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Cognitive Deficits in the R6/2 mouse model of Huntington's disease and their Amelioration with Donepezil

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The progressive neurodegenerative disorder Huntington's disease (HD), which is caused by a polyglutamine repeat expansion within the huntingtin protein, is characterized by movement disorders, cognitive impairment, and psychiatric symptoms. Of particular interest for our work, decreased cholinergic function has been reported in HD patients. The R6/2 (120 CAG repeats) mouse model of HD expresses a human transgene containing exon 1 of the mutant huntingtin gene and replicates many of the symptoms of the disease, including marked impairments in cognition and severe motor deficits; moreover, measures of cholinergic function have also been reported to be reduced in this model. We tested whether chronic treatment with the centrally acting reversible acetylcholinesterase inhibitor, donepezil, could improve the performance of R6/2 mice in a simple visual discrimination task, the two-choice swim tank. Mice were trained to swim towards a light cued platform located on one side of a water-filled tank and were tested on acquisition and reversal learning performance. Wild-type (WT) and R6/2 mice were administered donepezil or vehicle starting at 8 weeks of age and tested starting at 9 weeks of age. In the first dose-finding experiment, vehicle-treated R6/2 mice showed a significant deficit during acquisition and reversal as compared to vehicle-treated WT mice. Donepezil (0.6 mg/kg/day) improved reversal in the R6/2 group. In the second experiment, we confirmed the beneficial effect of donepezil (0.06 mg/kg/day) on reversal, and also found beneficial effects on acquisition. Donepezil had no effect on open-field activity measures or latency to reach the platform during the swim test. We suggest that the donepezil-induced improvements in cognitive function observed in the R6/2 transgenic model of HD may reflect amelioration of deficits in cholinergic function that have been reported previously in this model. Further work is required to confirm the findings of these interesting although preliminary studies.

Huntington's disease (HD) is a progressive neurodegenerative condition caused by an expanded CAG repeat in the huntingtin (*HTT*) gene. Clinical symptoms of HD include motor dysfunction, cognitive impairment and psychiatric disturbance (Bates, Harper, & Jones, 2002). The field was significantly advanced by the development of the first transgenic model of the disease, the R6/2 mouse, which expresses only the mutant exon 1 fragment of the human *HTT* gene (Mangiarini et al., 1996). The R6/2 is an animal model of HD with rapid disease progression, exhibiting motor function deficits from an early age, reduced body weight, and premature death (Mangiarini et al., 1996; Menalled et al., 2009). There are also numerous reports of cognitive deficits in R6/2 mice (Ciamei & Morton, 2009; Lione et al., 1999; Morton et al., 2005; Pallier et al., 2007; Picconi et al., 2006).

Reduced extracellular levels of striatal acetylcholine and abnormalities in potentiated responding of striatal cholinergic neurons have been reported in the R6/2 HD model (Farrar, Callahan, & Abercrombie, 2011; Picconi et al., 2006; Vetter et al., 2003); these deficits may contribute to the impaired striatal plasticity which likely contributes to cognitive dysfunction in this model. Out of the several preclinical cognitive assays, the two-choice swim tank has been proposed to be suitable for the characterization of cognitive impairment in R6/2 mice (Lione et al., 1999; Pallier et al., 2007). Given the high degree of interest in finding effective therapies for HD and the fact that the cognitive impairments associated with the condition are particularly disabling, we aimed to determine how effective current cognition enhancers would be in the R6/2 model. Centrally-acting acetylcholinesterase inhibitors have been shown to improve learning and memory in a number of animal models of cognitive dysfunction (Bontempi, Whelan, Risbrough, Lloyd, & Menzaghi, 2003; Dong et al., 2005; Van Dam, Coen, & De Deyn, 2008), and are a standard of treatment in patients exhibiting clinical signs of memory loss (Rodda, Morgan, & Walker, 2009; Tsuno, 2009). Moreover, Morton et al. (2005) demonstrated the acetylcholinesterase inhibitor, tacrine, administered in combination with moclobemide and creatine prevented cognitive decline in R6/2 mice, although the precise mechanism of this beneficial effect, specifically the extent to which it was mediated by tacrine, remains unclear.

The present studies tested the beneficial effects of chronic dosing with donepezil on cognitive deficits of R6/2 mice in the two-choice swim tank. The first experiment was conducted to identify an effective dose of donepezil. It included 11 days of acquisition and four days of reversal with a vehicle-injected WT control group. R6/2 mice were treated with vehicle, 0.3 or 0.6 mg/kg/day of donepezil. Based on the data from this study we trained a second group for 5 days of acquisition and a longer reversal (8 days) and tested the effects of 0.6 mg/kg/day of donepezil as compared to vehicle, in both WT and R6/2 mice.

