at 40–45 days of age as compared to normal rats (fig. 2). Specifically for the MK-801 study (fig. 2A), at 50–55 days the normal rats (n = 9) maintained a significantly higher ($p < 0.001$) activity level as compared to (+)MK-801 (n = 6)-, (–)MK–801 (n = 5)- or saline (n = 18)-injected mutants which did not significantly differ from each other (Tukey's; $p > 0.05$). Further, at 60–65 days the normal rats continued to yield significantly higher (one-way ANOVA; $p < 0.0001$) activity scores than mutants. At this time point, (+)MK-801-treated mutants exhibited a significantly higher activity level than saline-injected mutants ($p < 0.05$), but their activity level was

not significantly higher than (–)MK-801-treated mutants ($p > 0.05$). Furthermore, (–)MK-801-treated mutants exhibited a slightly higher but nonsignificant activity level than saline-injected mutants ($p > 0.05$). Average motor activity was recorded and analyzed for normal rats injected with saline (n = 9), (+)MK-801 (n = 7), and (–)MK-801 (n = 4). Oscillations in activity scores were observed at the different time points, but no significant differences ($p > 0.05$) were observed between any of the groups at any of the time points (data not shown).

Open-field activity levels were also recorded and analyzed for single- and double-injected ketamine mutants (fig. 2B). A statistically significant increase in activity was observed between single (n = 4)- and double-injected (n = 6) ketamine mutants (one-way ANOVA; $p < 0.005$) as compared to the saline-injected mutants at 50–55 and 60–65 days of age. No significant differences in activity levels were observed between single- and double-injected ketamine mutants. Similar to the normals injected with MK-801, no significant differences in activity levels throughout the experimental period were observed in normal controls injected with either of the ketamine paradigms (data not shown). Open-field activity tests were not performed on acute ketamine rats.

Effect of MK-801 and Ketamine on Cerebellar Purkinje Cell Survivorship

Qualitative analysis of sagittal sections through the cerebellum was conducted and is shown in figure 3. For this analysis all animals were sacrificed at 55 days of age, and the overall lamellar neuroanatomical structure was intact in all animals observed. Mutants treated with saline (fig. 3A) have lost approximately 40% of their Purkinje cells as compared to normal rats at this same age [Wagemann et al., 1990; Levine et al., 1992]. The cell loss was apparent by the large gaps in the Purkinje cell monolayer (arrows), which is characteristic of the disorder in this mutant at this age. In addition, many of the surviving Purkinje cells were smaller and shrunken in appearance, which may indicate necrotic neuronal degeneration. Figures 3B and 3C represent the (–)MK–801- and (+)MK-801-treated mutants, respectively. Although the Purkinje cell layer of MK-801-treated mutants was definitely abnormal, the severity of the disturbance seemed to be less than that of the saline-injected mutants at the same time point. In addition, similar histological observations were made in the Purkinje cell layer of double-injected ketamine mutants (data not shown). There were still gaps present in the Purkinje cell layers of the drug-injected mutants, but the gaps appeared to be smaller than in the

