Rats receiving systemic 3-nitropropionic acid demonstrate impairment of memory in Morris water maze

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A recent hypothesis for the etiology of Huntington's disease (HD) postulates that impaired mitochondrial energy production and/or the presence of reactive free radical species may lead to slow excitotoxic neuronal death. Consistent with this hypothesis, systemic administration of the mitochondrial toxin 3-nitropropionic acid (3-NP) in rats produces selective striatal neuropathology mimicking that seen in HD. Such injections of 3-NP additionally produce motor changes thought to model HD, but possible cognitive changes have not been well described. The present study explores this issue. Sixteen rats underwent acquisition training and subsequent memory assessment in a Morris water maze apparatus. Training over 5 consecutive days consisted of trials during which each rat could escape swimming by finding a permanently located submerged platform. Following training, the rats were divided into two groups and received daily intraperitoneal injections of either 3-NP (15 mg/kg) or saline vehicle for 7 days. On Day 8, a retention trial was conducted, in which the platform was removed and the rats were allowed to swim for 2 min. Swimming patterns were tracked and recorded. The rats receiving 3-NP had impaired memory of the platform location, as represented by decreased time swimming over the platform area, fewer entries into the area, and longer latency to entering the area. These results suggest that the 3-NP rat model produces cognitive dysfunctions that parallel HD dementia.

Huntington's disease (HD) is a neurodegenerative disease characterized by abnormal involuntary choreiform movements, progressive cognitive impairments, and specific neuropathology. The most severe pathological finding is degeneration of basal ganglia neurons, especially in the caudate nucleus, which results in decreased levels of the neurotransmitters GABA and substance P (Beal, 1992).

Interestingly, the HD gene mutation has recently been discovered and localized to chromosome 4, but the function of the normal gene product is still undetermined (Huntington's Disease Collaborative Group, 1993). In fact, the transcript is expressed widely in the brain without noticeable regionalization (Strong et al., 1993). However, a recent report has shown increased cellular accumulation of the mutant protein *huntingtin* in cells of the striatum that are known to be vulnerable to HD (Kosinski et al., 1997). Nevertheless, attempts to explain this

disease solely on the basis of genetics are presently insufficient (Schulz & Beal, 1994). Meanwhile, there is mounting evidence that the etiology of HD and other neurodegenerative diseases involves a defect in energy metabolism, which has led some to suggest that the genetic defect of HD may involve an abnormality of mitochondrial function (Schulz & Beal, 1994). One hypothesis postulates that impaired mitochondrial energy production and/or the presence of reactive free radical species may secondarily lead to slow excitotoxic neuronal death (Beal, 1992).

Consistent with this hypothesis, systemic administration of the mitochondrial toxin 3-nitropropionic acid (3-NP), an inhibitor of the Krebs cycle enzyme succinate dehydrogenase, produces neuropathology in rats that mimics that seen in HD, particularly in the striatum (Beal et al., 1993). In fact, 3-NP effects are localized to the striatum, with sparing of other brain regions, such as the hippocampus or the neocortex (Borlongan, Koutouzis, Randall, et al., 1995). 3-NP has also been shown to produce motor abnormalities thought to model HD. Specifically, chronic 3-NP administration in rats produces an initial hyperactivity, followed by progressive hypoactivity, which mimics the successive behavioral alterations

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seen in HD (Borlongan, Koutouzis, Freeman, Cahill, & Sanberg, 1995). This is a characteristic feature of 3-NP, which supports the suggestion that this model is an improved alternative to previous animal models of HD, such as those using the excitotoxins kainic acid and quinolinic acid. Interestingly, accidental ingestion of 3NP-containing fungal toxin by humans produces a syndrome similar to HD (Ludolph, He, Spencer, Hammerstad, & Sabri, 1991).