Method

Animals

WT and R6/2 transgenic mice carrying the N-terminal region of a mutant human huntingtin gene (Mangiarini et al., 1996) were used in this study. Mice were bred in our colony by crossing ovarian transplanted females on a CBAxC57BL/6 background (Jackson Laboratories; CAT # 006494) with C57BL/6 WT males (Jackson Laboratories; CAT # 000664). This breeding strategy was utilized to ensure that no mice were homozygous for the retinal degeneration 1 (rd1) mutation. Mice were genotyped before weaning by real-time PCR of tail snips. For mutant mice the CAG repeat length was analyzed by ABI 377 sequencer. Average CAG repeat length in this study was 118.1 ± 0.80. Mice were handled on 2 consecutive days (1 min each day) between 19-21 days of age. Animals were tail tattooed at 20-21 days of age and weaned at 21-22 days of age. Mice from multiple litters were used for each treatment group (equally divided between genders), and housed four mice/cage. In each cage, two WT mice of the same gender, but from different litters, were included to provide additional social stimulation. Body weights of the experimental mice were recorded biweekly during the period that mice were being dosed. All mice were housed in OptiMICE cages and supplied with a moderately enriched environment (Enviro-dri bedding, tunnel and a nylabone). Mice had free access to food and water and, in addition, all mice received wet powdered food placed on the floor of the cage (BioServ, Frenchtown, NJ). This additional food was replaced fresh daily and started from weaning. All testing was conducted during the light phase (0700-1900 h).

General Experimental Protocol

Dosing with either donepezil (Evotec, UK) or vehicle (1% Lutrol in 50 mM citrate) began at 8 weeks of age and continued through the completion of all behavioral testing at 10 weeks of age. Donepezil was prepared daily and administered i.p. 1 hour before testing. In the first study, all WT mice (n=20) were injected with vehicle: R6/2 were injected with vehicle ($n = 18$), donepezil at 0.3 mg/kg: ($n = 18$) or at 0.6 mg/kg ($n = 17$). In the second study mice were injected with either vehicle (WT: $n = 11$; R6/2: $n = 12$) or 0.6 mg/kg donepezil (WT: $n = 16$; R6/2: $n = 16$). In the second study mice were tested in the open field assay 1 h after treatment. In both studies, testing in the cued two-choice swim tank was conducted from 9 to 10 weeks of age. Acquisition lasted 8 and 5 days, and reversal 4 days and 9 days, in the first and second studies, respectively.

Open Field

On the first day of treatment, in the second study, mice were dosed and moved from the colony room to the open field experimental room to acclimate for 1 h. Mice were placed in the center of activity chambers (Med Associates Inc, St Albans, VT; 27 x 27 x 20.3 cm) equipped with infrared beams and their behavior was recorded for 30 min. Quantitative analysis was performed on total distance travelled, distance travelled in the center, and total rearing. Moreover, percent of total distance travelled in the center was evaluated as an index of anxiety-like behavior, with higher values on this measure interpreted as reduced anxiety-like behavior.

Cued Two-Choice Swim Test

The cued two-choice swim test involves acquisition and reversal of a dark-light visual discrimination. Animals were placed within a rectangular tank (76 cm x 30.5 cm x 30.5 cm) filled with water maintained at $25 \pm 1°C$. The water was rendered opaque by the addition of non-toxic white paint so the animals were unable to see the escape platform located 0.5 cm below the surface of the water at one end of the tank. A light source with a 25 W bulb was clipped to one side of the tank above the escape platform (7 cm in diameter with the borders slanted and roughened to help the rodents climb to it). The cue light was always paired with the platform during the initial acquisition phase to oppose the initial natural preference of mice for the dark side of the tank. The position of the light relative to the mouse entry point (left vs. right) was counterbalanced within each day's trials for every animal. On each trial, mice were placed into the middle of the tank and allowed to swim until they reached the platform or for up to 1 min. If an animal did not reach the platform within 1 min, the mouse was placed on it by the experimenter and allowed to remain there for 30 s on day 1, and 15 s on all subsequent days of testing, and then removed to a pre-warmed holding cage placed on a warming pad. During all acquisition days, mice were given 8 trials per day (4 blocks of 2 trials). Criterion performance during both acquisition and reversal stages was defined as mice attaining at least 75% correct choices for two consecutive days. In order to advance to reversal testing, mice were required to reach this criterion during acquisition. During the reversal phase of the test, the platform was placed on the dark side of the tank. On each trial during testing days (acquisition or reversal phase), the latency to reach the platform was recorded. A correct choice was scored if the animal initially turned in the direction of the platform and successfully mounted the platform. An incorrect choice was scored if the animal initially swam in the direction opposite the platform. A no choice was scored if the animal either did not make a choice, by swimming in the middle of the tank, or turned initially toward the platform but did not mount the platform. On each morning of testing, mice were dosed in the colony room and then moved to the experimental room, single-housed in small holding cages, and acclimated to the room for 60 min prior to the start of testing.

