Exercise attenuates neuropathology and has greater benefit on cognitive than motor deficits in the R6/1 Huntington's disease mouse model

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ABSTRACT

Huntington's disease (HD) is a neurodegenerative disease caused by a mutation within the huntingtin gene that induces degeneration within the striatal nuclei, progressing to widespread brain atrophy and death. The neurodegeneration produces symptoms that reflect a corticostriatal disconnection syndrome involving motor, cognitive and psychiatric disturbance. Environmental enrichment has been demonstrated to be beneficial to patients with neurological disorders, with exercise being central to this effect. Rodent studies have confirmed exercise-induced neurogenesis and increased growth factor levels in the brain and improved behavioural function. The present study sought to determine whether an extended regime of exercise could retard disease progression in the R6/1 mouse model of HD. The study was designed specifically with a translational focus, selecting behavioural assessments with high clinical predictive validity. We found that exercise improved gait function in both control and HD mice and selectively improved performance in the R6/1 mice on a motor co-ordination aspect of the balance beam task. Exercise also retarded the progression of cognitive dysfunction on water T-maze procedural and reversal learning probes presented serially to probe cognitive flexibility. In addition, exercise reduced striatal neuron loss in the R6/1 mice but increased striatal neuronal intra-nuclear inclusions, suggesting that the functional effects were striatally mediated. These results confirm and extend those from previous studies that demonstrate that HD may be amenable to exercise-mediated therapeutics, but suggest that the impact of such interventions may be primarily cognitive.

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Introduction

Huntington's disease (HD) is a neurodegenerative disease caused by a single mutation in the gene that codes for the protein huntingtin (The Huntington’s Disease Collaborative Research Group, 1993). HD is primarily characterised by the insidious and progressive neurodegeneration of the medium spiny neurons of the caudate nucleus and cortical atrophy with abnormalities in other brain regions occurring as the disease progresses (Aylward et al., 1998; Rosas et al., 2002, 2005; Tabrizi et al., 2009, 2011; van den Bogaard et al., 2011; Vonsattel et al., 1985). The disease induces motor, cognitive and psychiatric symptoms, which ultimately result in death around 15 years from onset.

There is considerable evidence that exercise or a more active lifestyle has a beneficial effect on the symptoms and prognosis of several disease states including Alzheimer’s disease (Verghese et al., 2003; Abbott et al., 2004; Rovio et al., 2005; Podewils et al., 2005; Larson et al., 2006), and Parkinson’s disease (Cruise et al., 2011; Gobbi et al., 2009; Muller and Muhlack, 2010; Nocera et al., 2010). These studies demonstrate an exercise-mediated improvement in the daily functioning of patients, sometimes after several years of follow-up studies. In addition to these palliative effects measured through cognitive and motor assessments, there is evidence that exercise may also slow the rate of disease neuropathology (Cruise et al., 2011; Gobbi et al., 2009; Muller and Muhlack, 2010; Nocera et al., 2010). To date only a single randomised controlled physical activity study in HD patients has been performed and demonstrated marked functional benefit with relatively little intervention (Khalil et al., 2013).

A small number of animal studies using mouse models of HD found that exercise may ameliorate some aspects of motor and cognitive dysfunction (Pang et al., 2006; van Dellen et al., 2008; Wood et al., 2011). R6/1 mice exposed to voluntary wheel running from a young age were found to demonstrate less body clasping when suspended by the tail, a delayed onset of motor dysfunction in a static beam test and produced a greater number of spontaneous alternations in a cognitive spatial alternation test, but wheel running had no effect on other transgene-induced functional deficits (Pang et al., 2006; van Dellen et al., 2008; Wood et al., 2011).
In other studies with normal mice (in both middle and old age) (Berchtold et al., 2010; van Praag et al., 1999, 2005), and mouse models of neurological dysfunction (Griesbach et al., 2009; Nichol et al., 2009) exercise was found to enhance learning. However, a study in mice bred to produce high levels of wheel running failed to see this effect (Rhodes et al., 2003). Studies on exercise and cognitive function use probes of hippocampus-mediated spatial learning in HD mouse lines (Pang et al., 2006; Potter et al., 2010). However, this type of probe has little relevance to the primary striatal neuropathology of HD. Hence, although there is some evidence to suggest that exercise in HD mouse lines may be of potential benefit to the clinical population, previous studies have typically used behavioural tests with low face validity and little translational relevance.

The present study was designed to determine whether life-long daily access to voluntary exercise was able to provide benefit to HD mice in a clinically relevant way that could be developed as a model therapeutic system. Hence, we sought to design a study with an emphasis on high predictive (translational) validity, such that the tests used in the animal study were correlates of those commonly used in clinical assessments of HD patients, and presently being used in the HD exercise study being run in Cardiff University (Khalil et al., 2013). The tests were chosen to be sensitive probes of cortico-striatal dysfunction, the principal cause of functional decline in HD. We also sought to determine the underlying mechanism of any beneficial effect of exercise in the HD mice by measuring striatal dopamine and BDNF activation, as both have been implicated in HD pathology (Augood et al., 1997; Giampa et al., 2013; Glass et al., 2000; Zuccato et al., 2001, 2008), and by a detailed stereological analyses of the striatum to determine atrophy, neuron numbers and inclusion pathology.

Material and methods

Animals

The R6/1 HD mouse line (Mangiarini et al., 1996) was chosen for the present study as it develops marked pathology over the course of 7 months and has been used in previous exercise studies (Pang et al., 2006; van Dellen et al., 2008). At the onset of the study, 67 mice were allocated to the experiment with 3 male mice subsequently being removed, two at 3 months of age and 1 at four months of age: one wildtype control mouse developed a cataract, and one R6/1 exercise mouse developed an anal prolapse with a second mouse from this group demonstrating excessive epileptic type of seizures. With the removal of the 3 mice, 64 R6/1 mice congeneric to a C57BL/6J background were used: Group 1 = wildtype control (7 males and 8 females); Group 2 = R6/1 control (9 males and 8 females); Group 3 = wildtype exercise (9 males and 8 females); Group 4 = R6/1 exercise (7 males and 8 females). The R6/1 mice in this study carried between 120 and 125 CAG repeats with a mean of 123 and were bred, tail-tipped and genotyped in-house. The mice had ad libitum access to food and water throughout the study period. The mice were housed at an ambient room temperature of 21 ± 1 °C at a humidity of 60 ± 1%. All experiments were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986 and local ethical review. The defined humane endpoint for the mice was 20% body weight loss, hence although some mice may live to 11 months in other studies, 7 months of age was taken as the final testing time point as a significant number of mice approached the humane endpoint. At the end of the experiment of the original 64 mice that began testing 52 mice remained, 9 non-exercised R6/1 mice and 3 exercised mice having died or been euthanized due to the predefined endpoints.

