Decreased hippocampal cell proliferation in R6/1 Huntington's mice

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In order to ascertain whether disturbances of neurogenesis occur in chronic neurodegenerative disorders, we assessed hippocampal cell proliferation in the R6/1 transgenic mouse model of Huntington's disease (HD). Using BrdU labelling for dividing cells at two different time points (5 and 20 weeks) in transgenic and wild type control mice, we have shown that cell proliferation in the hippocampus was similar in younger asymptomatic R6/1 mice and wild type controls, but that older R6/1 mice had significantly fewer $BrdU^+$ cells than controls. Such a decrease in cell proliferation may be relevant to some of the deficits seen in these mice, although further work is needed to prove this. *NeuroReport* 15:811–813 © 2004 Lippincott Williams & Wilkins.

Key words: Environmental enrichment; Hippocampus; Huntington's disease; *In vivo*; Neurogenesis; R6/1

INTRODUCTION

Huntington's disease (HD) is a progressive neurological condition that presents with a combination of motor, psychiatric and cognitive deficits. It is caused by a mutated form of the *huntingtin* gene, but the mechanism of cell loss is not fully understood. The striatum and cortex are affected in early stages of the disease and more recent evidence suggests hippocampal dysfunction as well, in both humans [1] and mice [2]. It is now widely accepted that neurogenesis occurs in adult mammals and these newly created cells form functional connections with existing circuits [3]. Recently, increased cell proliferation in the post-mortem brain of HD patients has been shown [4], although the extent to which these new cells form functional neurons is not known. Environmental enrichment is known to increase neurogenesis in the hippocampus [5] and to delay the onset of symptoms in the transgenic R6/1 mouse model of HD [6]. We, therefore, investigated cell turnover in the hippocampus of R6/1 transgenic mouse model of HD over time using bromodeoxyuridine (BrdU) under standard and enriched environments.

MATERIALS AND METHODS

Animals: Wild type CBA and R6/1 transgenic mice were used in accordance with the UK Animals (Scientific Procedures) Act 1986. Mice were separated into enriched or non-enriched environments at 4 weeks of age with enriched mice having various plastic and cardboard objects in their cage changed every 2 days. Cell turnover in these mice was detected at two time points using a 240 mg/kg i.p. injection of BrdU dissolved in saline every 12 h for 12 days, with mice being killed on day 13. One group of mice started injections at 5 weeks and another at 20 weeks. These mice were killed during weeks 7 and 22, respectively.

Behaviour analysis: At week 22, mice were placed on a rotarod (Ugo Basile, Comerio VA, USA model 7650) which accelerated from 4 to 40 r.p.m. in 3 min, and the speed at which the mice fell off was recorded.

Histology: Mice were perfused with PBS and 4% paraformaldehyde (PFA). Brains were removed and stored in PFA overnight and then transferred to a 30% sucrose solution. Brains were sectioned in the coronal plane at 40 μm intervals and staining was performed on free-floating sections. Incubation and washing solutions contained 0.2% Triton X-100 (Sigma; Poole, UK) in Tris-buffered saline (TBS). Sections were quenched for 10 min using 10% H_2O_2/10% methanol in distilled water. Sections were then treated with 2 M HCl for 30 min and then neutralized in phosphate buffered saline (PBS) prior to a 1 h block with 3% normal donkey serum (NDS; Dako, Ely, UK). The primary polyclonal anti-BrdU antibody (1:1000; Maine Biotechnology Services Inc., Maine, USA) in a 1% NDS solution was left on overnight at room temperature. After washing, incubation with a biotin-conjugated donkey anti-sheep IgG (1:200; Sigma) secondary antibody was followed by streptavidin-biotin-conjugated complex treatment (1:200; Dako, Ely, UK) and visualized with a Vector SG chromogen (Vector Laboratories, Burlingame, CA, USA). Sections were mounted on gelatinized glass slides, dehydrated using a series of ascending ethanol solutions (70%, 95% and 100%) and xylene, and then coverslipped.