Although many motor and pathological characteristics of the rat 3-NP model have been well described, its cognitive features need further examination. Impaired learning and memory has been demonstrated in kainic-acid-lesioned rats (Sanberg, Lehmann, & Fibiger, 1978), and impaired spatial learning has been demonstrated in the rat quinolinic acid model (Block, Kunkel, & Schwarz, 1993; Emerich, Bruhn, Chu, & Kordower, 1998; Haik-Creguer, Shear, Dong, Sabel, & Dunbar, 1997; Shear et al., 1997), but with regard to the 3-NP model, few studies have been done. We have demonstrated a contextual retention deficit in passive avoidance behavior in rats treated with systemic 3-NP (Borlongan, Koutouzis, Randall, et al., 1995), and Palfi et al. (1996) have shown impaired performance on the object retrieval detour task in baboons treated with chronic 3-NP.

Considering that dementia is a crucial part of HD, a further delineation of the cognitive impairment induced by 3-NP treatment of rats is needed. Accordingly, the present study investigated the ability of the 3-NP model to mimic the cognitive disturbances so often seen with HD. Specifically, we tested the behavioral effects of systemic subacute 3-NP administration on retention in a Morris water maze.

METHOD

Animals

Sixteen 19-week-old male Sprague-Dawley rats (380–410 g) were housed in wire cages containing groups of 4 in a room controlled for temperature (24°C) and humidity. A 12:12-h light:dark cycle was used, with lights on at 0700. The animals had Purina Rat Chow and water available ad lib. All the rats underwent acquisition training in a Morris water maze apparatus, then were randomly assigned to either the experimental (3-NP) or the control (saline) group.

Water Maze

The water maze apparatus consisted of a cylindrical vinyl pool 160 cm in diameter and 76 cm high, filled with chlorinated water maintained at approximately 25°C and measuring 50 cm deep. The pool was divided into four imaginary quadrants. In the center of the north quadrant was a 16 × 16 cm square transparent platform submerged 2.5 cm beneath the surface. A camera was mounted above the center of the pool, the input from which was fed to a video recorder and a computer with tracking capabilities (San Diego Instruments, San Diego). The pool and the surrounding walls both contained various high-contrast spatial cues of different sizes and shapes. During testing, the investigator stood at the west edge of the pool wearing a white lab coat. Testing was performed from 9 to 11 a.m. daily.

Testing

All the rats underwent training over 5 consecutive days, consisting of six swimming trials per day, during which each rat could escape by finding the submerged platform and, subsequently, would be allowed to rest for 60 sec on the platform before starting the next trial. The trials lasted a maximum of 60 sec, after which the rats that had not found the platform were placed on it and also allowed to rest there for 60 sec. Each rat was randomly started in the east, west, or south quadrant and started twice in each of these quadrants during 1 day's session. The rats were started by being placed at the edge of the pool in the center of the appropriate quadrant, facing the wall. For each trial, the computerized tracker recorded total distance traveled, as well as the distance traveled in each quadrant, the run time, the dwell time in each quadrant, entries into each quadrant, and latency to finding the platform. Following training, the rats were randomly divided into two groups of 8 and were intraperitoneally injected daily for 7 days with 15 mg/kg of 3-NP (Sigma chemicals; dissolved in 0.9% saline and pH adjusted to 7.4 with NaOH) or with an equal volume of saline vehicle. On Day 8, a *retention* trial was conducted, in which the platform was removed and the rats were allowed to swim for 2 min. All the rats were started in the south quadrant and were tested for only one trial, during which they were tracked for memory of platform location. Latency to entering platform area, entries into that area, and time spent in that area were used as indices of retention.

Histological Analysis

One day following behavioral testing, the rats were administered a lethal dose of sodium pentobarbital and transcardially perfused with saline, followed by phosphate-buffered 4% paraformaldehyde. Brains were sectioned at 50 μ m on a vibrating microtome and stained for Nissl substance, using cresyl violet, and for the presence of acetylcholinesterase (AChE).

Statistical Analysis

Results are expressed as means $\pm$ standard errors of the mean. The data from the acquisition phase of the study were analyzed, using a two-way analysis of variance (ANOVA) with repeated measures, with group membership in the later retention trial and performance on each training day as the main effect variables. For retention parameters, a one-tailed Student's *t* test was used.

RESULTS

Learning

Prior to 3-NP administration, there were no significant differences in the ability of the two groups to find the submerged platform during the learning phase of the study. Since both groups of animals acquired the task in the same manner, differences between the groups must be attributed to a deficit in retention.