Exercise administration

Exercise was administered daily (14 h/day, 5 days/week), from post-weaning (5 weeks of age) by individual housing of the mice overnight (17:30-09:30) in cages (34 cm × 28 cm × 1 cm) fitted with an ENV-044 tabulating running wheel, connected wirelessly to a DIG-804 interface hub and laptop computer running the SOF-861 wheel analysis software (Med Associates, St. Albans, VT, USA). By day, the mice were returned to their grouped housing conditions in the same sized cages to prevent the development of abnormal behaviours caused by continual social isolation. Non-exercised mice were treated in the same way as the R6/1 mice but without access to a running wheel to be consistent with previous studies (Pang et al., 2006; van Dellen et al., 2008).

Behavioural testing

The mice were tested at regular monthly intervals (every 4 weeks) from 8 weeks of age through to 28 weeks of age throughout their lives on a number of tests of motor function and tested twice on a water T-maze attentional shift task at an early (9 weeks) and a late (22 weeks) disease time point. For the motor tests data analyses were taken from data points between 4 and 7 months of age to control for sex differences in wheel running levels in the R6/1 mouse (see below). Behavioural tests were selected for their high translational value and consequently have clinically relevant human correlates (rotarod and gait analysis ≡ kinetic and kinematic gait assessments; grip strength ≡ dynamometry; motor activity ≡ self report and direct accelerometer based measures of daily physical activity; balance beam ≡ clinical balance tests and force plate measure; water T-maze ≡ procedural learning/attentional probe). All tests were performed blind to genotype.

Body weight

Body weight was measured weekly to assess the general health of the animals. Body weight is also an indicator of disease development in HD, as mice that are gene carriers tend to lose weight relative to their wildtype littermates.

Rotarod

Motor coordination was tested on the accelerating version of the rotarod test using a standard apparatus (Ugo Basile, Varese, Italy), as described previously (Brooks et al., 2004, 2012c, 2012d, 2012f). During test sessions, the mice are allowed three trials, with data from the final 2 being collected.

Balance beam

An elevated bridge (balance beam) apparatus was used to measure balance and motor coordination as described previously (Brooks et al., 2012a). A tapered balance beam (1.5 cm to 0.5 cm) with a ledge running its length (Schallert, 2006) was used to determine whether the mice had a balance impairment. The balance beam was 100 cm in length and angled at 17° with the start point at the low end and a goal box at the high end. The start point was 15 cm from one end of the beam, and the end point was 10 cm from the other. The beam was also fitted with a ledge that ran the full length at 2 cm below the height of the running surface, and protruded 0.5 cm on either side of the beam to prevent the animals from falling, and to aid in the identification of foot-slips. Mice were trained to run the beam prior to testing. During testing, each animal was given a trial run, and then two experimental runs from which the data was collected. At the onset of testing the mouse was placed on the extreme end of the beam (low end), facing away from the beam. The mouse must turn towards the beam in order to run it, and reach the goal box. The time it took the mouse to turn on the end of the beam was taken as a measure of motor coordination. The running/walking was then timed from the start point to the end...
foot-slips were counted as measure of balance. The point of the beam to determine the ability of the mice to traverse it, and the measures when the animal was either accelerating or decelerating. Four separate parameters were measured; stride length; hind and fore paw base width; overlap between fore and hind paws. For each set of prints three consecutive examples were taken for each of the measured parameters. Footprints were always recorded mid-run to avoid taking three consecutive examples for each of the measured parameters. Grip strength was used to line the runway floor. The mice were permitted to walk to the goal box from the opposite end of the runway thus allowing their footprints to leave footfall patterns on the white paper. To measure grip strength a standard stainless steel mouse cage lid (43 × 26.5 cm) with lateral 1 mm diameter rungs was used in accordance with previous studies (Brooks et al., 2012b). The mouse was placed on the cage lid which was held flat in the horizontal plane 20 cm above a softened (with a folded towel) bench surface and the lid was then slowly inverted through 180° along the horizontal axis so that the mouse cluing upside-down to the cage lid. The mouse was then timed to fall for a maximum of 1 min.

Open field motor activity

16 Perspex activity cages (42 × 26 × 19 cm) fitted with infra-red beams (Med Associates, St. Albans, VT) were used. Mice were placed in the cages between 10:00 h and 12:00 h where they remained under their normal 12 h light/dark cycle, for 32 h with free access to food and water throughout. The first 30 min of data was collected to measure transfer activation with the 24 h circadian cycle of activity being used to assess motor activation.

Water T-maze set-switching task

A water T-maze swimming task was used to measure different aspects of discrimination learning and cognitive flexibility as described previously (Brooks et al., 2012b). As the procedure takes around 4 weeks to run, it was used only twice, at 9 and 22 weeks of age. The labour intensity of the task also dictates the number of mice that can be run daily which on the procedure described below is 40. We determined to run balanced groups of 10 mice comprising of 5 male and 5 female mice, with these 40 mice running the task at both time points to reduce the stress associated with unfamiliar exposure to the water. On analyses of the first data set we found that we had inadvertently included a male exercised wildtype mouse in the non-exercised wildtype group resulting in unbalanced wildtype groups (wildtype = 4 males/5 females; wildtype exercise = 6 males/5 females; R6/1 = 5 males/5 females; R6/1 exercise = 5 males/5 females). On the second run of the procedure at 22 weeks of age 2 of the R6/1 animals with wheels had died (1 male and female), and could not be replaced as this group of 40 mice had had previous exposure to the test, resulting in a group of 8 mice.