Statistics

Factorial ANOVA was used to examine the effects of genotype and treatment on measures of open field activity and average number of trials to criterion during acquisition and reversal of the two-choice swim tank. A mixed model ANOVA was used to assess effects of genotype, treatment and testing day for measures of percent correct choice and latency to make a correct choice during the reversal phase. Percent correct data were analyzed for all days of testing, but latency data for the first day of reversal testing were excluded from analyses due to the high number of animals that failed to make any correct choices on day 1 of reversal. Data for the proportion of mice to reach criterion during acquisition were analyzed using a Kaplan-Meier survival test.

Results

Experiment 1

2-choice swim tank acquisition. Treatment with donepezil significantly increased the number of R6/2 mice reaching criterion (Logrank/Mantel-Cox, $p < 0.05$; Figure 1A). The beneficial effects of donepezil treatment were observed at 0.6 mg/kg/day (Logrank/Mantel-Cox, $p < 0.05$ but not at 0.3 mg/kg dose (Logrank/Mantel-Cox, $p > 0.30$). Day-by-day chi-square comparisons further indicated an effect of the 0.6 mg/kg dose that was evident on Days 3, 4 and 7 (see Table 1).

During 8 days of acquisition testing, 5 vehicle-treated R6/2 mice, 3 R6/2 mice treated with 0.3 mg/kg donepezil and 1 mouse treated with 0.6 mg/kg donepezil failed to reach criterion. For the mice that reached criterion, there was no difference between vehicle-treated WT and vehicle-treated R6/2 mice in the average number of trials to reach criterion (Genotype effect: $F(1,31) = 0.10$, n.s.). Comparison of the donepezil-treated R6/2 groups indicated no effect of Treatment on acquisition ($F(2,41) = 1.53$, n.s.; Figure 1B).
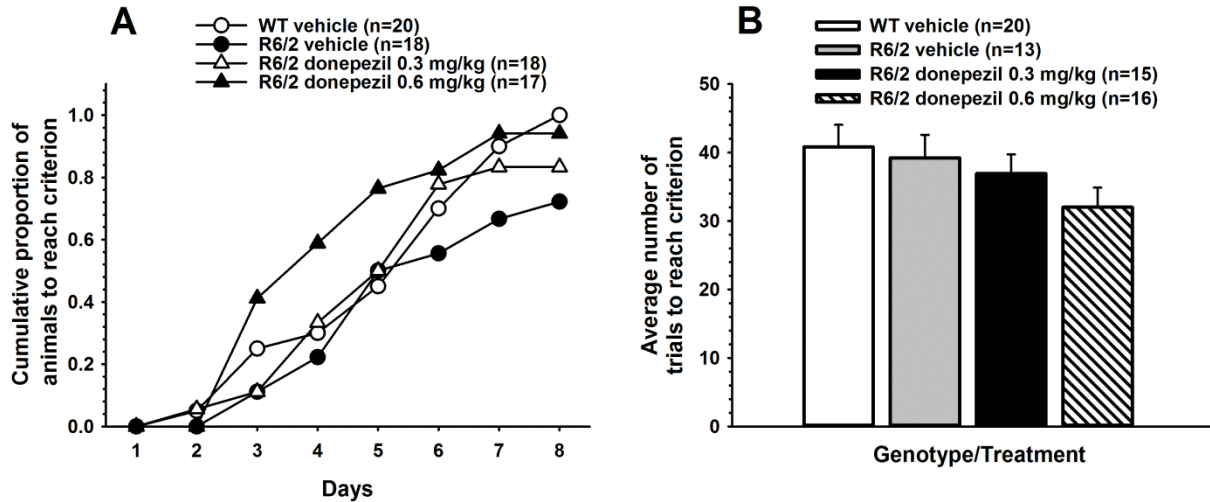
Figure 1. Effects of donepezil (0.3 or 0.6 mg/kg/day) on acquisition of the 2-Choice Swim Tank. The graphs show the cumulative proportion of wild-type (WT) and R6/2 mice to reach criterion during the acquisition phase (A), and the mean (± SEM) number of trials to criterion during acquisition (B).

