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Behavioral Recovery Patterns in Rats Receiving the NMDA Receptor Antagonist MDL 100,453 Immediately Post-Stroke

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MARKGRAF, C. G., M. P. JOHNSON, D. L. BRAUN AND M. V. BICKERS. *Behavioral recovery patterns in rats receiving the NMDA receptor antagonist MDL 100,453 immediately post-stroke.* PHARMACOL BIOCHEM BEHAV **56**(3) 391–397, 1997.—Rats were given MDL 100,453 ((R)-4-Oxo-5-phosphononorvaline) in a pre-determined neuroprotective dose consisting of a bolus of 24.8 mg/kg followed by an infusion of 1.05 mg/kg*h for 24 h (MDL group; $n = 8$) or saline of the same volume (SALINE group; $n = 8$) 30 min. after the onset of a 90 min. period of middle cerebral artery occlusion. Eight animals underwent SHAM surgery. Rats were evaluated post-operatively for 14 days using seven neurological tests, including water maze. SALINE animals exhibited a pattern of neurological impairment compared to SHAMs (poor performance in five of the six motor/reflex tests) peaking five days post-injury. Relative to the SALINE group, the MDL group exhibited significantly improved outcome on two of the tests and a pattern of improved behavior on the remainder of the battery. By day 14 post-ischemia, all groups exhibited recovery on the motor/reflex tests. Learning ability was disrupted in the SALINE group on days 17 and 18, whereas the MDL group's performance was not distinguishable from the SHAM group in the water maze. Thus, a neuroprotective dose of MDL 100,453 produced a pattern of behavioral sparing in the immediate post-ischemic days that was uniquely different than saline. The addition of behavioral outcome measures to histological neuroprotection data adds significantly to the ability to better evaluate a putative neuroprotective compound. **Copyright © 1997 Elsevier Science Inc.**

NMDA Focal ischemia Rat Behavior Water maze

THE competitive NMDA receptor antagonist MDL 100,453 ((R)-4-Oxo-5-phosphononorvaline) has been shown to be neuroprotective when given post-ischemia in rodent models of focal cerebral ischemia (15,19, Johnson, unpublished data). Efficacy has been shown in the embolic (monofilament) middle cerebral artery (MCA) occlusion model (37), a model producing ischemia and infarction in the MCA territory by occluding the origin of the MCA with an intraluminar monofilament. The dosing regimen to achieve significant neuronal salvage has been determined to be an i.v. bolus injection followed by infusion (15). An optimal dose of the compound has also been determined: plasma levels of 400 pmol/5l provide approximately 50% protection, whereas plasma levels of 200 pmol/5l do not affect infarct size (19). Each of the above studies was conducted using a survival period of 24 h following MCA occlusion, as the experimental infarct is mature enough to visualize at this time using 2,3,5-triphenyltetrazolium (TTC) (18) and the infarct volume can be easily quantified.

Stroke, however, is a complex disease process, and brain injury continues to evolve and change over at least 7 days (20), possibly up to 42 days after the ischemic insult (27). There are many brain systems affected by ischemia, and thus many ways to assess outcome following experimental cerebral ischemia in addition to measuring infarct volume at 24 h. For example, changes in regional blood flow have been mapped in the ischemic brain over time (4); elevations in extracellular amino acids such as glutamate have been measured in the acute post-ischemic period (11) and shown to be decreased by treatment that is neuroprotective (10); diffusion-weighted images from a magnetic resonance imager (MRI) have been used to show the development of focal ischemia in the rat brain (25,28) and also used to show a reduction in ischemic

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damage with post-ischemic treatment (24). One outcome measure that is noninvasive, is easily performed and can be used repeatedly in the same subject is behavior. In fact, in clinical trials designed to test new stroke drugs, behavioral outcome measures are one of the means for determining efficacy, along with mortality measurements (5,8,13,14,16,17,21,32,34).