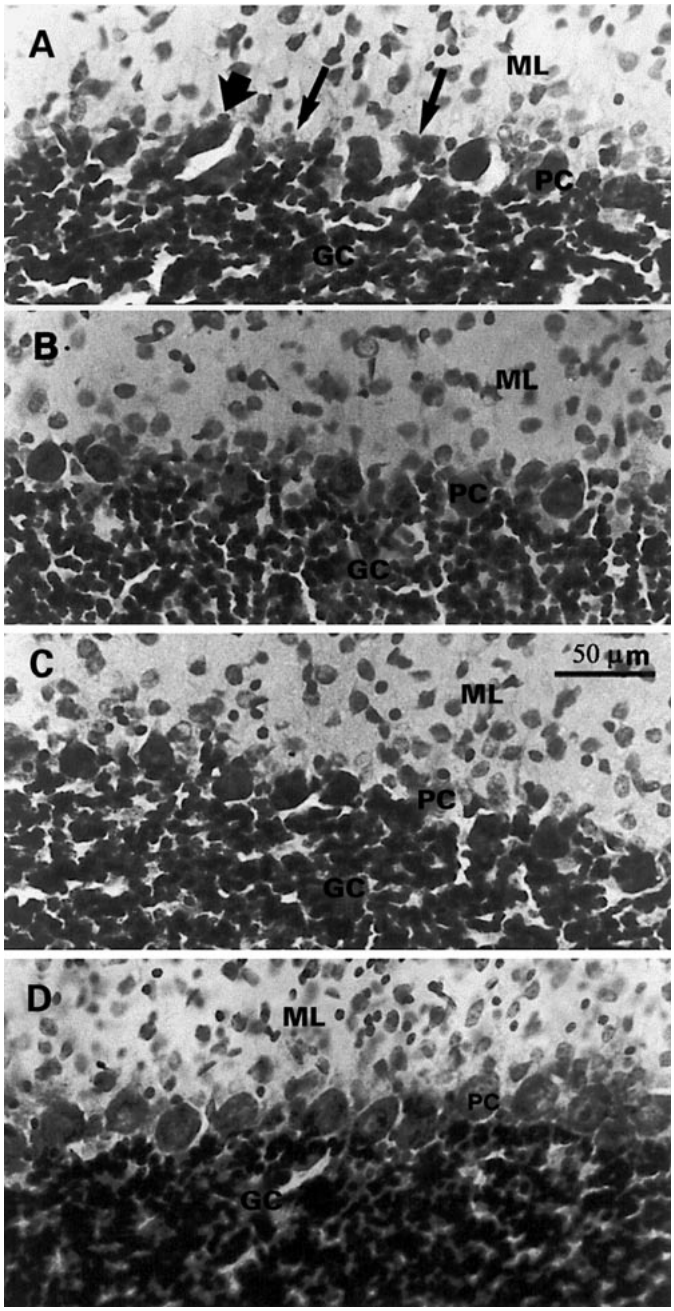

Fig. 3. Photomicrographs of cresyl-violet-stained 50-µm sagittal sections of the cerebellum of mutant rats treated with saline (**A**), (–)MK-801 (**B**), (+)MK-801 (**C**), and a normal rat that received saline (**D**). All animals represented were sacrificed at 55 days of age. The observed Purkinje cell loss (over 40%; **A**) is characteristic of this stage of the disorder in the mutant. The degree of deterioration in the saline mutant is shown by gaps in the Purkinje cell layer, indicating cell death (thin arrow). The cells that are present in the saline mutant exhibit a necrotic morphology compared to normals (thick arrow). The drug-treated mutants have approximately 70% of their cerebellar Purkinje cells surviving at this age. ML = Molecular layer; GC = granule cell layer; PC = Purkinje cell. Scale bar is shown in the top right corner of **C**.