Table 1

Chi square p-values for comparisons of WT vehicle, and compound-treated R6/2 groups with the R6/2 vehicle group on each day of testing

Treatment/genotype comparisons	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Vehicle R6/2 vs. Vehicle WT	0.34	0.27	0.59	0.76	0.36	0.08	0.01*
Vehicle R6/2 vs. 0.3 mg/kg donepezil R6/2	0.31	ND	0.46	ND	0.16	0.25	0.42
Vehicle R6/2 vs. 0.6 mg/kg donepezil R6/2	ND	0.04*	0.03*	0.11	0.09	0.04*	0.09

Note. ND indicates "not determined", due to identical proportions of mice attaining criterion in the two compared groups at that specific time-point.
* $p < .05$.

2-choice swim tank reversal. Only a subset of mice completed the four days of reversal: WT vehicle ($n = 6$), R6/2 vehicle ($n = 4$), R6/2 donepezil 0.3 mg/kg ($n = 6$), R6/2 donepezil 0.6 mg/kg ($n = 10$). Vehicle-treated WT mice showed significantly greater percent correct choices during the four days of reversal testing when compared to vehicle-treated R6/2 mice (Genotype main effect: $F_{(1,8)} = 27.01$, $p < 0.001$; Days main effect ($F_{(3, 24)} = 2.13$, n.s.; Genotype x Days interaction: $F_{(3, 24)} = 0.71$, n.s.). In the R6/2 mice, treatment with donepezil significantly increased percent correct choices during reversal ($F_{(2, 17)} = 4.07$, $p < 0.05$; Treatment x Day interaction: $F_{(6, 51)} = 1.77$, n.s.); this effect was observed only in the 0.6 mg/kg group ($p < 0.003$; Figure 2A). Number of trials to criterion was not analyzed due to the low number of test days (four reversal days) that resulted in very few subjects reaching criterion.

Choice latencies were significantly lower in vehicle-treated WT versus R6/2 mice (Genotype main effect: $F_{(1, 8)} = 29.54$, $p < 0.001$), irrespective of day (Day main effect: $F_{(2, 16)} = 0.21$, n.s.; Genotype x Day: $F_{(2, 16)} = 0.18$, n.s.). Choice latencies tended to decrease across the four reversal test days in R6/2 mice treated with 0.6 mg/kg donepezil compared to vehicle, although the effect did not reach significance (Treatment x Day interaction: $F_{(4, 34)} = 2.64$, $p < 0.051$; Treatment: $F_{(2,17)} = 2.65$, $p = 0.10$; Day main effect ($F_{(2, 34)} = 0.38$, n.s.; Figure 2B).
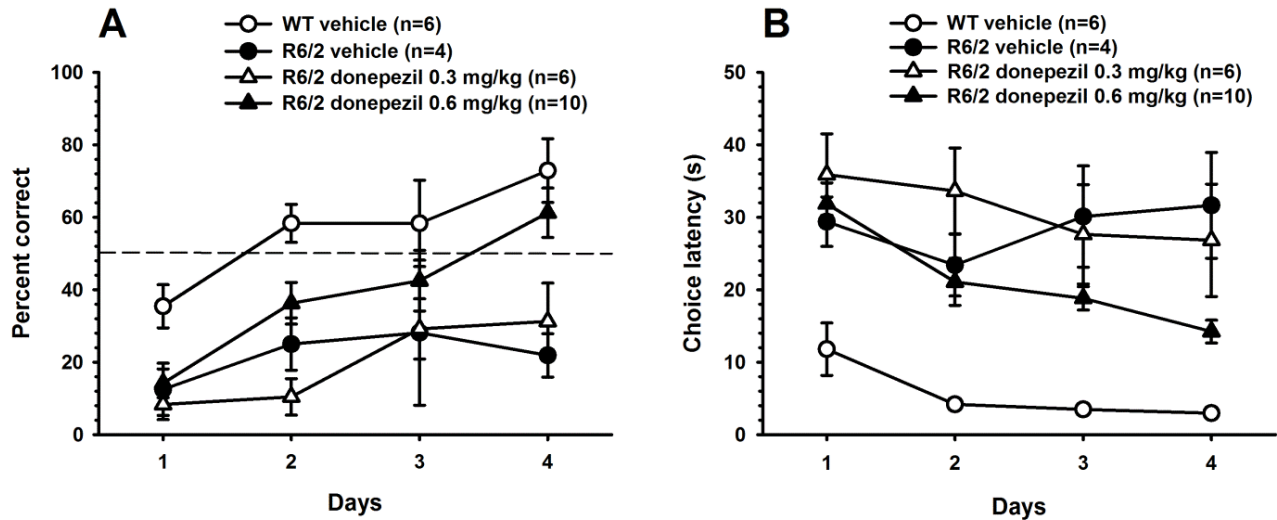
Figure 2. Effects of donepezil on reversal testing in 2-Choice Swim Tank. Graphs depict percent correct choices (A) and latency to choose (B). Data are expressed as means ± SEM.