Behavioral test batteries used in clinical settings are typically comprised of a scale that rates the patient's reflex and involuntary behaviors (i.e., limb ataxia; level of consciousness) and cognitive processes (i.e., aphasias and memory loss). A comparable battery of tests has been described for use in rodent models of cerebral ischemia (20,21), with tests that evaluate limb ataxias, grooming, locomotion, muscle strength and learning and memory. This battery is more extensive than is often used, however. One test that is used by itself as an adjunct to infarct volume is the postural reflex test first described by Bederson et al. (2). Mean abnormal posture as measured by this easily performed test correlates well with infarct size (2,9). Increasingly, more sophisticated behavioral tests are being used to evaluate post-stroke rats, such as measuring cogntive abilities using the water maze (1,12) and evaluating sensorimotor integration using the sensitive elicited limb placing test (7).

In order to more fully characterize the neuroprotective properties of MDL 100,453, animals were given a pre-determined neuroprotective dose of the compound or an equivalent volume of saline in the immediate post-ischemic period following occlusion of the middle cerebral artery (MCA) and evaluated for 18 days using the battery described above. The patterns of deficit and recovery for post-stroke animals are described here, along with how these patterns are altered by a neuroprotective dose of MDL 100,453.

MATERIALS AND METHODS

All procedures were approved by the Institutional Animal Care Committee prior to starting the study. The surgical methods and detailed descriptions of the behavioral tests have been published previously (22,37) and hence will be briefly mentioned here.

Surgery

Male Sprague–Dawley rats were obtained from Charles River Breeders and were allowed to acclimatize for one week, during which time they were maintained on a 12 h light: dark schedule and allowed free access to both food and water. At the time of surgery, they weighed between 270–310 g. Each rat was anesthetized in 5% halothane in O_2:N_2O (1:2 ratio) and maintained on 1.5% halothane in O_2:N_2O by face mask. Body temperature was monitored via a rectal probe and maintained at 37.0 ± .5°C. The ventral neck region was exposed and a midline incision was made. An indwelling saline-filled venous catheter (PE 50) was placed in the left jugular vein and exteriorized through the back of the neck. The left common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were exposed and dissected free from the surrounding fascia. A 3-0 nylon monofilament with a heat-rounded tip as described by Zea Longa et al. (37), was placed in the ECA, secured with two ties and advanced into the ICA for at least 20 mm, until a slight resistance was felt: this position corresponds to the origin of the middle cerebral artery (MCA) and the monofilament thus blocks the flow to the MCA territory. The neck wound was closed and the animal was allowed to awaken from anesthesia. Ninety minutes later, the rat was re-anesthetized and the monofilament was removed to the point of the CCA/ICA bifurcation to restore blood flow to the MCA territory. Body temperature was monitored during reperfusion. The SHAM group consisted of operated animals in whom the monofilament had been introduced into the ECA in the manner described above, but it was not advanced into the ICA.

Drug Administration

MDL 100,453 was manufactured at the Marion Merrell Dow Research Institute. Prior to administration, it was dissolved in 1 molar equivalent of 1N NaOH and q.s. with saline to the desired volume. Thirty minutes post-MCA occlusion, a bolus of 24.8 mg/kg MDL 100,453 was given i.v. through the previously placed catheter, followed immediately by an infusion (1.05 mg/kg*min) for 24 h to the MDL group (n = 8). The SALINE group (n = 8) was given an equal volume bolus and infusion for 24 h of physiological saline (0.9%). Animals in the SHAM group were randomly divided in half and four animals received MDL 100,453 at the same dose as the MDL group, and four animals received saline in the same volume as the SALINE group. Upon cessation of the infusion the next day, the jugular catheter was removed under surgical anesthesia and the rat returned to an individual home cage for the duration of the study.

Behavioral Testing

Beginning on post-ischemia (PI) day 2 and continuing for five testing sessions until PI day 14, the rats were weighed and given the battery of six behavioral tests described below. On PI day 17 and 18, the rats received training in a water maze.

Posture Reflex Test. This test measures postural reflexes and is conducted by first hanging the rat by the base of the tail 1 m above the floor and observing posture. Abnormal posture is indicated by flexion of the contralateral limb and shoulder, a more severe deficit is indicated by failing to resist a lateral push to the contralateral side (2). A score of 0 is given for normal posture; a score of 1 is given for abnormal posture only; a score of 2 is given for abnormal posture and decreased resistance to lateral push.