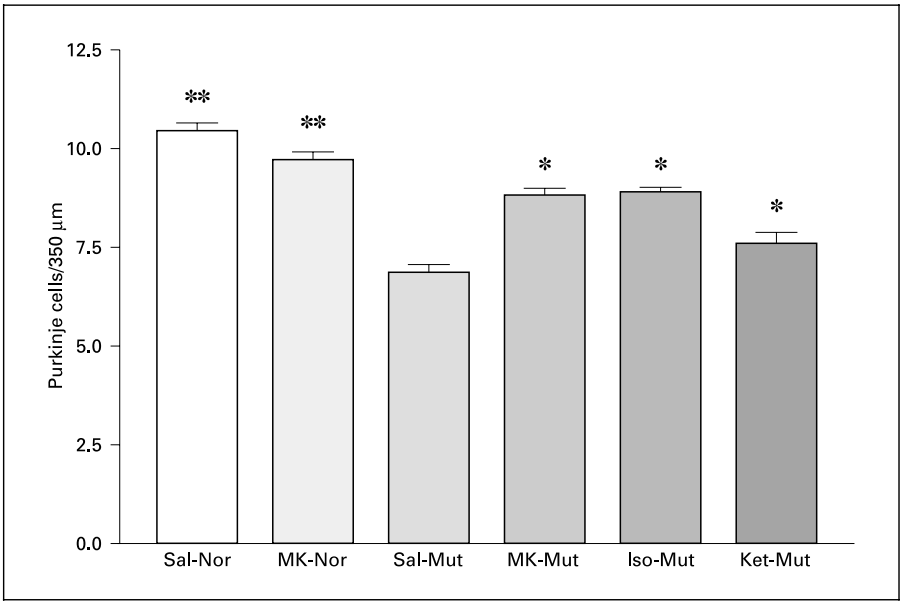

Fig. 4. Mean Purkinje cell counts analyzed from mutant and normal rats injected with NMDA antagonists or saline. No statistically significant differences were observed in the number of Purkinje cells among normal rats injected with saline or the NMDA antagonists. All mutants showed a statistically significant decrease in Purkinje cells per transect with respect to the normals (ANOVA; $p < 0.001$). * $p < 0.001$, mutants injected with NMDA antagonists vs. saline-injected mutants. ** $p < 0.001$, normals vs. mutants (ANOVA and Tukey's multiple comparison analysis; $p < 0.001$). All values are means ± SEM. n = 5 for all treatments and n = 125 transects counted per animal. Sal-Nor = Saline normal; MK-Nor = (+)MK-801 normal; Sal-Mut = saline mutant; MK-Mut = (+)MK-801 mutant; Iso-Mut = (–)MK-801 mutant; Ket-Mut = ketamine mutant.

saline-injected mutants. Moreover, the morphology of the surviving Purkinje cells in the drug-treated as compared to the saline-treated mutants appeared to be healthier and more consistent with unaffected cells.

Quantitative histological analysis of the cerebellar Purkinje cells was performed on mutant and normal animals treated with double-injected ketamine, (–)MK-801, (+)MK-801, or saline (fig. 4; n = 5, 125 transects analyzed for all groups). All normal animals (NMDA antagonist or saline injected) had a significantly higher (one-way ANOVA; $p < 0.005$) number of Purkinje cells per 350 µm transect than all mutants regardless of treatment. Normal saline-injected rats had an average of 10.4 ± 0.2 cells per transect while saline-injected mutants had an average of 6.8 ± 0.2 cells per transect (35% decrease). (+)MK-801-treated mutants displayed 8.8 ± 0.15 cells per transect (22.8% increase over saline mutants) and (–)MK-801 mutants exhibited 8.9 ± 0.1 cells per transect (23.6% increase over saline mutants) which were both significantly higher (Tukey's; $p < 0.001$) than saline-injected mutants. Double-injected ketamine mutants exhibited 7.7 ± 0.2 cells per transect (11.7% increase over saline mutants). Normals injected with (+)MK-801 and (–)MK-801 (data not shown) had slightly lower cell counts (9.7 ± 0.2 and 9.4 ± 0.2 cells per transect, respectively) than normals injected with saline. Despite the minor variation in cell counts, differences between all normal groups were not statistically significant (Tukey's; $p > 0.05$).

Effect of Ketamine and MK-801 on Survivorship of Hippocampal CA3c Pyramidal Cell Neurons

Qualitative analysis was performed on the hippocampus to examine possible neuroprotective effects of ketamine and MK-801 in this region (n = 5 per treatment). Figure 5 displays photomicrographs of coronal sections through a typical hippocampus of 1 control and 3 mutant 55-day-old rats that received drug or saline. Normal saline (fig. 5D), and normal (+)MK-801, (–)MK-801 and ketamine (data not shown)-treated rats all showed the characteristic hippocampal laminae with no apparent cell loss throughout the different layers. At 55 days of age, a mutant injected with saline (fig. 5A) lost a large number of CA3c pyramidal cells in the hilar region of the hippocampus (arrow). The hippocampus of the (–)MK-801 (fig. 5B), (+)MK-801 (fig. 5C), and double-injected ketamine (data not shown)-treated mutants appeared to have a much greater density of CA3c pyramidal cells (arrows) that extend further into the hilar region than the saline-injected mutant. These qualitative observations were consistent among all analyzed groups.