Experiment 2

Locomotor activity. R6/2 mice exhibited reduced activity as measured by total distance traveled ($F(1, 34) = 9.55$, $p < 0.01$), distance traveled in the center ($F(1, 34) = 7.79$, $p < 0.01$) and rearing ($F(1, 34) = 7.36$, $p < 0.05$) in the open field compared to WT mice at 8 weeks of age. Donepezil (0.6 mg/kg) did not affect open field activity in either WT or R6/2 mice ($Fs < 1.7$, n.s.). There was no effect of genotype on percent of total distance in the center (main effect: $F(1, 34) = 1.69$, n.s.; interaction: $F(1, 34) = 0.05$, n.s.), the effect of donepezil treatment on this measure was marginal, but not significant ($F(1, 34) = 3.64$, $p < 0.07$). Data are shown in Figure 3.

2-choice swim tank acquisition. All R6/2 and vehicle-treated WT mice reached the acquisition criterion over five days of testing whereas one donepezil-treated WT mouse failed to reach criterion during this period. The cumulative proportion of mice successfully acquiring the task is shown in Figure 4A. Treatment with donepezil exhibited a marginal increase in the number of mice reaching criterion, although this effect fell short of significance (Logrank/Mantel-Cox, $p < 0.064$). The possible beneficial effects of donepezil treatment were observed in the R6/2 mice (Logrank/Mantel-Cox, $p < 0.093$) rather than WT control mice (Logrank/Mantel-Cox, $p > 0.53$). There was no significant effect of genotype on the number of trials to reach criterion ($F(1, 34) = 0.03$, n.s.; Figure 4B). There was a marginal but non-significant decrease in the number of trials required to reach criterion in donepezil- versus vehicle-treated mice (Treatment main effect: $F(1, 34) = 3.69$, $p < 0.063$), regardless of genotype (Genotype x Treatment interaction: $F(1, 34) = 1.77$, n.s.).

2-choice swim tank reversal. Across nine days of reversal testing, two WT mice (one per treatment group) and four R6/2 mice (two per treatment group) failed to reach the reversal criterion. Donepezil treatment significantly increased percent correct choices, particularly in R6/2 mice (Genotype x Treatment interaction: $F(1, 34) = 4.59$, $p < 0.05$; Treatment main effect: $F(1, 34) = 4.24$, $p < 0.05$; Genotype main effect: $F(1, 34) = 8.04$, $p < 0.01$; Figure 5A). All groups of mice improved over the course of testing (Day main effect: $F(8, 272) = 55.47$, $p < 0.001$), regardless of treatment or genotype. Surprisingly, the average number of trials to reach criterion (Figure 5B) during the reversal phase did not show an effect of either genotype or

treatment (Genotype main effect: $F(1, 28) = 0.76$, n.s.; Treatment main effect: $F(1, 28) = 1.85$, n.s.; Genotype x Treatment interaction: $F(1, 28) = 1.25$, n.s.).