Retention

There was a significant decrease in the performance of 3-NP-lesioned rats, as compared with controls. Figures 1A–1C show the relative values for the three parameters used to assess memory in the Morris water maze task: latency to entering the platform area, entries into the platform area, and time spent swimming over the platform area. Mean latency to entering the platform area for the control animals was 25.79 ± 7.45 sec, versus 68.74 ± 15.47 sec for the lesioned rats [$t = 2.5, p < .05$].

Figure 1. Morris water maze parameters assessing memory of platform position. Panel A shows that the mean latency to entering the platform area was delayed by >40 sec in 3-NP-lesioned rats versus control rats. Panel B shows the difference in the number of entries into the platform area. Control rats entered the platform area more than three times as often as those in the 3-NP group during the trial. Panel C demonstrates that the time spent swimming directly over the platform area also differed by a factor greater than three.

Entries into the platform area numbered 6.63 ± 1.12 for the control rats, versus 1.75 ± 0.62 for those in the 3-NP group [$t = -3.82, p < .005$]. Time spent actually swimming over the platform area amounted to 1.90 ± 0.50 sec for the control animals, as compared with 0.55 ± 0.21 sec for those in the 3-NP group [$t = -2.54, p < .05$]. A comparison of individual performances in terms of latency to entering the platform area on the last day of learning, rather than during the retention trial, revealed a 428% increase in latency over baseline for 3-NP-treated animals and a 268% increase over baseline for the control animals. Empirically, the control rats tended to swim a shorter and more direct course to the platform area than did the 3-NP-lesioned animals (Figure 2).

Swim Speed

Figure 3 shows that the swim speeds for each group were almost identical: 31.78 ± 2.15 sec for the controls, versus 32.66 ± 1.86 sec for the 3-NP rats. This non-significant difference indicates that 3-NP-treated rats were not deficient in their locomotor behavior (i.e., swimming), as compared with saline-treated rats. Therefore, locomotor effects cannot be used to explain memory abnormalities.

Histology

AChE and Nissl-stained brain sections showed no obvious lesions in the striatum or other areas when viewed under the microscope.

Figure 2. Typical swim patterns for rats in each group. The 3-NP-lesioned rats swam a longer and more convoluted course before first entering the platform area, as compared with the control rats. Figures represent the courses of rats that most closely matched the mean latency for their respective groups.

CONCLUSIONS

Our previous work has shown that the 3-NP model is more sophisticated than older models in its ability to mimic the motor characteristics of HD. The effects of 3-NP on cognitive function have remained largely uncharacterized, but the present behavioral study makes inroads into this area by demonstrating decreased retention during a Morris water maze task in 3-NP-treated rats.

Since both groups of animals acquired the task in the same manner, noted deficits can be attributed to impaired memory of the platform location. One might argue that motivational or proprioceptive deficits underlay the alterations in the performance of the 3-NP-treated rats. Because the rats were tested behaviorally only 24 h

after the last 3-NP treatment, it is even possible that they were experiencing acute drug effects, and not any pathology modeling HD. However, previous studies have shown that a single injection of 3-NP (at doses higher than the present dose; Nishino et al., 1997) leads to no observable changes in locomotor activity or cognitive task performance. Therefore, the observed dysfunction in the present study suggests a direct effect of 3-NP on memory. Recently, a chronic regimen of 3-NP has been utilized to demonstrate similar memory impairments in monkeys (Palfi et al., 1996).

Noted deficits were not due to any motor impairment, since there was no difference in the swim speeds between groups. This observation would seem to contradict previous reports (Borlongan, Koutouzis, Freeman, et al., 1995; Koutouzis, Borlongan, Freeman, Cahill, & Sanberg, 1994) showing hypoactivity in 3-NP-treated rats, but the stressful conditions of the water maze trial and its concomitant excitation mechanisms (e.g., catecholamine surge) may be sufficient to explain the observed activity (Mabry, Gold, & McCarty, 1995). Because such stress-related factors are not present in the Digiscan box (the apparatus used to measure locomotor activity in the previous studies), these variables might explain the different behavioral outcomes between the water maze and general spontaneous locomotor tests.