The water T-maze apparatus was constructed of clear Perspex. All arms were 30 cm high and 7 cm wide, the stem 21.5 cm long and the two perpendicular side arms each 37 cm long. The apparatus was filled with water at 23 ± 2 °C to a depth of 22.5 cm. An escape platform could be placed at the end of either side arm (6 × 6 × 21.5 cm high), coloured white, and designed to sit snugly in to the arm of the maze at 1 cm below the water surface. The water was coloured white with pasteurised milk so as to make the platform invisible from within the maze. Exterior to the maze, two standard anglepoise lamps fitted with 40 W bulbs, were positioned to illuminate the end of either arm. At any time only one of the lamps was turned on. The mice were tested successively on a series of visual and directional discrimination tasks. In each case training continued over several days until they reached criterion (see below), when they were then switched to the next task in the series.

At the 9-week stage each animal was run 12 trials per day, but by 22 weeks the mice underwent 6 trials per day to reduce the physical demands on the R6/1 mice. The mice of each cohort were tested in rotation, such that each mouse was dried and returned to its home cage after each trial, and with an inter-trial interval of approximately 15 min or longer. On each trial, the response choice was determined when the animal’s body fully entered the arm. If the choice was correct (entering the arm containing the escape platform) the mouse was allowed to swim and climb on to the platform, and was removed after 10 s. If the animal performed an incorrect choice, the animal was constrained to swim in the incorrect arm for 10 s, using a plastic blocking panel that fitted precisely to the width of the maze. All training employed a correction procedure whereby after 10 s the blocking panel was turned through 90° to open the arm and simultaneously block the stem of the T-maze and the mouse was permitted to find the platform in the correct arm of the maze. In order for the mice to advance through the experimental stages they had to achieve 9 correct trials from any sequence of 10 trials (>90% accuracy) for each stage. The data for analyses was the number of trials required to reach criteria and the percentage of correct choices.

Six test stages were used. Each of the stages required the animal to learn a different rule in order to reach criteria and move to the next stage of the task. As this test aimed to probe for attention-mediated cognitive flexibility and procedural learning deficits, currently irrelevant dimensions, which may become relevant later, were always present on each trial. Briefly the six stages were:

i. Simple directional discrimination where the mouse had to always turn in the same direction, with the irrelevant dimension (lit lamp) randomly assigned to either arm on each trial.

ii. Reversal of the directional learning task such that mice trained to turn right, now must go left and vice versa. The irrelevant light stimulus remains irrelevant and continued to be randomly presented.

iii. Shift from the directional rule to a light/dark discrimination based on the rule “move towards/away from the light”. In this stage direction became irrelevant.

iv. Reversal of the light/dark rule, such that an animal trained to move to light, had now to move towards the dark and vice versa.

v. Shift away from the light/dark discrimination back to the original directional rule (Stage 1), with the light cues continuing to be present and randomised, but irrelevant.

vi. Maze rotation manipulation was introduced to determine whether the animals were utilizing the turn or place strategies to solve the directional learning stages of the task which may have confounding effects on the interpretation of experimental results.

Neuropathological assessment

Eight mice from each group were randomly selected and perfused intracardially for 3 min with pH = 7.4 phosphate buffered saline (PBS), after a systemic injection of (0.2 ml) Euthatal (Merial, Merz, Essex, UK). Following the PBS flush, the mice were trans fused with 4% paraformaldehyde (PFA) in a 0.1 M PBS solution for 5 min. The brains were then removed and fixed in a 4% PFA solution for 4 h, prior to being placed in a 25% sucrose/PBS solution until they sank. The brains were sectioned into 1:12 series, 40 μm coronal slices on a freezing sledge microtome (Leitz Bright Series 8000, Germany) and stored in antifreeze at −20° until used. For histological analyses, the 1:12 slices were Nissl stained with cresyl violet (Sigma) as a cellular marker and S830 (Prof.
Cresyl fast violet

Sections were mounted on gelatine coated glass slides (Fisher Scientific, UK) and left to dry for 24 h at 37 °C, prior to dehydration in ethanol solutions of 70%, 90% and 100% for 5 min each, after which the tissue was delipidised in a 20% chloroform/20% ethanol (1:1 v/v) solution for 20 min. The tissue was then rehydrated in descending concentrations of ethanol (100%, 90%, 70%) for 5 min each prior to immersion in distilled water for a further 5 min. They were then stained for 5 min with cresyl violet (0.7% in distilled water with 0.5% sodium acetate; Sigma). The slides were then rinsed in distilled water and dehydrated in the ascending ethanol concentrations, cleared in xylene (VWR, Darmstadt, Germany) for 10 min and cover-slipped with DPX mounting medium (RA Lamb, Somerset, UK).

Immunohistochemistry

Slices were placed in a pH = 7.4 Tris buffered saline (TBS) and washed twice. They were then incubated in methanol containing 3% H2O2 for 5 min to inhibit peroxidase activity prior to placement in TBS. A 3% horse serum solution in TBS was used to block non-specific binding sites prior to incubation of the sections with the S830 (1:25,000), TH (1:2000) and BDNF (1:500) antibodies at room temperature overnight. The sections were then washed several times in TBS and incubated with horse anti-goat or horse anti-rabbit secondary antibody at a 1:200 concentration (Vector Laboratories, Burlingame, CA, USA) for 2 h at room temperature. The sections were washed several times and exposed to biotin–streptavidin kit according to the manufacturer’s instructions (Vector). Finally, the sections were washed in TBS again and a 3,3′-diaminobenzidine (DAB) kit (Sigma) was applied prior to the sections being mounted on gelatine coated slides, dehydrated and cover slipped. CV staining and S830 staining were used for the basis of stereological assessment and light intensity measures on the TH and BDNF slices to determine the level of antibody binding were assessed with Image-J (Softonic.com).