Latencies to make a correct choice were significantly shorter in WT compared to R6/2 mice (Genotype main effect: $F(1, 32)=95.10$, $p < 0.0001$); there were no significant main or interaction effects of Days or Treatment on latency. Average latency across all days of testing is shown in Table 2.
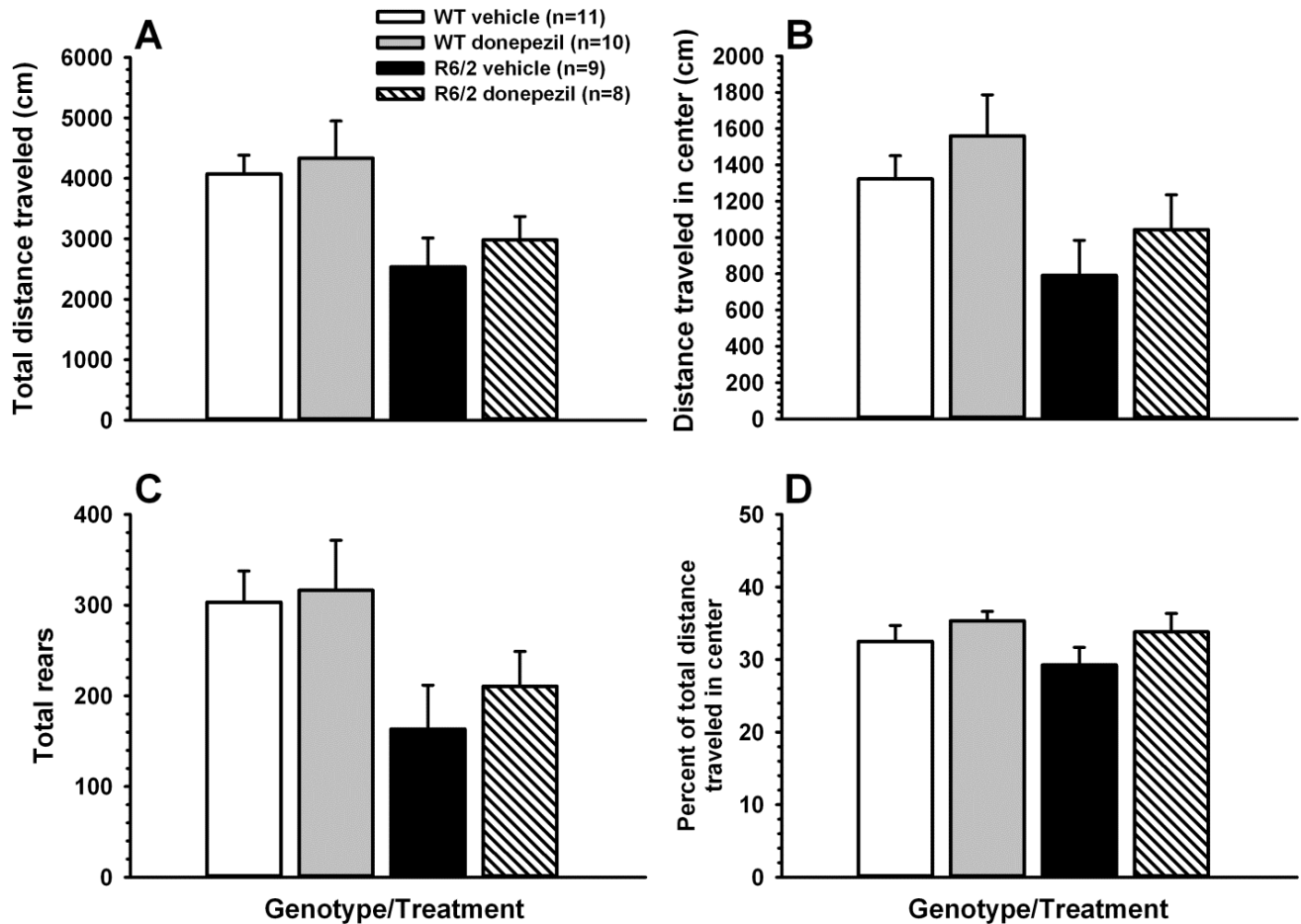