Fluorescent doubled-labelling for BrdU and NeuN (1:200; Chemicon) followed a similar procedure as above. Donkey

anti-sheep IgG FITC (1:200; Sigma) and horse anti-mouse biotin-conjugate IgG (1:200; Vector Laboratories) secondary antibodies were used. Finally, streptavidin-TRITC (1:200, Cambridge Bioscience, Cambridge, UK) was added and left for 2 h in the dark. Sections were washed 3 times and coverslipped using Vectashield mounting medium (Vector Laboratories). Staining with the anti-huntingtin antibody (S803, 1:1000; gift from Gill Bates) followed a similar procedure but without HCl treatment.

Stereology and microscopy: The Olympus CAST-Grid system (version 1.09; Denmark) was used for volumetric measurements and quantification of BrdU-positive cells. Areas of the hippocampus were sampled at random and cell counts were used to determine the total number of BrdU positive cells in each structure. Volumetric measurements were determined by measuring the striatum and overlying cortex on sections stained for acetylcholinesterase (modified Koelle method) in a 1 in 6 series. The volume was then calculated by summing these values and adjusting for the sampling frequency and section thickness. A fluorescent microscope (Axiovert 135; Zeiss, Germany) was used to identify cells that stained for both BrdU and NeuN. Images were collected under a $\times 40$ objective and analyzed using LaserSharp software (Biorad, version 3.2).

Statistical analysis: A 2-way ANOVA (2 environments $\times$ 2 genotypes) was used to test for differences in the histological and behavioural dependent variables. In all tests, α was set at 0.05. BrdU values for the 22-week mice were $\log_{10}$-transformed prior to analysis as the variance was greater for those groups with higher means [7]. Graphs display untransformed data.

RESULTS

Behavioural tests at 22 weeks showed a significant effect of environment ($p = 0.03$), but not genotype ($p = 0.430$), with mice in the enriched environment falling off the rotarod at higher speeds and thus showing better motor performance than mice in the standard environment, irrespective of genotype (Fig. 1a).

There were no significant differences in hemispheric volumes between younger R6/1 (mean ($\pm$ s.d.) $76.5 \pm 3.0\,mm^3$) and control mice ($73.5 \pm 2.4\,mm^3$; $p = 0.13$). In the 22-week mice there was a significant effect of genotype, with the R6/1 mice having smaller hemispheric volumes (Fig. 1b; $p < 0.001$), as reported previously [6]. There were no significant differences in hippocampal cell proliferation in the 7-week mice (Fig. 1c) between environments ($p = 0.19$) or genotypes ($p = 0.38$). In the 22-week group there was still no significant difference between environments ($p = 0.26$) but there was between genotypes ($p = 0.03$), with R6/1 mice having fewer $BrdU^+$ cells in the hippocampus (Fig. 1d, Fig. 2a,b).

To determine the fate of recently divided cells, $\sim$150 $BrdU^+$ cells in the hippocampus of each animal were examined for concomitant NeuN labelling using a confocal microscope (Fig. 2e). No cells were found to be unambigu-