Elicited Limb Placing. This test selectively measures sensorimotor integration of a tactile stimulus to the forepaw and a motor response of placing the limb on a table's surface (6). A score of 0 is given for normal, immediate placing behavior; a score of 1 is given for delayed placing and a score of 2 is given for the absence of placing to a tactile stimulus. The scores for two tactile stimuli trials to each forelimb (dorsal and lateral surfaces) are summed for a maximal deficit score of 4 for each limb.

Limb Adduction. This test measures locomotion and balance by examining the rat in an open Plexiglas cage for rearing bouts for a period of 3 minutes (31). Rearing up on the hind legs using both limbs for support against the wall is a normal exploratory behavior in rats; rats with ischemic damage to one hemisphere of the brain will adduct the contralateral forelimb when rearing. The percent of rearing bouts in which the contralateral limb is adducted is compared to the normal sham operated animals' behavior.

Grooming. This test measures the number of grooming bouts ocurring during a 10 minute observation period, and the percent of those bouts that are completed (3). Grooming is elicited by misting the rat's face with water and consists of

four well-defined phases which, in the intact animal, always proceed in order. Rats with ischemic brain damage engage in fewer bouts of grooming and fewer of those grooming bouts are completed through the four phases.

Foot Fault Test. This test measures coordination, sensorimotor integration and spontaneous locomotion (30): the rat is placed on an elevated grid with 3 cm^2 openings and allowed to move freely for 2 minutes. The number of missteps in which a forelimb or hindlimb falls through a grid opening are counted and expressed as a percent of the total steps taken during the trial. Intact animals make few foot faults and these are typically symmetrical. That is, equally as many missteps occur with the contralateral limb as with the ipsilateral limb. An animal with ischemic brain damage will make more contralateral foot faults.

Vertical Screen Test. This test measures muscle strength and grip strength. The rat is placed upon a movable screen (40 x 60 cm) when it is in the horizontal position. The screen is slowly raised to the vertical position and the rat's ability to grip the screen for 5 seconds is noted. A score of 0 is given for normal performance, i.e., holding onto the screen for 5 sec.; a score of 1 is given if the rat falls during the 5 sec. test period and a score of 2 is given if the rat falls off immediately.

Water Maze. This test measures cognitive spatial learning and memory by requiring the rat to locate a hidden platform using distal spatial cues (26). It is conducted in a round tank (4 ft diameter) painted black and filled with clear water. A clear Plexiglas platform (10 cm diameter) is located in one quadrant, covered by 2 cm water and is invisible to the swimming rat. The rat is released from one of three start locations around the pool and allowed 60 sec. to locate the platform by swimming. Once the escape platform has been located, the rat is allowed 10 sec. on the platform. If the rat is unable to locate the platform by the end of the 60 sec. trial, it is placed upon the platform for 10 sec. The trial is then concluded and the rat is allowed a 2 min. inter-trial interval in a dry cage. On PI days 17 and 18, each rat received 6 trials per day; 2 starts from each location, given in a random order so that the order of start locations was not predictable. The latency (in seconds) to reach the platform and the path length were recorded by a camera suspended above the pool and analyzed using Prototype Systems software (Boulder, CO).

Statistical Analyses

Scores for each test for each day were analyzed separately using an ANOVA and where appropriate, post hoc comparisons were made using Scheffe test. For three of the tests (posture reflex; elicited limb placing and foot fault) on day 14, the SHAM group had mean ± SEM scores of 0 ± 0, therefore a nonparametric test was necessary and Kruskal-Wallis was applied. Water maze data were analyzed using a split-plot ANOVA with post hoc comparisons using a Scheffe test.

RESULTS

Upon examination of the two subsets of the SHAM group (one receiving MDL 100,453 and the other receiving saline during the first 24 h of the post-surgical period), there were no differences between them on any behavior test, so the two subsets were pooled and considered one group (N = 8) for the remainder of the analyses.

Only two animals died in the post-ischemic period, both of those in the SALINE group. Although a chi square test did not show that this number was significantly different from the SHAM or MDL groups, it is a pattern suggestive of the protective effects of MDL 100,453. These animals died within 24 h after surgery and were thus excluded from the following analyses.