Figure 3. Open field activity on the initial day of dosing with 0.6 mg/kg donepezil or vehicle in wild-type and R6/2 mice. The graphs show total distance traveled (A), distance traveled in the center (B), total rearing (C) and percent of total distance traveled in the center (D). Data are presented as mean ± SEM.
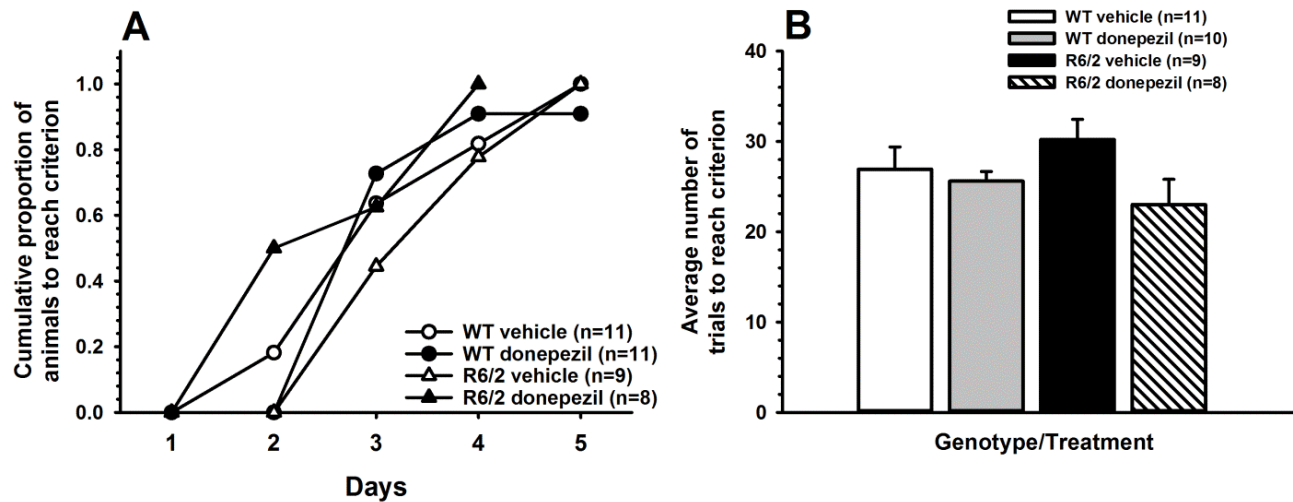
Figure 4. Effects of donepezil (0.6 mg/kg/day) on acquisition in the 2-Choice Swim Tank in R6/2 and wild-type mice. Graphs depict the proportion of mice acquiring the task across 5 days of acquisition (A), and the mean (± SEM) number of trials to reach criterion (B).
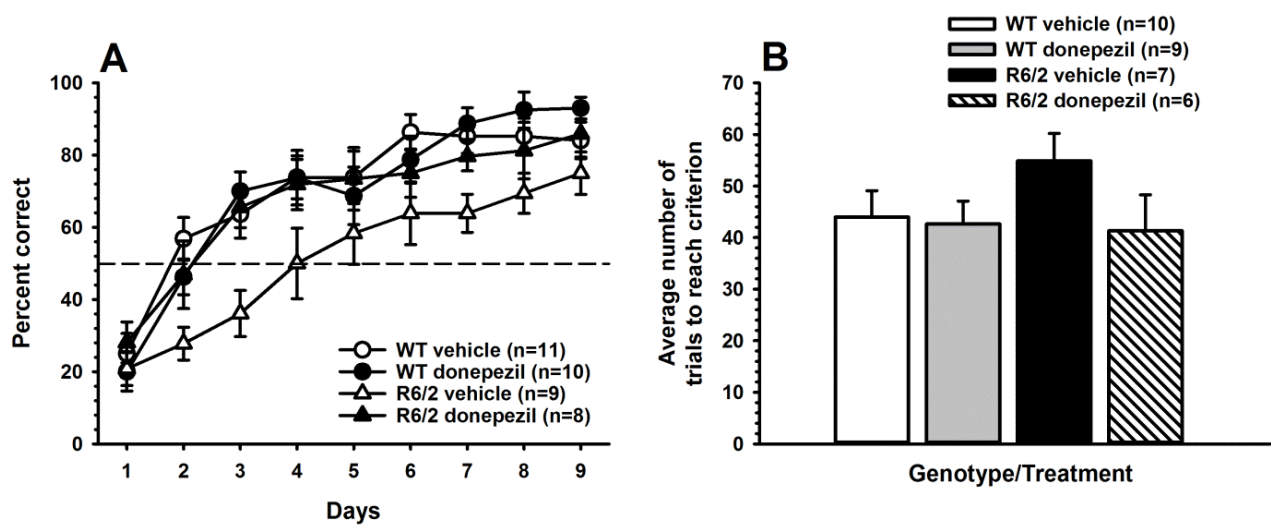

Figure 5. Effects of donepezil (0.6 mg/kg/day) on reversal in the 2-Choice Swim Tank in R6/2 and WT mice. Graphs depict percent correct choices (A) and number of trials to criterion (B). Data are presented as mean ± SEM.

Table 2

Latency to choose in the 2-Choice Swim Tank in R6/2 and WT mice

Treatment, genotype	Mean latency (s)
Vehicle, WT	3.69 ± 0.24
Vehicle, R6/2	14.72 ± 0.90
Donepezil, WT	5.65 ± 0.60
Donepezil, R6/2	11.33 ± 0.63

Note. Latencies are expressed as mean ± SEM, in seconds.