Histological and stereological assessments

All stereological assessments of striatal tissue were taken from single 40 μm coronal sections of striatum at ~0.38 mm anterior of bregma as it provides good landmarks to produce consistency between animals and provides a good representation of the striatum and cortex. Two dimensional stereology was undertaken using the Olympus C.A.S.T. Grid system v1.6 in combination with an Olympus BX50 microscope (Olympus Optical Company Ltd., Tokyo, Japan). Data from both striata (see Fig. 1) on each analysed CV section were taken that represented striatal size, cortical thickness (×4 magnification) and neuronal number (×40 magnification with a 622 μm2 counting frame). S830 sections were used to assess neuronal inclusion number (×40 magnification with 622 μm2 counting frame) which were randomly sampled from the tissue using 30 sampling areas, and neuronal nuclei diameter at their widest point being taken as measures of their respective size (×40 magnification) taking on average 46 measurements of neuronal inclusions and neuronal nuclei from randomly sampled regions on the slice.

Optical density assessment was used for the assessment of striatal TH and striatal and hippocampal BDNF contents. The sections were stained together at the same time to ensure consistency. Eight images of the striatum and hippocampus from each hemisphere from 4 sections) from each mouse were photographed ensuring that lighting and microscope settings were constant throughout. For each image the whole of the corpus callosum from the same hemisphere was photographed in order to ensure that the contrast and the whole of the striatum or hippocampus on the same hemisphere. The optical density measure for each striatum or hippocampus was then subtracted from the corpus callosum standard for each slice. These values then meant for each animal to produce the data used for analyses.

Statistical analyses

GenStat version 10 (VSN International, Hemel Hempstead, UK) was used to run all of the statistical analyses. For the behavioural data, split-plot analyses of variance (ANOVA) were conducted on each dataset with Exercise, Sex, and Genotype as between-subject factors, and Age and one or more behavioural parameters as within-subject factors. Although full analyses were run, for clarity only Genotype × Exercise interactions were reported as these were of primary interest and the effects of sex and age were negligible where present. Post-hoc analyses compared group differences using Newman–Keuls’ and Sidak’s test, as appropriate to correct for multiple comparisons. Statistics for bodyweights and exercise were taken over the entire experimental period. For the motor tests analysis was over months 4–7 to take account of differences in the levels of exercise between the sexes in the R6/1 groups (see Fig. 2). For analyses of the 22 week water T-maze experiments 2-way ANOVAs (Genotype × Exercise) were conducted separately on the first 2 maze manipulations (direction and direction reversal), since the number of mice completing each separate task.
fell below critical levels for parametric analyses from stage 3 onwards. For the analyses of the neuropathology, split plot analyses of variance were used with Hemisphere (side) as a single within-subject factor, and Exercise and Genotype as between-subject factors. As there were no effects of hemisphere, later data collection for neuronal nucleus and inclusion size were restricted to the left side of the brain and analysed with t-tests comparing the R6/1 groups across exercise.

Results

After the 28 weeks of behavioural assessment 12 of 32 of the R6/1 mice (9 non-exercise group, 3 exercise group) had died or were euthanized due to a predetermined 20% weight loss end point (see below). No wildtype mice died within this period. As a consequence late stage behavioural tests and the neuropathological analyses are based on correspondingly reduced group sizes.

Wheel running over the experimental period

To ensure that the mice exposed to the running wheels did undertake exercise, levels of wheel running (the number of turns of the wheel) were recorded for each mouse of the exercise groups (Fig. 2A). The wildtype mice produced greater level of wheel running than their R6/1 counterparts ($F_{1,28} = 20.36, p < 0.001$) and female mice ran further than male mice ($F_{1,28} = 10.84, p < 0.01$). As the mice aged the R6/1s became less active ($Age \times Genotype: F_{12,326} = 7.90, p < 0.001$), but the male and female wildtype animals maintained a consistently high level of performance relative to their same-sexed R6/1 counterparts ($Age \times Genotype \times Sex: F_{12,326} = 7.90, p < 0.001$).

The effect of exercise on mortality and body weight

The effect of exercise on mortality rate was measured (Fig. 2B). Over the course of the study no wildtype mice died but 12 R6/1 (3 exercised and 9 control) littermates died (Genotype: $F_{1,56} = 18.11, p < 0.001$), with wheel running not affecting lifespan of the mice as a whole (Exercise: $F_{1,56} = 2.25, \text{n.s.}$) or differentially by genotype (Exercise $\times$ Genotype: $F_{1,56} = 1.34, p = 0.25, \text{n.s.}$). Chi squared analyses of death rates across all four groups found significant differences between the groups (χ² = 11.69, p = 0.01) but no effect of wheel running (WT, $\chi^2 = 0.01, p = 0.97, \text{n.s.}$; R6/1, $\chi^2 = 0.01, p = 0.97, \text{n.s.}$).

Mouse weights were recorded as an index of general health (Figs. 2C, D) with a 20% weight loss regarded as the humane end-point for the subjects. At the onset of the study there was little difference in weight between the sex-matched wildtype and R6/1 mice. As the mice aged the male and female wildtype mice retained their weight relative to their R6/1 counterparts (Genotype $\times$ Sex $\times$ Age, $F_{19,1033} = 41.66$,...
Data are the meaned mouse scores with standard errors from 4 to 7 months of testing from the manipulations described in the Materials and methods section. Analyses of base-widths found broader base-widths in the hindlimbs of the wildtype mice (Genotype, $F_{1,56} = 6.30, p < 0.05$), but not forelimbs (Genotype, $F_{1,56} = 1.35, n.s.$). Exercise increased base-width in the forelimbs of the mice (Exercise, $F_{1,56} = 16.67, p < 0.001$), but not the hindlimbs (Exercise, $F_{1,56} = 0.42, n.s.$). No significant interactions between genotype and exercise were returned for either the fore-limb (Exercise × Genotype, $F_{1,56} = 0.69, n.s.$), or hindlimb measure (Exercise × Genotype, $F_{1,56} = 0.94, n.s.$). The gait analysis data suggests that exercise has some beneficial effects on gait, especially stride length, but that these effects were not specific to a genotype.