Discussion

Consistent with previous studies in our laboratory, all mutants used in this study possessed behavioral and histological disturbances, which resulted in impaired physical capabilities (onset around postnatal day 30–40) and

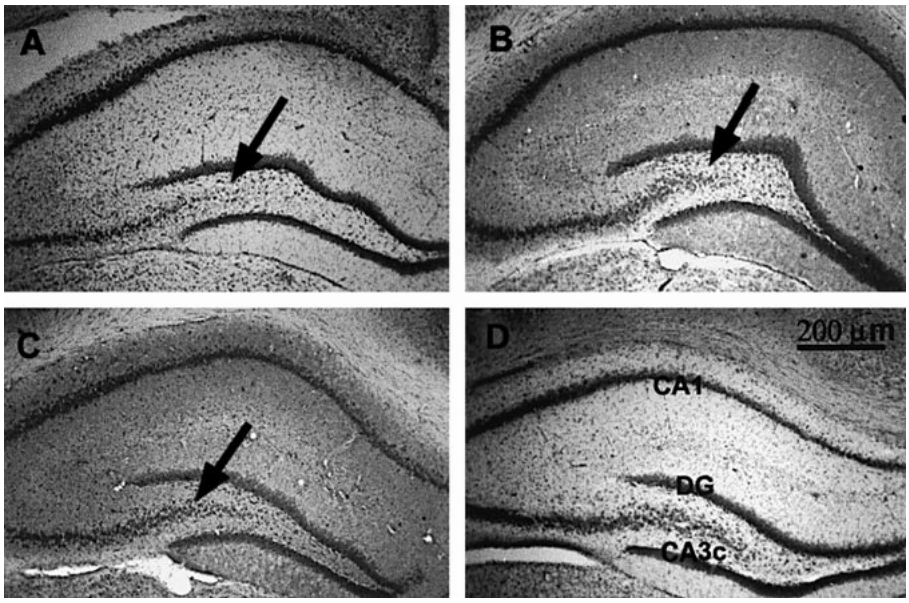

Fig. 5. Cresyl-violet-stained sections of a mutant (**A–C**) and normal (**D**) hippocampus at 55 days of postnatal age. **A**, **D** Saline injections. **B** (–)MK-801, **C** (+)MK-801. In the mutant treated with saline (**A**) CA3c pyramidal cell degeneration is severe and is accompanied by apparent collapse (arrow in **A**) of the dentate gyrus (DG). The NMDA antagonist MK-801 appeared to offer a noticeable degree of neuroprotection as determined by the increased cell density and extension into the hilus of CA3c pyramidal cells (arrows in **B** and **C**) as compared to the saline mutant. No cell loss was apparent in the normal hippocampus (**D**). Scale bar is shown in the top right corner of **D**.

early mortality (between postnatal days 60–90) as compared to their normal siblings. Since the mutants were identified by behavioral and physical characteristics, all mutants at the onset of treatment (30 days) were smaller and showed fore limb tremor compared to their normal siblings, which implies that physiological abnormalities were already present. All mutants underwent the characteristic progressive physical deterioration, culminating in hind limb rigidity, inability to move and feed, and eventually death.

The present study provides evidence that administration of NMDA antagonists (MK-801 and ketamine) to the *spastic* HW mutant can attenuate progressive neurodegeneration. Our study indicates that the neuroprotection offered by these antagonists can increase the life span of this mutant, and that MK-801 appears to be the more effective of the two antagonists. Also, treatment with MK-801 and ketamine appears to slow the progression of motor skill deterioration in the mutants as determined by monitoring activity levels in an open-field test.

Binding affinity may be a possible determinant for the degree of neuroprotective efficacy that an NMDA antagonist has on treating the HW disturbance. With the exception of Purkinje cell counts in the cerebellum, intermediate effects between the (+)MK-801 and saline-treated mutants were observed when the less effective isomer (–)MK-801 (seven times weaker binding affinity) [Wong et al., 1986; Moring et al., 1994] was used to treat the mutants. Further, administration of ketamine, which has a 300-fold lower binding affinity to the NMDA receptor as compared to MK-801 [Wong et al., 1988], resulted in a reduced, yet intermediate level of neuroprotection in the cerebellum.

The various drug administration paradigms utilized in the ketamine study suggested that there was not much difference in neuroprotective effectiveness between giving a large dose of ketamine (50 mg/kg) once at 30 days of age or giving moderate doses (10 mg/kg) more often. In a pilot MK-801 study, we showed that if chronic weekly injections began at 35 days or later, no increase in longevity was observed as compared to saline-injected mutants [unpubl. observations]. These results suggest that there may be a developmental window when NMDA antagonists are most effective in this mutant.