Interestingly, preliminary histological analysis in this experiment, using stains for AChE and Nissl substance, revealed no obvious brain pathology. The daily dosing schedule employed for this experiment is unreported in the literature, but Nishino, Shimano, Kumazaki, and Sakurai (1995) reported an experiment using the same concentration of drug administered three times per week. They observed minimal lesions of the striatum in only a small percentage of the animals after 1 and 2 weeks. Yet our experiment showed no lesions. One explanation

Figure 3. Swim speeds during the retention trial. The swim speeds of both groups were not significantly different.

might be that the exercise associated with the learning phase of the experiment provided a neuroprotective effect against 3-NP. Similar results have been observed in different lesion models (Christie & Dalrymple-Alford, 1995; Grabowski, Sorensen, Mattsson, Zimmer, & Johansson, 1995), but clearly this is an intriguing issue that warrants further study. Our new *subacute* 3-NP model may also represent a syndrome similar to that of early HD, in which clinical disability is noted but histopathologic changes are not observed (Myers et al., 1988). Thus, 3-NP-induced behavioral pathology appears to occur prior to any overt striatal lesions. However, further studies should look for more sensitive neurochemical alterations. Specifically, markers for GABA, substance P, somatostatin, or NADPH could have been tested (Beal et al., 1993). Others have looked at levels of succinate dehydrogenase (SDH) activity or terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL; Alexi, Hughes, Knusel, & Tobin, 1998). Jenkins et al. (1996) have used spectroscopic imaging to reveal metabolic damage caused by 3-NP administration. They suggested that impairment of oxidative metabolism, caused by 3-NP, precedes damage noticeable even with this sensitive imaging technique.

Although no overt lesions were seen with this previously unreported administration schedule, multiple studies have demonstrated that various 3-NP administration regimens cause selective striatal lesions (Beal et al., 1993; Borlongan, Koutouzis, Randall, et al., 1995; Koutouzis et al., 1994; Nishino et al., 1995; Shimano et al., 1995). In addition to its role in controlling motor behavior, the striatum has been implicated in passive avoidance learning and memory (Sanberg et al., 1978), and we have demonstrated that 3-NP-specific striatal damage is sufficient to disrupt passive avoidance retention (Koutouzis et al., 1994). The present study supports these previous reports but also identifies memory retention deficits in rats undergoing testing in a more complex task that allows measurement of multiple variables, as well as qualitative comparison. Notably, the impairment in this study was not limited to nighttime testing, as in past investigations with passive avoidance (Borlongan, Koutouzis, Randall, et al., 1995). Interestingly, preliminary work we have done shows no impairment of learning in 3-NP-lesioned rats during the Morris water maze task (unpublished observations). This is consistent with previous results showing no difficulty with acquisition during passive avoidance in 3-NP-treated animals (Borlongan, Koutouzis, Randall, et al., 1995).

Dementia is observed in human patients with basal ganglia degeneration owing to HD (Butters, Salmon, & Heindel, 1994). Although memory deficits specifically have traditionally been thought of as a late manifestation of HD, recent reports suggest that certain memory deficits are present early on, and even in asymptomatic HD gene carriers (Brandt, Bylsma, Aylward, Rothlind, &

Gow, 1995; Hahn-Barma et al., 1998; Lawrence et al., 1996; Rosenberg, Sorensen, & Christensen, 1995). The demonstration of impaired memory performance in rats after 3-NP administration further strengthens the argument that 3-NP produces a more representative model of HD; not only does this model show similarities to HD in motor dysfunction and neuropathology, but also in cognitive dysfunction.

Although gene therapy may be on the horizon for treatment of diseases such as HD, it will not be available for some time. Until then, we are dependent on animal models that accurately represent the biological and clinical features of HD to test and develop new therapies. The subcortical dementia associated with HD is indisputably debilitating for patients. With the added ability to evaluate experimental treatments of HD with this model, based not only on improvement of motor coordination but also on improvement of cognitive function, future investigations may be enhanced.

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