Balance beam

For this test 5 separate ANOVAs were applied to the dependent variables (Latency to turn; Latency to cross; Forelimb slips; Hindlimb slips), to determine overall performance on the test. All measures demonstrated significant performance deficits in the R6/1 mice that worsened with age (Latency to turn, Genotype $F_{1,56} = 70.91, p < 0.001$; Latency to cross: Genotype, $F_{1,56} = 223.40, p < 0.001$; Forelimb slips, Genotype, $F_{1,56} = 406.23, p < 0.001$; Hindlimb slips, Genotype, $F_{1,56} = 501.41, p < 0.001$). The latency to turn measure identified a marked beneficial effect of exercise in the R6/1 mice (Genotype × Exercise, $F_{1,56} = 8.31, p < 0.01$), and a generally beneficial effect of exercise on the mice (Exercise, $F_{1,56} = 4.131, p < 0.05$). No other effects of exercise were found on this task.

The effect of exercise on stimulus-response learning and cognitive flexibility

At 9 weeks of age the wildtype mice learned the 6 rules of the water T-maze procedure in significantly fewer trials than the R6/1 mice (Fig. 3A; Genotype, $F_{1,39} = 17.35, p < 0.001$). Wheel running had no effect on the performance of any of the mouse groups at 9 weeks of age (Exercise, $F_{1,39} = 0.05, p = 0.82, n.s.$), and there was no demonstration of behavioural modification between the genotypes due to the wheel running intervention (Genotype × Exercise, $F_{1,39} = 0.01, n.s.$).

When the trials to criteria were analysed as the mean number of trials required for learning each rule, clear genotype differences were found at 9 weeks of age (Genotype, $F_{1,31} = 18.19, p = 0.01$), but no overall effect of wheel running (Exercise, $F_{1,31} = 0.001, n.s.$), or a differential effect of wheel running on the genotypes (Genotype × Exercise, $F_{1,31} = 0.03, n.s.$) was found. Wheel running did not modify the performance of the mice as a whole on the learning of the rules (Exercise × Rule, $F_{3,154} = 0.22, n.s.$).

At 22 weeks of age, many of the transgenic carrier mice dropped out of testing for failing to attain the necessary criteria for progression to the next stage of testing (see numbers in the bars on Fig. 3B). As a

Table 1

<table>
<thead>
<tr>
<th>Motor behaviours (4–7 months of age)</th>
<th>Wild-type mice</th>
<th>R6/1 transgenic mice</th>
<th>Genotype</th>
<th>Exercise</th>
<th>G × E</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>No exercise</td>
<td>Exercise</td>
<td>No exercise</td>
<td>Exercise</td>
<td>(F_{1,56})</td>
</tr>
<tr>
<td>Grip strength (inverted lid)</td>
<td>56.51 ± 1.61</td>
<td>58.66 ± 0.66</td>
<td>31.54 ± 3.42</td>
<td>34.85 ± 3.06</td>
<td>86.53***</td>
</tr>
<tr>
<td>Rotorad</td>
<td>115.18 ± 4.64</td>
<td>128.43 ± 6.11</td>
<td>37.81 ± 3.72</td>
<td>50.06 ± 4.46</td>
<td>158.67***</td>
</tr>
<tr>
<td>Loco activity (30 min)</td>
<td>681.3 ± 39.2</td>
<td>650.2 ± 29.3</td>
<td>353.7 ± 16.7</td>
<td>313.8 ± 16.9</td>
<td>130.93***</td>
</tr>
<tr>
<td>Loco activity (24 h)</td>
<td>48.62 ± 39.5</td>
<td>52.4 ± 40.9</td>
<td>68.0 ± 34.7</td>
<td>814.6 ± 33.3</td>
<td>11.01*</td>
</tr>
<tr>
<td>Footprint stride length</td>
<td>6.80 ± 0.07</td>
<td>8.11 ± 0.11</td>
<td>6.72 ± 0.06</td>
<td>7.90 ± 0.12</td>
<td>0.08</td>
</tr>
<tr>
<td>Footprint overlap</td>
<td>0.32 ± 0.03</td>
<td>0.35 ± 0.03</td>
<td>0.25 ± 0.03</td>
<td>0.38 ± 0.03</td>
<td>0.04</td>
</tr>
<tr>
<td>Footprint hindpaw base</td>
<td>2.61 ± 0.03</td>
<td>2.67 ± 0.03</td>
<td>2.59 ± 0.03</td>
<td>2.53 ± 0.03</td>
<td>6.30*</td>
</tr>
<tr>
<td>Footprint forepaw base</td>
<td>1.33 ± 0.03</td>
<td>1.41 ± 0.02</td>
<td>1.34 ± 0.02</td>
<td>1.46 ± 0.02</td>
<td>1.35</td>
</tr>
<tr>
<td>Bridge turn (s)</td>
<td>2.55 ± 0.14</td>
<td>4.01 ± 0.06</td>
<td>18.62 ± 1.82</td>
<td>15.33 ± 1.80</td>
<td>70.91***</td>
</tr>
<tr>
<td>Bridge cross (s)</td>
<td>5.75 ± 0.68</td>
<td>5.36 ± 0.24</td>
<td>20.04 ± 2.93</td>
<td>22.74 ± 2.73</td>
<td>223.40***</td>
</tr>
<tr>
<td>Bridge forepaw slips</td>
<td>0.85 ± 0.15</td>
<td>0.67 ± 0.09</td>
<td>4.81 ± 0.56</td>
<td>5.06 ± 0.60</td>
<td>0.02</td>
</tr>
<tr>
<td>Bridge hindpaw slips</td>
<td>2.85 ± 0.40</td>
<td>2.01 ± 0.19</td>
<td>11.16 ± 0.96</td>
<td>12.24 ± 1.15</td>
<td>501.41***</td>
</tr>
</tbody>
</table>