There was a large weight loss for both the SALINE and MDL groups on day 2 after MCA occlusion: 40.16 ± 7.4 and 49.8 ± 5.6 g. respectively, statistically greater than the SHAM group's weight loss of 17.8 ± 3.6 g. [$F(2, 21) = 9.74, p < .001$], with Scheffe tests demonstrating that the SALINE and MDL groups' weight loss did not differ from one another. By day 5 post-occlusion, the weight loss differences had equalized between the three groups, with ANOVA not showing a significant effect of group. Body temperature did not differ during surgery between any of the groups, and remained normothermic at reperfusion (60 min. into drug infusion).

Behavioral deficits for the SALINE group were maximal on day 5 for all tests except for grooming bouts, in which deficits were maximal on day 2, although the difference in deficit scores between day 2 and day 5 were slight on all tests. For clarity, the results are presented and analyzed on the day that SALINE group deficits were maximal and the day by which maximal recovery had occurred, which for all tests was day 14.

Grooming Test

Figure 1 shows the groups' scores for day 2 (when deficits were maximal) and for day 14, when all groups had returned to baseline performance. On this test, the lower the score, the greater the impairment. ANOVA performed on the scores for day 2 showed a significant effect of group [$F(2, 21) = 2.64$, $p = .05$], and a post hoc Scheffe test revealed that the SALINE group was significantly different from SHAM group, while the MDL treated group was not. ANOVA performed on the scores for day 14 was not significant, indicating that there was no difference between groups by this time point.

Contralateral Limb Adduction

Table 1 shows the groups' scores for day 5, when deficits were maximal, and day 14 when recovery had occurred. On this test, the greater the score, the greater the deficit. On day 5, although the SHAM group had the lowest score, the SALINE group the highest score and the MDL group had an intermediate score, the ANOVA comparing these scores did not reach statistical significance. The scores on day 14 were not significantly different either. The scores for the ipsilateral limb were not different between groups for any test session.

Foot Fault Test

Table 2 shows the scores for day 5 when deficits were maximal and day 14 when recovery was maximal. On this test, the higher the score, the greater is the impairment. While the SALINE group had noticeably higher scores than either the SHAM or MDL groups on day 5, the ANOVA did not reach significance. The scores for day 14 were not significantly different between groups, as revealed by Kruskal-Wallis test.

Contralateral Limb Placing

Figure 2 shows the scores for day 5 when deficits were maximal and day 14 when recovery was greatest. On this test, the higher the score the greater the impairment. The ANOVA revealed that on day 2, the groups' scores differed from each

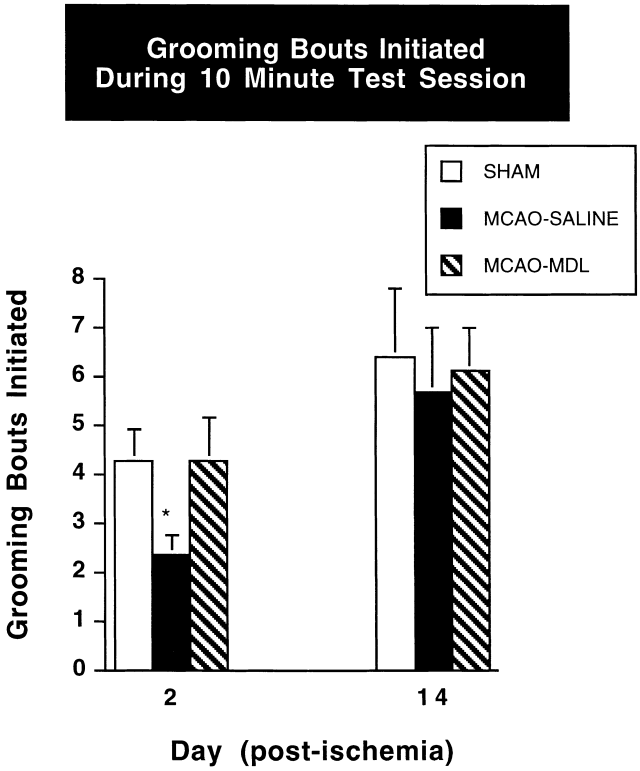

FIG. 1. Mean ± S.E.M. for the three group's grooming bouts initiated during the 10 minute test sessions on Day 5 post-ischemia when deficits were maximal and on Day 14 when maximal recovery was observed.