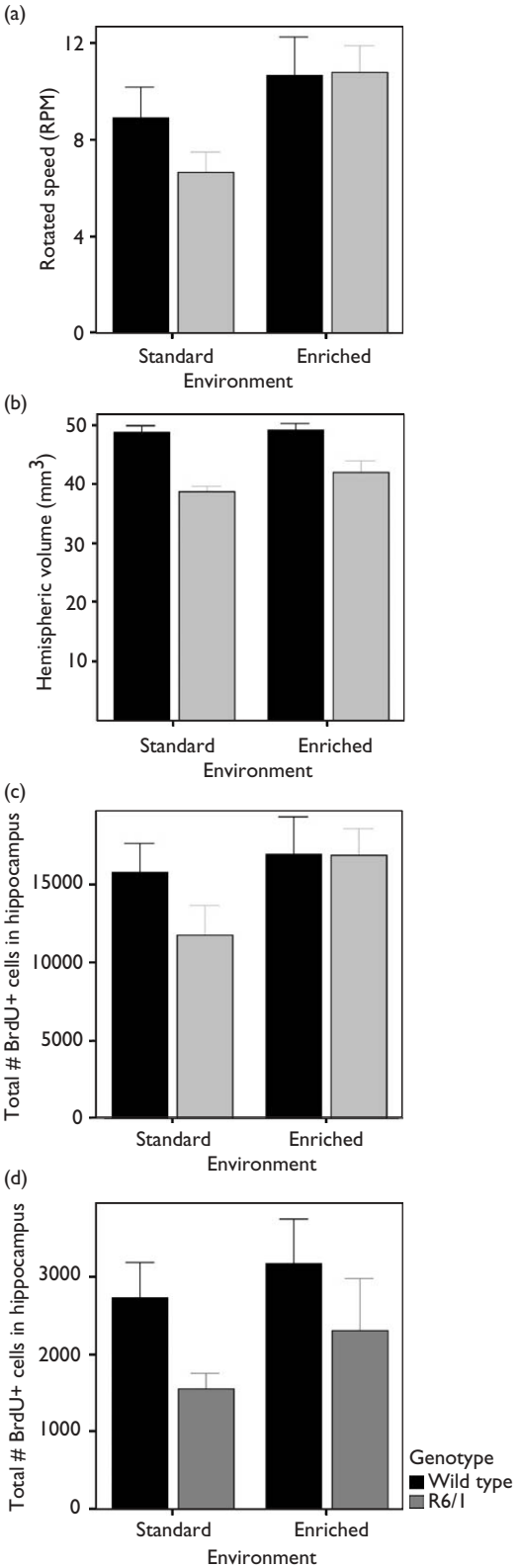

Fig. 1. Histological and behavioural results analyzed with a 2-way ANOVA. (**a**) At 22 weeks enriched mice had better motor ability and fell off the rotarod at higher speeds than mice in the standard condition ($p = 0.03$), but there was no difference between R6/1 and controls ($p = 0.430$). (**b**) Older symptomatic R6/1 mice had significant hemispheric atrophy compared to controls ($p < 0.001$) which was not altered by environmental enrichment. (**c**) There were no significant effects of genotype or environment in the number of $BrdU^+$ cells in the hippocampus in the 7-week mice. (**d**) There was an overall decrease in the number of $BrdU^+$ cells in the 22-week mice compared to the 7-week, and in addition, the R6/1 mice had significantly less $BrdU^+$ cells than the control ($p = 0.03$), irrespective of environmental enrichment. In neither the R6/1 nor control mice was there a significant increase in $BrdU^+$ cells with environmental enrichment.