Data are the meaned mouse scores with standard errors from 4 to 7 months of testing from the manipulations described in the Materials and methods section. $p > 0.05$. $p < 0.05$. $p < 0.01$. $p < 0.001$.
consequence, the analysis of the performance for the water T-maze procedure at 22 weeks of age was split into discrete ANOVAs for the first two rules (direction and direction reversal) where sufficient numbers of mice per group were present (n = 6/group as a minimum), with the dependent variables of "trials to criteria" (Fig. 3B) and "% correct choices" (Fig. 3C) being analysed. Due to senescence only 77.8% of wild type mice completed all six stages of the task, in exercised wild type mice this was 63.6% due to fewer of the latter group proceeding beyond the light reversal task. For the R6/1 carriers, no mice regardless of exercise completed 5 of the six tasks within the 90 trial limit for each individual task. In total, 75% of exercised R6/1 mice and 70% non-exercised mice completed the direction learning stage, with 75% of exercised R6/1 but only 20% of non-exercised R6/1 mice completing the direction reversal stage.

Fig. 3. At 9 weeks old, R6/1 mice demonstrated impairments on the reversal tasks (A). At 22 weeks of age the wildtype mice completed more rules than the R6/1 mice, and the R6/1 exercised mice completed the direction reversal task in fewer trials than the non-exercised R6/1 mice (B). Only 10% of non-exercised R6/1 mice made it through to the light discrimination stage, compared to 75% of the exercised R6/1 mice (mouse numbers indicated in the bars). When the percentage of correct trials was analysed the exercised R6/1 mice with exercise produced a higher percentage of correct trials than the R6/1 mice non-exercised mice on the direction learning and direction reversal tasks. Significance taken at p < 0.05. ** denotes difference between genotypes at p < 0.01, and § denotes differences between the R6/1 groups at p < 0.01.
Only 2 R6/1 mice completed the light discrimination stage with both being from the exercise group.

For the trials to criteria measure on the direction task (Fig. 3B), a significant effect of genotype was found (Genotype, F1,34 = 13.2, p < 0.001), but no effect of exercise (Exercise, F1,34 = 1.45, n.s.) or interaction effect (Genotype × Exercise, F1,34 = 1.86, n.s.) was returned. However, analyses of the percentage of correct trials (Fig. 3C) returned a significant interaction effect (Genotype × Exercise, F1,34 = 4.8, p < 0.05), and a significant main effect of genotype (Genotype, F1,34 = 20.9, p < 0.001) and exercise (Exercise, F1,34 = 1.45, n.s.). Pair-wise post-hoc analyses demonstrated that the exercised R6/1 mice produced a significantly greater proportion of correct trials (71.8%) than the non-exercised R6/1 mice (52.4%). There were no significant differences between the wild type exercised (85.9%) and the non-exercised wild type (92.3%) mice, but both groups demonstrated a significantly greater proportion of correct trials when compared with the R6/1 non-exercised mice.

The analyses of the direction reversal phase of testing demonstrated robust significance across the groups on both the “trials to criteria” and “percentage correct trials” measures. For the trials to criteria measure (Fig. 3B), main effects of genotype (Genotype, F1,29 = 20.9, p < 0.001), and exercise (Exercise, F1,29 = 30.44, p < 0.001) were returned with a significant interaction of the two factors (Genotype × Exercise, F1,29 = 29.9, p < 0.001). Post-hoc analyses found that the exercised R6/1 mice reached criteria in fewer trials than the non-exercised R6/1 mice. There was no significant difference between the number of trials that exercised and non-exercised wild type mice required to reach criteria, with both groups differing significantly from the non-exercised R6/1 mice. The proportion of correct trials (Fig. 3C) achieved by the mice also differed significantly across the groups with a significant interaction (Genotype × Exercise, F1,29 = 29.8, p < 0.001) and main effect of exercise (Exercise, F1,29 = 30.44, p < 0.001) being returned. Post-hoc analyses revealed that the exercised R6/1 mice produced the greatest proportion of correct responses across all four experimental groups (73%) compared to the non-exercised R6/1 mice that made lowest proportion (20.5%), with the exercised (54.5%) and non-exercised (59.8%) wild type mice demonstrating similar levels of performance.

The effect of exercise on neuropathology

The R6/1 mice exhibited striatal atrophy relative to their wildtype littermates (Figs. 4A–E: Genotype, F1,28 = 22.75, p < 0.001). Clear increases in ventricular size were observed in the R6/1 mice resulting in a decrease in the dorsal striatum, and therefore striatal surface area +0.38 mm anterior to bregma. Exercise failed to affect striatal area in either the wildtype animals or the R6/1 mice (Exercise, F1,28 = 0.79; Genotype × Exercise, F1,28 = 2.40, both n.s.). Wildtype mice were found to have thicker cortices (Figs. 4F–J) than the R6/1 mice (Genotype, F1,26 = 9.51, p < 0.01) but again, exercise did not influence this significantly in either group (Fig. 4B, Exercise, F1,26 = 1.04; Genotype × Exercise, F1,26 = 0.01; both n.s.).

At ×40 magnification neuronal and non-neuronal cells looked normal in all mouse groups. Analyses of striatal neuron counts found that R6/1 mice demonstrated significant neuronal loss relative to their wildtype littermates (Genotype, F1,28 = 5.85, p < 0.05) which was significantly reduced in the exercise group (Figs. 5A–E: Genotype × Exercise, F1,28 = 2.40, p < 0.05). Pair-wise post hoc analyses demonstrated that the neuronal loss in the R6/1 mice not exposed to the running wheels was significantly reduced compared to wildtype mice without wheels. In contrast, no difference in neuron number was found between the R6/1 mice with the running wheels and the wildtype groups suggestive of a relative sparing of neurons due to the exercise intervention in this R6/1 group. Stereological analyses of neuronal intra-nuclear inclusion formations in the R6/1 mouse groups at the neuronal level (Figs. 5F, J–M) demonstrated that the exercised R6/1 mice had a greater inclusion load than the R6/1 mice that had not had exercise (t25 = 2.68, p < 0.05), despite there being
no significant difference between neuron counts. In the non-exercised mice 99.8% of striatal cells contained neuronal intra-nuclear inclusions compared with 93.3% in the exercised mice. Analyses of inclusion formations extraneous to the neuronal nuclei (Figs. 5G, J–M) found that non-exercised R6/1 mice demonstrated a significantly greater number than were present in the exercised R6/1 mice (t14 = 2.67, p < 0.05). Examination of striatal neurons in the non-exercised and exercise R6/1 striatum (Figs. 5H–M) indicated that there was no difference between the R6/1 groups in nucleus size (t14 = 1.49, n.s.), but that there were larger neuronal inclusions in the R6/1 exercise mice which were confirmed by quantitative analysis (t14 = 3.4, p < 0.01).