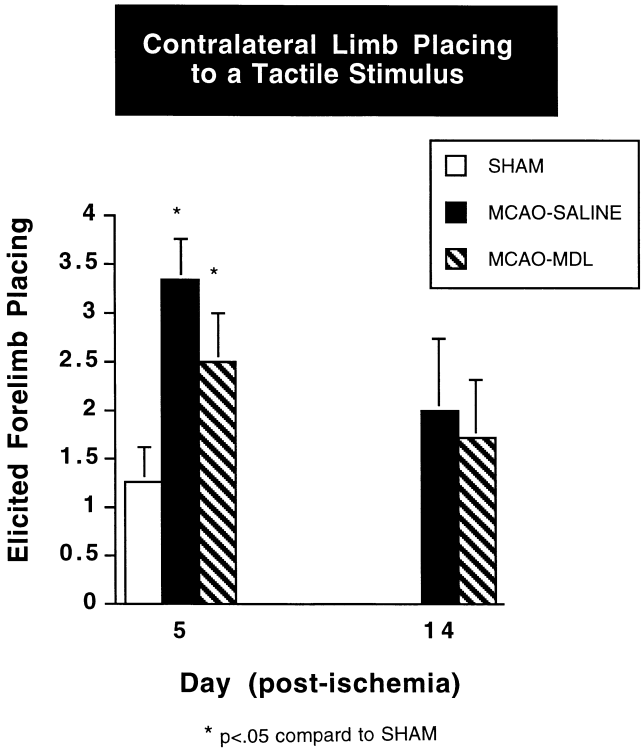

FIG. 2. Mean ± S.E.M. for the three group's elicited limb placing scores initiated during the 10 minute test sessions on Day 2 post-ischemia when deficits were maximal and on Day 14 when maximal recovery was observed.

other [$F(2, 21) = 5.49$, $p < .01$], and post hoc Scheffe tests showed that the SALINE group was significantly impaired compared to the SHAM group ($p < .05$) as was the MDL-treated group ($p < .05$). However, the two ischemic groups were not different from each other. By day 14, the pattern was the same: a Kruskal-Wallis test showed a significant group effect ($p < .05$) and post hoc comparisons showed that the ischemic groups were each different from SHAM (p's shows the scores for day 5 when deficits were maximal and day 14 when recovery was maximal. ANOVA performed on the scores indicated that there was a significant difference between the groups on day 2 [$F(2, 21) = 5.77$, $p < .05$], and post hoc Scheffe tests showed that both SALINE and MDL treated groups were different from SHAM ($p < .05$ each) but not different from each other. By day 14, although the scores for all groups had decreased, the pattern was the same: Kruskal-Wallis test showed that a group difference existed ($p < .05$) and post hoc comparisons revealed that SALINE and MDL treated groups differed from SHAM but not from each other.

Vertical Screen Test

The scores on this test showed no difference between groups on either day 2 or day 14. This pattern of normal behavior in even the SALINE treated ischemic rats indicates that the animals are capable of performing some tasks; they are selectively impaired in the tests presented above.

Water Maze Test

Figure 4 shows the performance of the groups on the water maze test: latency to find the platform on post-ischemic days 17 and 18. Split-plot ANOVA performed on the latency scores over the two days showed a significant effect of day [$F(2,$

TABLE 1

CONTRALATERAL LIMB ADDUCTION

Group	Day 5*	Day 14*
SHAM	44.5 ± 12.78	17.37 ± 3.32
SALINE	41.17 ± 13.44	43.33 ± 12.1
MDL	61.25 ± 15.3	33.25 ± 10.1

*Mean ± SEM % contralateral limb adduction during the 3 min test session.

TABLE 2

CONTRALATERAL FOOT FAULT INDEX

Group	Day 5*	Day 14*
SHAM	1.63 ± 1.49	0 ± 0
SALINE	6.83 ± 2.7	2.5 ± 2.13
MDL	2.0 ± 1.48	3.63 ± 1.68

*Mean ± SEM % contralateral foot faults during the 2 min test session.