The improved survivorship of Purkinje cells and CA3c pyramidal cells observed in this study suggests that excitotoxicity via NMDA receptors may be involved directly or indirectly in the HW pathology. NMDA receptors are found in abundance in the two major affected brain regions (cerebellum and hippocampus). Glutamate receptors, especially the NMDA receptor, have been postulated to be involved in various dysfunctions of the central nervous system [Meldrum and Garthwaite, 1990]. Originally, it was shown by Olney [1969] that activation of excitatory amino acid receptors plays a role in glutamate-induced excitotoxicity, and it was later shown that this excitotoxicity could be attenuated by the addition of NMDA antagonists [Rothman, 1984]. In addition, both

affected cells in the mutant have been shown to be highly susceptible to glutamate-induced excitotoxicity [Brorson et al., 1994; Brusa et al., 1995].

The neuroprotection and increased survivorship of the Purkinje cells in the mutants treated with MK-801 and ketamine are most likely the major contributing factors for increased activity levels after 50 days of age. Similar anomalies to this mutant, such as tremors, ataxia, and spasticity, have been seen in other animals with cerebellar disturbances [Ito, 1984]. For example, mutation of the staggerer gene in mice causes severe ataxia that is correlated with a decrease in the Purkinje cell population and an overall neurodegeneration of the cerebellar cortex [Zanjani et al., 1992]. Purkinje cell anomalies similar to these ultimately render the animal unable to move, feed, or breathe. Since the cerebellum is involved in motor function, it is the cell loss in this region that is also the suspected source of mortality in the *spastic* HW mutants. Therefore, the ability of NMDA antagonists to slow down neurodegeneration in this brain region appears to be the basis for the observed increase in motor skills and possibly longevity.

The exact mechanisms by which these antagonists exert their efficacy is unclear from this study, but can be speculated upon. Previous research on this mutant rat strain has generated a large body of evidence that it is suffering from glutamate-induced excitotoxicity [Levine et al., 1992; Margulies et al., 1993; Cohen et al., 1997]. One possible hypothesis generated from these studies is that there may be an alteration in glutamate receptor assembly in affected areas. The 2nd and 3rd postnatal weeks, when the first symptoms arise in this mutant, are important developmental periods when the composition of glutamate receptors undergoes changes in abundance and distribution from the developmental to the adult state [Ben-Ari et al., 1997]. This transition has also been implicated to be activity dependent. The binding of one of these NMDA antagonists to the receptor could alter cellular properties, such as calcium abundance or neuronal excitability, which in excess have both been shown to result in cellular injury and death [Rothman and Olney, 1986; Choi, 1995].

In the hippocampus, NMDA receptors are abundantly present on the spines of the dendrites of pyramidal cell neurons [Kandel et al., 1991]. MK-801 may function to protect these neurons by blocking the NMDA channels directly, decreasing the amount of calcium ion influx. Several in vitro studies on rat hippocampal slices have demonstrated that treatment with MK-801 prior to inducing epileptic acitivity (by pharmacological or electrophysiological methods) blocked Ca^{2+} entry and subsequently reduced neurodegeneration [Miller et al., 1996; Thompson et al., 1996].

The proposed mechanism of neuroprotection by the NMDA antagonists in the cerebellum of the HW mutant may be more complicated than simply blocking the NMDA channels on the Purkinje cell. Although studies suggest that there are NMDA receptors on adult Purkinje cells [Mount et al., 1993; Garcia et al., 1996], a large body of evidence suggests that these cells lack functional NMDA receptors [Crepel et al., 1982; Johnson et al., 1993]. Purkinje cells receive input from two excitatory afferents, the parallel fibers (granule cell axons) and climbing fibers (inferior olivary nucleus cell axons). When climbing fibers stimulate the Purkinje cell there is a massive, synchronous depolarization of the cell that activates voltage-sensitive calcium channels in the dendritic membrane. The activation of these channels results in the generation of an action potential, triggering a large influx of calcium into the Purkinje cell. Although calcium may not be entering the cell directly through NMDA channels, elevated calcium levels may exert toxic effects via similar intracellular cytotoxic pathways. Purkinje cells also undergo periods of long-term depression after being stimulated by the parallel fibers, which leads to dis-inhibition of these GABAergic neurons and an increase in sensitivity to excitatory signals from the neurons in the deep cerebellar nuclei [Burt, 1993]. This event may also lead to an increase in calcium ion influx and subsequent degeneration. Therefore, a disturbance in the glutamatergic system or NMDA receptor distribution upstream in the communication pathway may result in calcium toxicity in susceptible Purkinje cells, manifesting in the observed behavioral symptoms.