Discussion

We report here a positive effect of chronic treatment with the reversible acetylcholinesterase inhibitor donepezil on cognitive function in the R6/2 mouse. Specifically, repeated daily administration of donepezil at 0.6 mg/kg in R6/2 mice significantly increased accuracy in a reversal task in the two-choice swim tank, but failed to significantly decrease the number of trials to reach criterion, compared to vehicle-treated controls. The R6/2 deficit observed in this study, consisting of a modest or no impairment during the acquisition phase and a marked deficit during the reversal phase, is typical of our previous observations at 9 weeks of age (data not shown). The comparatively more severe deficit observed in R6/2 mice during the reversal vs. acquisition phases is consistent with an impaired ability of these mice to adapt to changing contingencies. Deficits in cognitive flexibility and executive function have been widely reported in HD (Aron, et al., 2003; Backman, Robins-Wahlin, Lundin, Ginovart, & Farde, 1997; Rodrigues, et al., 2009) and constitute an important area of study in the development of potential therapies for HD.

Defects in cholinergic transmission have been reported in the R6/2 model of HD. These include decreased potentiated acetylcholine efflux and alterations in synaptic striatal plasticity (Farrar et al., 2011; Picconi et al., 2006; Vetter et al., 2003), including a lack of long-term potentiation (LTP) in cholinergic striatal interneurons (Picconi et al., 2006). The closely-related R6/1 mouse also exhibits decreased vesicular acetylcholine transporter and choline acetyltransferase mRNA and protein levels (Smith et al., 2006). Alternatively, donepezil appears to possess neuroprotective properties against glutamate excitotoxicity, possibly via effects on $\alpha 7$ nicotinic receptors (Shen et al., 2010). In addition, the acetylcholinesterase inhibitor, galantamine, reduced striatal degeneration in the 3-nitropropionic acid model of HD, an effect possibly mediated via nicotinic acetylcholine receptors (Park, Lee, Im, Chu, & Kim, 2008). A reported decrease in acetylcholinesterase activity in the R6/1 murine HD model (Smith et al., 2006) suggests a possible limitation in the utility of acetylcholinesterase inhibition as a therapeutic approach, and a similar characteristic reduction in acetylcholinesterase activity of HD patients could explain the apparently limited efficacy of this approach in the small HD patient populations tested to date. For example, Rivastigmine produced a trend for improved cognitive performance in two small studies (de Tommaso, Difruscolo, Sciruicchio, Specchio, & Livrea, 2007; Rot, Kobal, Sever, Pirtosek, & Mesec, 2002); however, other investigators have found no positive effects associated with donepezil treatment (Cubo et al., 2006; Fernandez, Friedman, Grace, & Beason-Hazen, 2000). In contrast to the conflicting results observed in HD patients, acetylcholinesterase inhibitors are the current first line of treatment for patients with Alzheimer's disease (Rodda et al., 2009) (Tsuno, 2009). Similarly, in Parkinson's patients, acetylcholinesterase inhibitors have improved cognitive performance in multiple studies (Ravina et al., 2005; Reading, Luce, & McKeith, 2001; Schmitt, Farlow, Meng, Tekin, & Olin, 2010). Nonetheless, it is possible that acetylcholinesterase activity is diminished as a result of a primary deficit in cholinergic signaling.

In conclusion, we suggest that donepezil and other acetylcholinesterase inhibitors deserve further study regarding their positive effects on the cognitive performance of R6/2 mice. An important first step is to follow up the present preliminary data, exploring multiple doses of donepezil in a full-scale study that is sufficiently powered to detect putative effects on number of trials to reach criterion in addition to percent correct. The version of the swim T-maze used in these studies seems sensitive to the mouse preference for dark rather than well-lit places. Thus, it is possible that anxiolytic effects such as those shown in patients treated with donepezil (Gauthier et al., 2002), as well as the non-significant tendency for donepezil to increase relative distance traveled in the center of the open field in the present study, may simulate or potentiate cognitive benefits. Moreover, given thermoregulatory problems in HD (e.g., lower basal temperature in N171 mice (Weydt et al., 2006), and our observations of increased loss of temperature in the 240 CAG R6/2 after testing in a wet T-maze test [unpublished]), it would be important to repeat the study using a dry version of this discrimination reversal test, such as the food-reinforced T-maze employed by Lione et al. (1999). However, it

should be noted that R6/2 mice may be differentially sensitive to the incentive to perform the task (i.e., water escape versus food reinforcement). In addition, cognitive assays that assess learning and cognitive flexibility via different sensory modalities and/or reinforcement types are a logical extension of the current studies. Finally, due to the rapidly progressive nature of the disease process in R6/2 mice, and the associated difficulty of assessing changes in cognition in this line, other animal models of HD should be utilized to confirm cognitive improvement with acetylcholinesterase inhibitors.

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