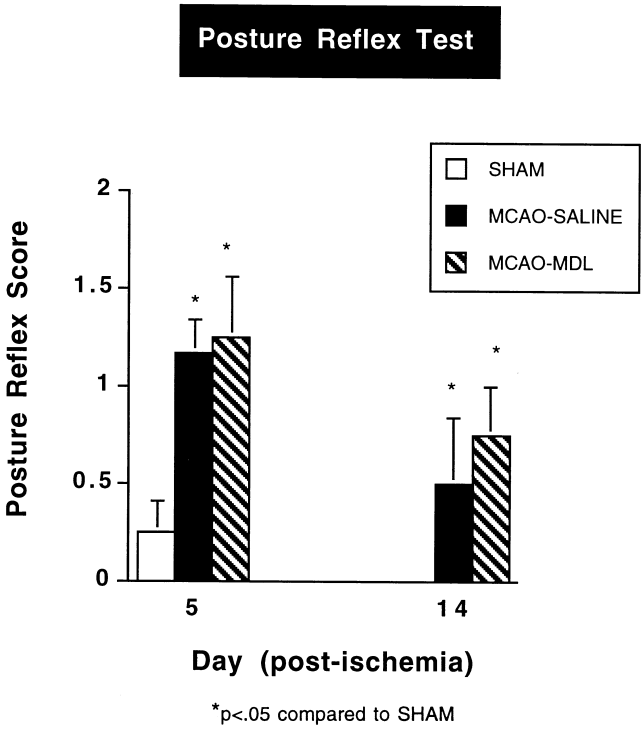

FIG. 3. Mean ± S.E.M. for the three group's posture reflex scores on Day 5 post-ischemia when deficits were maximal and on Day 14 when maximal recovery was observed.

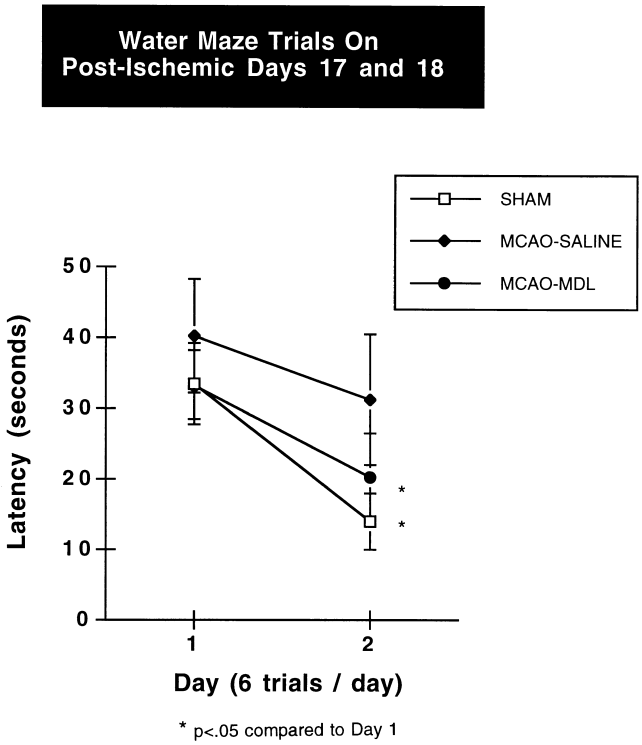

FIG. 4. Mean ± S.E.M. for the three group's latencies in finding the platform in the water maze test, given on Days 17 and 18.

2) = 9.92, $p < .001$], but not of group. Post hoc Scheffe tests showed that the SHAM group showed a significant decrease in latency (i.e., learning occurred) between day 17 and day 18 as evidenced by shorter latencies to find the platform. The SALINE group did not exhibit any learning curve: there was no decrease in latency to find the platform over days ($p > .05$) but the MDL-treated group did show a decrease in latency: Scheffe test showed that the MDL-treated group more quickly found the platform on day 18 than day 17. Like the SHAM group, learning the platform's location occurred in the MDL group. The path length scores for the three groups demonstrated the same patterns as the latency scores, as shown by split-plot ANOVA. That is, no significant effect of group was seen, but there was an effect of day [$F(2, 2) = 13.9$; $p < .001$]. Taken together with the latency data, these data indicate that the groups were equally efficient in their abilities to swim, i.e., deficits in latency to find the platform are due to an inability to learn rather than an inability to physically navigate the maze.