While some previous studies have suggested that MK-801 may have neurotoxic effects in other brain regions, such as the cingulate cortex [Fix, 1994], more recent studies suggest that MK-801 does not induce apoptotic neurodegeneration after postnatal day 30 in the rat [Ikonomidou et al., 1999]. Nissl-stained coronal sections of the cingulate cortex in both mutant and normal animals of all treatments did not reveal any gross cell loss or alteration in cytoarchitectonics (data not shown). Since the injections began on postnatal day 30, the animals may have passed the developmental time period when cells are most sensitive to MK-801-induced neurotoxicity. Also, if MK-801 did induce neurotoxicity, then the extent of neuroprotection in this study was underestimated.

Our laboratory has also evaluated the effectiveness of non-NMDA antagonists on attenuating the disturbance in

this mutant, which have yielded results that suggest a more moderate level of protection as compared to MK-801 [Nisim et al., 1999]. The two non-NMDA antagonists GYKI-52466 and CNQX were administered to these mutants in higher doses more frequently than MK-801 and were shown to provide less neuroprotection. Since non-NMDA receptors have been shown to contribute to the activation of NMDA receptors by depolarizing the membrane and removing the magnesium blockade from the NMDA receptor, it is not surprising that non-NMDA antagonists may have neuroprotective effects. By blocking the non-NMDA receptors these agents may be working upstream to prevent the opening and consequential influx of calcium through the NMDA receptors as well as suppressing overall excitability of the brain. There are several explanations for the neuroprotective effects of these non-NMDA antagonists being more moderate than MK-801. First, if the primary neuropathology of the disorder involves the NMDA receptor, then reduced neuroprotection by non-NMDA antagonists would be expected. Second, the different pharmacological properties of the drugs may play an important role in the degree of neuroprotection. For example, the NMDA antagonist ketamine used in higher doses (10 mg/kg) more frequently yielded modest neuroprotective effects compared to MK-801 (1 mg/kg). These results suggest that MK-801 may have unique binding properties that distinguish it as a more effective neuroprotectant. Finally, if the cells express high levels of the non-NMDA-type glutamate receptor with the subunit composition shown to be responsible for high calcium influxes (i.e. receptors with low levels of GluR2 expression) [Hume et al., 1991], non-NMDA antagonists may be able to offer limited neuroprotection by reducing calcium influx into the cell directly.

Systemic administration of these NMDA antagonists may possibly affect nonglutamatergic neurotransmitter systems [Deutsch et al., 1995; Asin et al., 1996]. Thus, it cannot be excluded that these compounds may be exerting neuroprotective effects via alternative mechanisms. However, our laboratory has evidence that no system other than glutamate is involved in this neurodegeneration. For example, biweekly administration of $GABA_A$ agonists or antagonists did not have any effect on longevity, behavior, or Purkinje cell counts [Abdullah and Cohen, 1997].

In conclusion, this study demonstrates the ability of the NMDA antagonists MK-801 and ketamine to selectively attenuate the neurodegeneration observed in the cerebellum and hippocampus of the *spastic* HW mutant. This study also supports using this mutant as a model of excitotoxicity. Overall, localization of the mutation responsible for the pathology in this rat may provide a wealth of information about the biochemical mechanism of excitotoxicity as well as pharmacological agents that are most effective in the treatment of its characteristic disorders.

Acknowledgments

We would like to thank Janelle Asai and Toni Uhlendorf for their technical expertise. We also thank the following students for their help counting cells: Mohammed Abdullah, Avi Nisim, Agata Pikula, Kun Du Rhee and Klaus Yi. This work was supported by CSUN Foundation grants to K.L.B. and R.W.C.

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