When the striatum was stained with TH to determine dopamine content (Figs. 6A–D, M), clear genotype specific differences were demonstrated (Genotype, F1,28 = 41.04, p < 0.001), in the absence of an exercise effect (Exercise, F1,28 = 0.01, n.s.), or interaction between genotype and exercise (Genotype × Exercise, F1,28 = 0.01, n.s.). The same pattern was found for striatal BDNF levels (Figs. 6E–H, N), with a significant reduction in striatal BDNF in the R6/1 mice relative to the wildtype animals (Genotype, F1,28 = 4.53, p < 0.05), but no effect of exercise (Exercise, F1,28 = 0.01, n.s.) and no genotype/exercise interaction (Genotype × Exercise, F1,28 = 0.01, n.s.). For the hippocampal BDNF levels (Figs. 6L–O), a genotype specific reduction was again found in the R6/1 mice relative to wildtype levels (Genotype, F1,28 = 5.94, p < 0.05), but no effect of exercise (Exercise, F1,28 = 0.73, n.s.), or exercise interaction with genotype was found (Genotype × Exercise, F1,28 = 1.04, n.s.).

**Discussion**

The aims of the present study were to determine if life-long exposure to voluntary exercise can modify or ameliorate the neuropathological course and functional decline in R6/1 HD mice, and to determine the viability of exercise-based interventions as translational therapeutic systems. In the motor analyses of the present study we found several main effects of exercise but only one effect ("latency to turn" on the balance beam) where exercise was differentially beneficial to the R6/1 mice over their wildtype littermates. Previous studies looking at exercise in the R6/1 mouse demonstrated relatively mild effects of exercise on motor and neurological readouts (Pang et al., 2006; van Dellen et al., 2008) with one report that 10 min of enforced daily exercise on the rotarod was only beneficial to female R6/2 mice with prior experience of the test (Wood et al., 2011). Taken together these studies suggest that exercise in HD mice has a modest effect on motor outcomes in contrast to the findings in patients that demonstrate significant symptom relief with relatively little intervention (Khalil et al., 2013). This apparent difference in efficacy probably represents our lack of knowledge regarding the optimisation of the exercise administration in terms not only of the quantity of the intervention, but also its nature.
(aerobic or non-aerobic, exercise or physiotherapy), and highlights the need for model experimental systems to optimise the experimental approach in mice. It should also be noted that excessive exercise may also be detrimental to the development of HD in people as marathon running was found to enhance the disease process (Kosinski et al., 2007), and mice bred to produce high levels of exercise also failed to improve their learning (Rhodes et al., 2003), indicating that exercise administration requires optimisation.

The present study demonstrated a clear benefit on cognitive probes of corticostriatal function, procedural and reversal learning, both clinically relevant measures of cognitive dysfunction mediated via striatal circuitry (Clarke et al., 2008; Hampshire et al., 2012; Palencia and Ragozzino, 2004), and dysfunctional in HD patients (Lawrence et al., 1996, 1998, 1999). Also of note from the motor tasks, was the “latency to turn” measure on the balance beam on which exercise exerted the greatest therapeutic benefit. On reviewing the videos of the results we suspect that we were measuring motor planning/initiation rather than motor coordination as we originally thought, as observation of the mice found that the differences in the latencies of the mice reflected the time between being placed on the beam and the onset of movement, rather than the speed of moving per se, suggesting that in HD mice, exercise more specifically modifies function in the cognitive rather than the motor domain. It should be noted however, that this is conjecture as we did not directly measure “motor planning”.

The cognitive enhancing effects of exercise in other neurological disorders have been documented elsewhere (Cruise et al., 2011; Marzolini et al., 2013; Vreugdenhil et al., 2012; Yaguez et al., 2011) and are consistent with the present and previous results in the R6/1 mouse line (Pang et al., 2006). The limited (10 min per day) and forced exercise regime used by Wood et al. (2011) was found to be detrimental in the female R6/2 mice to learning on the initial testing stage in the Lashley III maze, with this negative effect disappearing on the subsequent re-test trials. Given the relatively minor exercise intervention in the latter study the lack of any therapeutic benefit

Fig. 6. The striata of control (C) and exercised (D) R6/1 mice demonstrated an absence of TH staining relative to their wildtype control (A) and exercised (B) littermates, but no effect of exercise was demonstrated (M). BDNF levels were reduced in R6/1 striatum in control (G) and exercised (H) R6/1 mice, and also in hippocampus of control (K) and exercised (L) R6/1 mice relative to the wildtype control striatal and hippocampal levels (EJ respective) and wildtype exercised striatal and hippocampal levels (FJ respectively), but no effect of exercise was demonstrated in striatum (N) or hippocampus (O).
perhaps may not be unexpected. Taken as a whole the results from the present study add further strength to the translational value of exercise based model therapeutic systems for HD.