CONCLUSIONS

A neuroprotective dose of the competitive NMDA antagonist MDL 100,453 given to rats 30 minutes after the occlusion of a major cerebral artery produced a pattern of behavioral sparing that was uniquely different than saline treated rats in the immediate post-ischemic days, although the changes were not of a large magnitude. Brains were collected for histological analysis in the present study, but the samples were not able to be used due to an error in processing the tissue. However, the dosing regimen used in the present study had previously been shown to achieve 50% neuroprotection in a model of MCA occlusion produced by the same method (monofilament) in the same strain of rat (Sprague–Dawley; (19). On two of the seven behavioral tests, MDL 100,453 treated rats had behavioral scores that were indistinguishable from normal, sham-operated rats. On all of the tests except one, MDL-treated rats had improved behavioral function compared to saline treated rats, although these differences did not always reach statistical significance. The vertical screen test, which measures muscle strength, did not show differences between any of the groups on any test session, a result that is consistent with previously reported results following focal ischemia in rats (22, 23).

MDL 100,453 treatment improved behavioral outcome by several measures in the early days following MCA occlusion. However, since the saline-treated rats showed rapid spontaneous recovery in all the tests of the sensorimotor battery, reaching normal performance by the last test session on four of six tests, it is impossible to evaluate the effect of MDL 100,453 on performance in the later test sessions. It appears that in this model of 90 minutes of MCA occlusion followed by reperfusion in the Sprague–Dawley rat, behavioral deficits are not as prolonged for the saline treated control group as have been reported for rats undergoing untreated permanent MCA occlusion (22,23,27). Thus, a pattern of sustained behavioral recovery in the MDL 100,453 treated group would be difficult to evaluate compared to the rapidly-recovering saline treated group in the present study. The improved performance of the MDL group in the water maze compared to the SALINE group on post-ischemic days 17 and 18 indicates the existence of a persistent cognitive deficit which can be revealed by more sophisticated tests.

While histopathological outcome couold not be measured

in the present study, the dose chosen for MDL 100,453 has been shown to be neuroprotective in a similar model of focal ischemia in the same strain of rat (19) and the current behavioral results show a pattern of normalized scores in the early post-ischemic days that is consistent with neuroprotection. In a previous study examining a group of rats with varying infarct sizes, a direct relationship was seen between infarct size and severity of behavioral impairment (23). This finding suggests that less severe behavioral deficits are predictive of smaller infarcts, as would be expected with neuroprotective treatment. Similarly, using simple one-test batteries, histological neuroprotection has been associated with improved behavioral outcome produced by treatment with NMDA antagonists (29,33) calcium channel blockers (6,9) and a thyrotropin-releasing hormone analog (36).

However, behavioral improvement does not always parallel reduction in brain damage. Behavioral improvements independent of histological changes were described in the studies of Yamaguchi et al. (35) in which post-ischemic treatment with a muscarinic agent did not offer neuroprotection as measured by changes in infarct volume, but afforded significant behavioral improvement as measured by passive step-through avoidance and neurological scores.

These findings underscore the importance of using both histopathological and behavioral endpoints in evaluating neuroprotective drugs in experimental models of stroke. The addition of behavioral outcome measures to histological neuroprotection data can add to the ability to better evaluate a putative neuroprotective compound. Variants of behavioral test batteries are routinely used clinically for monitoring patient outcome (5,8) and for determining efficacy in clinical stroke trials (13,16,17). The extension of these techniques to animal models of cerebral ischemia will allow a more direct comparison of promising treatments from the laboratory to the clinic.

The results of the current study, together with the documented infarct reduction of this compound (15,19, Johnson, unpublished data), present evidence that MDL 100,453 is a neuroprotective compound that exhibits both histologically protective activity and improvements in behavioral outcome in the first two weeks following focal cerebral ischemia.

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