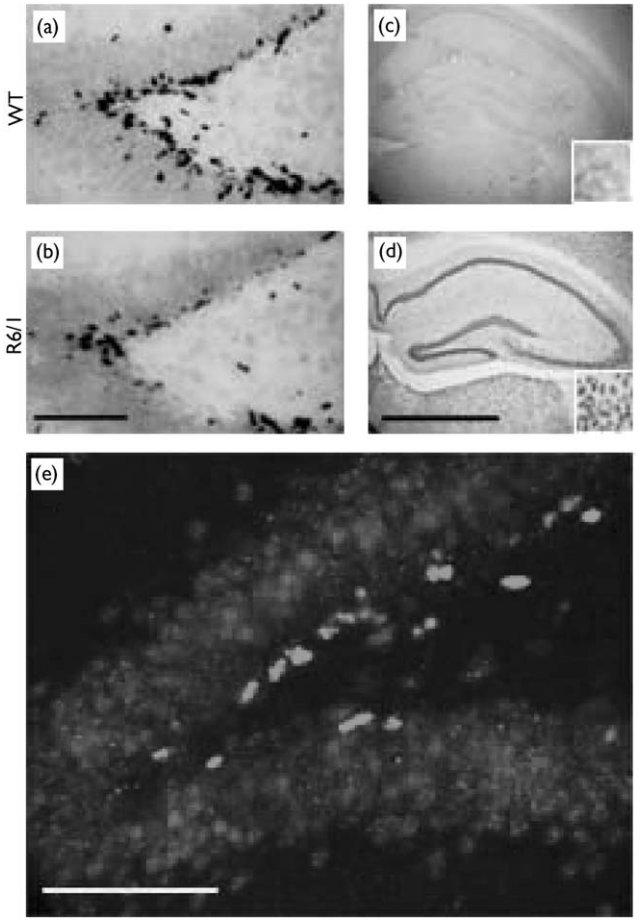

Fig. 2. BrdU (**a**,**b**), anti-huntingtin (S803; **c**,**d**) and BrdU (white) + NeuN (gray; **e**) immunochemistry in the hippocampus of non-enriched wild-type (WT) and transgenic mice (R6/1) at 7 weeks. (b) Decreased cell proliferation can be seen in dentate gyrus of the R6/1 mice. (c,d) Note the nuclear huntingtin inclusions in the R6/1 but not the wild-type mice. (e) Confocal image showing no double-labelling of BrdU and NeuN antibodies. Bar = 0.1 mm (a,b,e), 1 mm (c,d).

ously double-labelled. Expression and accumulation of the transgenic protein was confirmed by the anti-huntingtin (S803) antibody, which selectively stained cells throughout the brain only in the transgenic mice (Fig. 2c,d).

DISCUSSION

This study has shown that older symptomatic R6/1 transgenic mice have decreased cell proliferation in the hippocampus compared to wild type mice while pre-symptomatic 7-week R6/1 mice had only slightly fewer $BrdU^+$ cells than wild type mice when both were reared in a standard environment. However, when reared in an enriched environment the number of $BrdU^+$ cells was similar in the R6/1 and wild type mice. In no cases were double-labelled cells seen, but the study concentrated on acute cell turnover so insufficient time had elapsed to allow for differentiation. In addition, the R6/1 and control mice had similar motor abilities at 22 weeks as assessed using the rotarod test. Motor performance was not related to changes in the number of $BrdU^+$ cells, arguing against any direct causal link but importantly the decrease in $BrdU^+$ cells seen in the R6/1 mice could not be due to a lack of physical activity as a consequence of an inability to move about.

This study also demonstrated, as others have previously, that R6/1 transgenic mice have significant cortical and subcortical atrophy compared with controls and that enrichment enhances the motor performance of both groups of mice. However this study did not show an increase in $BrdU^+$ cells with environmental enrichment irrespective of genotype as has been previously reported by Kempermann *et al.* [8], although, the enriched mice did tend to have a greater number of $BrdU^+$ cells. The larger effect size of environmental enrichment observed in the Kempermann study could be due to the running wheel which mice had access to in that (but not our) study, since running alone increases neurogenesis [9]. Further, Kempermann *et al.* used a different protocol and investigated neurogenesis 4 weeks after BrdU injections while this study examined cell proliferation acutely 13 days after BrdU injections, at a time before the $BrdU^+$ cells could have differentiated, accounting for the absence of neuronal double-labelling in this study.

CONCLUSION

This study demonstrates that younger pre-symptomatic R6/1 mice have similar levels of cell proliferation in the hippocampus as wild type controls but that proliferation is less in older transgenic mice compared to equivalent aged wild type controls. Therefore, it is possible that cell turnover in the hippocampus of R6/1 mice may contribute to the progressive hippocampal dysfunction observed in this transgenic line, but further work is needed to prove this.

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