As HD is primarily a disease of the caudate nucleus (although it affects many other brain and peripheral tissues), our histological analyses of the mice focused on the striatum. The present study demonstrated exercise-induced sparing of striatal neurons in the absence of overt striatal atrophy, which may account for the beneficial effects of exercise in the R6/1 mice. It should be noted that our statistical analyses demonstrated a significant reduction in striatal neurons in the non-exercised R6/1 mice relative to wildtype control animals but no difference between exercised and non-exercised R6/1 mice in a direct comparison. Whilst a significant difference between the two R6/1 groups would be desirable, this may represent an unrealistic expectation for enrichment types of study but some sparing of neurons may be a more accurate reflection of the potential of this type of intervention. The neuropathological analyses also found cortical atrophy in the R6/1 mice relative to wildtype animals that has been reported in the related R6/2 line (Sawiat et al., 2009). It should be noted that the study by Potter et al. (2010), which found exercise to be deleterious to the N171-82Q HD mouse line, also reported increased striatal atrophy in the mutant gene carriers that had had exercise. Whilst the results from the present and latter studies are not consistent, both studies implicate a striatally mediated mechanism for their findings in the absence of evidence for a hippocampal based mechanism which has been previously posited as being causative (see below). However, the results from Potter et al.’s (2010) study should be treated with caution, as significant effects of exercise were inferred from post-hoc analyses of main effects of genotype, in the absence of significant genotype × exercise interactions, making rigorous interpretation difficult.

The previous enrichment studies in the R6/1 mouse suggest that the observed functional rescue of motor deficits was mediated by increases in neurogenesis and/or hippocampal or striatal BDNF levels (Lazic et al., 2006; Spires et al., 2004a; Zajac et al., 2010). This avenue of research was stimulated by the finding that BDNF is underexpressed in HD patients and animals (Cho et al., 2007; Ferrer et al., 2000; Simpson et al., 2011; Zuccato et al., 2001, 2005, 2008). Indeed, body clumping akin to that seen in HD mice may be related to reduced BDNF in the striatum (Baquet et al., 2004), and BDNF administration has been demonstrated to retard disease progression generally and reverse the increased GABAergic function of medium spiny neurons in R6/2 mice (Cepeda et al., 2004; Giampa et al., 2013). Studies using HD mice to assess the beneficial effects of exercise have typically used hippocampal-based spatial learning probes to measure cognitive dysfunction in striatally compromised HD mouse lines (Pang et al., 2006; Potter et al., 2010), and whilst these mouse lines have clear hippocampal-mediated deficits, the hippocampal focus in the application of the tests may detract from the validity of the results in relation to HD. Regardless, we found a reduction of striatal and hippocampal BDNF in the R6/1 mice relative to the wildtype animals but no effect of exercise on BDNF levels in either striatum, consistent with some studies (Cepeda et al., 2010; Pang et al., 2006), or in the hippocampus in contrast to other studies (Pang et al., 2006). It is difficult to reconcile these discrepancies with reference to the role that BDNF plays in mediating the observed functional effects of exercise in HD. The discrepancies between the present study and the previous studies may represent methodological differences either in the sensitivity of the analyses methodology (image-J in the present study versus the more sensitive western blotting for example), but it should be considered that the optical density methods used in the present study were sufficiently sensitive to demonstrate genotypic reductions in TH and BDNF levels consistent with other studies (Lerner et al., 2012; Spires et al., 2004b). More likely differences in the application of the exercise may be more important, as in the original studies the exercised mice had access to the running wheels but for 24 h of the day (Pang et al., 2006; van Dellen et al., 2008), rather than the 14 h that the animals in the present study had. In addition, there were differences in background strain used which the influence of which is often underestimated, with the original studies on enrichment in the R6/1 mice using the CBA × B6 mouse (Pang et al., 2006) to produce the experimental cohorts whereas the present study used the congenic C57B/6 R6/1 line. Background strain is a known modifier of disease in HD mice (Van Rammolak et al., 2007). In all three experiments the animals spent time group housed although in the present study the mice were housed individually whilst they had access to the wheels. Crucially, no measure of exercise was reported in the earlier studies making cross comparisons difficult on this crucial parameter. In all likelihood, a combination of these factors probably accounts for the differences between the studies. However, the issue remains as to whether the hippocampus and BDNF and/or neurogenesis are central to the retardation of cognitive decline in tests that probe procedural and reversal learning, and whilst there is evidence that these brain regions are functionally linked (Ben Yakov and Duda, 2011; Jacquet et al., 2013; Voermans et al., 2004), and HD transgenic mice and patients have been found to compensate for striatal dysfunction with hippocampal-mediated strategies (Gamei and Morton, 2009; Voermans et al., 2004), the evidence presented in the present study and elsewhere (see above) is more suggestive of a striatally-mediated mechanism that is independent of BDNF.

Clear genotypic differences in striatal TH content were found with Image-J in agreement with previous mouse and human studies that found reduced dopamine content in striatum (Cummings et al., 2006; Glass et al., 2000; Lerner et al., 2012), but exercise failed to modify these levels consistent with the relative lack of benefit that exercise produced on the motor tasks. These data also suggest that striatal dopamine did not contribute to the cognitive sparing properties that exercise induced.

The most surprising aspect of the present study was that within the neuronal population of the exercised R6/1 mice the intraneuronal inclusions were larger and more prevalent than in the R6/1 control group. These data may suggest that the larger inclusion size represents a more efficient compensatory mechanism in neurons if the inclusions are considered to be neuroprotective as has been hypothesised (Arrasate et al., 2004; Miller et al., 2010; Morton et al., 2000). An alternative explanation would be that in the non-exercised R6/1 mice the neurons have died depositing previous intra-nuclear inclusions in the neuropil as extra-neuronal inclusions, consistent with our findings of increased extra nuclear inclusions in the non-exercised mice. Thus, inclusion bodies can exist in different forms within and without the neuronal nuclei, and in non-neuronal cells (Davies et al., 1997; Morton et al., 2000; Turmaine et al., 2000). In the absence of specific double staining of inclusions with distinct cellular markers it is impossible to verify the precise loci in which the numerous non-nucleic inclusions exist.

The present study found that chronic exercise preferentially improved cognitive function, reduced striatal cell loss and increased neuronal intra-nuclear inclusion size and number in the R6/1 mouse line, providing further evidence of therapeutic benefit of exercise in HD. Future studies using the present intervention system will focus on optimising this intervention strategy by determining the extent and nature of the exercise required to maximise these effects.

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References


